Plasmodium falciparum: *in vitro* chloroquine susceptibility and allele-specific PCR detection of *Pfmdr1* $^{Asn}86^{Tyr}$ polymorphism in Lambarene, Gabon

M. P. GROBUSCH[1,2,3], I. S. ADAGU[2]*, P. G. KREMSNER[3,4] *and* D. C. WARHURST[2]

[1] *II. Medizinische Klinik, Virchow-Klinikum, Humboldt-Universität, 13353 Berlin, Germany*
[2] *Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK*
[3] *Research Laboratory, Albert Schweitzer Hospital, Lambarene, Gabon*
[4] *Department of Parasitology, Institute of Tropical Medicine, University of Tübingen, Germany*

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SUMMARY

Plasmodium falciparum resistance to chloroquine has been described in many parts of the world particularly in Africa where malaria is endemic. High levels of chloroquine resistance in our study area, Lambarene–Gabon, has led to the use of an alternative regimen for treatment and prevention of *P. falciparum* infection. In this study, we examined the *in vitro* chloroquine sensitivity of 15 isolates from this area and assessed the prevalence of a putative chloroquine resistance associated *Pfmdr1* polymorphism ($^{Asn}86^{Tyr}$) using a novel allele-specific polymerase chain reaction (PCR). Only 4 of the isolates examined were chloroquine sensitive. The allele-specific PCR shows that all 15 isolates carried the variant (86^{Tyr}) codon. Eleven of these were resistant to chloroquine suggesting a 73% agreement between chloroquine resistance phenotype and the point mutation. This molecular marker was examined in a further 73 Gabonese isolates, where 58 (79·5%) showed 86^{Tyr} and 15 (20·5%) showed 86^{Asn}. In all, 4 (4·5%) of the 88 isolates assessed carry both mutant and wild-type codons, suggesting mixed parasite populations. The incomplete agreement found between chloroquine resistance phenotype and *Pfmdr1* (86^{Tyr}) polymorphism would support the view that other genetic factors as well as *Pfmdr1* may be involved in chloroquine resistance. While our results suggest a high prevalence of 86^{Tyr} polymorphism in Lambarene, the $^{Asp}1246^{Tyr}$ polymorphism (a point mutation which to date has only been associated with South American *P. falciparum*) seems to be absent in our study area.

Key words: *Plasmodium falciparum*, chloroquine resistance, allele specific PCR, *Pfmdr1* polymorphisms, Gabon.

INTRODUCTION

While the mechanism of *Plasmodium falciparum* resistance to dihydrofolate reductase inhibitors is generally agreed, it is not yet clear how this parasite becomes resistant to blood schizontocides, particularly chloroquine. Biochemical studies have associated chloroquine resistance with reduced accumulation of the drug in the parasite digestive vacuole – the putative site of action of the drug. The reduced drug accumulation has been linked to a deficient uptake mechanism and/or enhanced drug efflux (Bray *et al*. 1992). Chloroquine resistance has been associated with changes in a 400 kb region of chromosome 7 (Wellems *et al*. 1990; Wellems, Walker-Jonah & Panton, 1991). Polymorphisms in a multidrug-resistant gene homologue of *P. falciparum* (*Pfmdr1*) on chromosome 5 have also been associated with the chloroquine resistance phenotype (Foote *et al*. 1990). Although Wellems' reports (Wellems *et al*. 1990, 1991) and those of Barnes *et al*. (1992), Awad-El-Kariem, Miles & Warhurst (1992) and Wilson *et al*. (1993) suggest apparent lack of association between chloroquine resistance phenotype and point mutations in the *Pfmdr1* gene, there is increasing evidence that this gene does play a role (see Van Es *et al*. 1994*a*, *b*; Peel *et al*. 1994; Adagu *et al*. 1995*a*, 1996, 1997; Volkman, Cowman & Wirth, 1995; Basco *et al*. 1995; Ruetz *et al*. 1996). The $^{Asn}86^{Tyr}$ polymorphism has been associated with chloroquine resistance in Nigerian (Adagu *et al*. 1995*a*, 1997), Guinea Bissauian (Adagu *et al*. 1996), Gambian (Duraisingh *et al*. 1997) and Malaysian (Cox-Singh *et al*. 1995) isolates of *P. falciparum*. Other studies have associated polymorphisms in codon 1042 and 1246 of the *Pfmdr1* gene with *in vitro* chloroquine resistance in South American isolates (Foote *et al*. 1990; Povoa *et al*. 1997). Strains of *P. falciparum* from the Gabon were reported to be highly resistant to chloroquine, 100% (43/43) *in vitro* chloroquine resistance was observed in the Moyen Ogooue province around Lambarene (Winkler *et al*. 1994). Similar levels of resistance were observed in other studies (Kremsner *et al*. 1994; Philipps *et al*. 1997). Isolates from this highly chloroquine resistant area

* Corresponding author: Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK. Tel: +44 171 927 2341. Fax: +44 171 636 8739. E-mail: I.Adagu@LSHTM.ac.uk

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of Central Africa have not been examined for any resistance-related genetic variation, therefore we conducted an *in vitro* chloroquine susceptibility study of *P. falciparum* from Lambarene–Gabon and examined these and further isolates for presence or absence of polymorphisms (in codon 86 and 1246 of the *Pfmdr1* gene) putatively associated with chloroquine resistance.

MATERIALS AND METHODS

Study area

Albert Schweitzer Hospital, Lambarene, Gabon is the site where the *in vitro* drug test and blood sample collection on glass fibre membrane were carried out in July 1996 and genetic studies were conducted at the Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine. Malaria in Lambarene is stable, predominantly hyperendemic and affects all age groups, particularly children (Wildling *et al*. 1995). Transmission appears to be all year round with small seasonal fluctuations. In our study site, the hospital village, Lambarene, transmission varies between the short rainy, dry and long rainy seasons. Peak transmission occurs during the short rainy season (see Wildling *et al*. 1995). Although *P. falciparum* is the predominant parasite in this area, there are cases of *P. malariae* and *P. ovale* infections. *Anopheles gambiae* is the principal vector.

Patients

Children (3 months and above) presenting with febrile illness to the Paediatric Out Patient Department of Albert Schweitzer Hospital and adult patients of the internal and surgical wards suspected of having malaria were selected and examined for *P. falciparum* infection. Those who were microscopically positive for *P. falciparum* were informed of the study and oral consent was obtained before blood sample collection which was carried out before appropriate treatment was instituted. Although parasitaemia in these patients ranged from 10 to $>100\,000$ parasites/μl of blood, samples for the *in vitro* test were taken only from those with a parasitaemia between 1000 and 100 000 parasites/μl of blood. Ethical approval for the sample collection and the *in vitro* drug assay of the samples was obtained from the ethics committee of the International Foundation of the Albert Schweitzer Hospital.

Sample collection

In all patients except those whose samples were to be assayed for *in vitro* sensitivity to chloroquine, a 100 μl blood sample was withdrawn by finger prick into a heparinized 100 μl capillary tube. This was spotted (5 spots of approximately 20 μl) onto a 2·5 cm glass-fibre membrane disc supported on a 5·5 cm Whatman filter paper disc. Both discs were labelled and blood spots were allowed to air dry before transferring the discs to a $9\times11{\cdot}5$ cm resealable plastic bag carrying the same label. Samples were stored at 4 °C until the end of the study and were transported at ambient temperature from Lambarene to London where cold storage was resumed until needed (Adagu, 1996).

In vitro *drug test*

Blood samples were prepared according to the WHO *in vitro* microtest procedure (mark II) (WHO, 1990). Standard chloroquine test kits were obtained from the World Health Organization, Geneva, Switzerland. Analysis of the inhibitory concentrations (IC_{50} and IC_{95}) preventing schizont maturation was carried out using XLFit, an add-in for EXCEL (PC) using a non-linear regression (Levenberg–Marquardt algorithm). An $IC_{95}\geqslant5{\cdot}7$ pmol chloroquine/well (114 nM) was taken to indicate resistance.

Allele specific polymerase chain reaction (*AS–PCR*)

The method of Warhurst, Awad-El-Kariem & Miles (1991) was adopted in preparing blood samples spotted on glass-fibre membrane. A sector of the prepared membrane was used in PCR. Oligonucleotides were selected to flank regions of the *Pfmdr1* gene containing a single base change at residues 86 and 1246 (see Frean *et al*. 1992). The allele-specific oligonucleotide or inner forward primer (DCW-3, specific for 86^{Tyr}) was designed with the 3′ terminal nucleotide corresponding to the single base change. A base immediately upstream to this change was mismatched to enhance adequate blockade of amplification of non-target sequence (Wang *et al*. 1995). Wild-type (WT) specific oligonucleotide, forward, (DCW-4, specific for 86^{Asn}) was similarly designed (Table 1). For the allele-specific PCR for codon 86 a new outer forward oligonucleotide (ISA-2) was designed to replace that described by Frean *et al*. (1992) leading to amplification of a 329 bp fragment of the gene. See Table 1 for the sequences of the reverse oligonucleotide (DCW-2 (same as that of Frean *et al*. 1992)) and the forward primers described above. The semi-nested reaction was carried out in one tube. Amplifications were performed in standard PCR buffer, first for 8 cycles, at 94 °C for 30 sec; 50 °C for 30 sec and 72 °C for 30 sec then for another 32 cycles at 94 °C for 30 sec; 45 °C for 30 sec and 72 °C for 30 sec. Primers were used at 0·3 μM (ISA-2) and 0·5 μM (DCW-2, DCW-3, DCW-4). PCR products were resolved on a 2 % agarose gel for documentation.

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Table 1. Primer sequences

Primer	Sequence*
ISA-2	5′..AGA TGG TAA CCT CAG TAT CA..3′
DCW-2	5′..TTA CAT CCA TAC AAT AAC TTG..3′
DCW-3	5′..GTG TAA TAT TAA AGA ACA TCT..3′
DCW-4	5′..GTG TAA TAT TAA AGA ACA TAA..3′

* All primers were obtained from R & D Systems. See text for primer concentrations.

Table 2. *In vitro* sensitivity of 15 *Plasmodium falciparum* isolates from Lambarene, Gabon (1996)

Samples	Parasitaemia/μl	IC_{50} (nM)	IC_{95} (nM)	Status (R/S)*
TA007	1500	81·7	887	R
MR009	3500	7·4	35·6	S
BU010	2500	45·9	106	S
MR056	13500	28·5	44·3	S
MA059	5600	23·7	68·5	S
BP070	100000	94·7	253	R
EK071	2500	58·9	484	R
NS080	22000	145	165	R
MJ080	45000	48·2	158	R
MU092	4000	19·1	1866	R
BM100	19500	7·3	169	R
MA102	17500	24·1	> 10000	R
AS105	60000	309	505	R
MH110	22000	184	354	R
BG116	35500	171	451	R

* R, resistant; S, sensitive.

PCR/restriction fragment length polymorphism (RFLP)

PCR amplification followed by RFLP of the region of *Pfmdr1* flanking codon 86 and 1246 were carried out as reported earlier (Frean *et al*. 1992; Awad-El-Kariem *et al*. 1992; Adagu *et al*. 1995*a*). Only the 15 samples characterized for *in vitro* sensitivity to chloroquine were examined by the PCR/RFLP method. See Frean *et al*. (1992) for primer sequences and the reaction conditions. In this study, however, PCR products were incubated with *Afl*III endonuclease (5′..AC(A/G)(T/C)GT) (Boehringer Mannheim) instead of *Nsp*1 used in the previous studies. To detect wild-type, the *Apo*1 restriction enzyme was used, (G(A)AATTT(C)) (GIBCO BRL) cutting the PCR product and producing a gel RFLP pattern exactly opposite to that produced by *Afl*III (Duraisingh *et al*. 1997). The amplified region of the gene containing codon 1246 polymorphism was incubated with *Eco*RV and analysis of both PCR and restriction digestion products was as described by Frean *et al*. (1992).

RESULTS

In vitro *test*

In all, blood samples from 196 patients infected with *P. falciparum* were collected and spotted on glass-fibre membrane. Of these, 22 (11%) were obtained from patients from provinces other than the Moyen Ogooue. Of the 196 samples, the first 36 (18·4%) which satisfied the criteria for the *in vitro* micro test (mark II) (WHO, 1990) were assayed for sensitivity to chloroquine. Fifteen of the 36 isolates (41·7%) showed 10% or more schizont maturation in the control well – a criterion allowing the interpretation of the test results. The remaining 58·3% did not satisfy this criterion and were therefore not included in the *in vitro* test analysis. Only 1 of the 15 parasite samples was isolated from a patient from Haut Ogooue, the remaining 14 were from Moyen Ogooue. Although both IC_{50} and IC_{95} values were obtained for all the parasites, resistance determination was based on the IC_{95} cut-off value (114 nM) as this is comparable to the WHO M.I.C. cut-off point for the micro *in vitro* test. Based on the 114 nM

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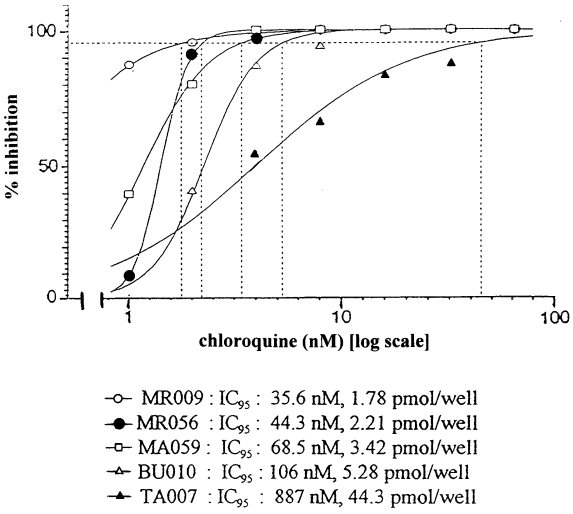

Fig. 1. Log dose versus percentage inhibition curves for response to chloroquine in 4 chloroquine-sensitive and 1 resistant isolate of *Plasmodium falciparum* from Lambarene, Gabon.

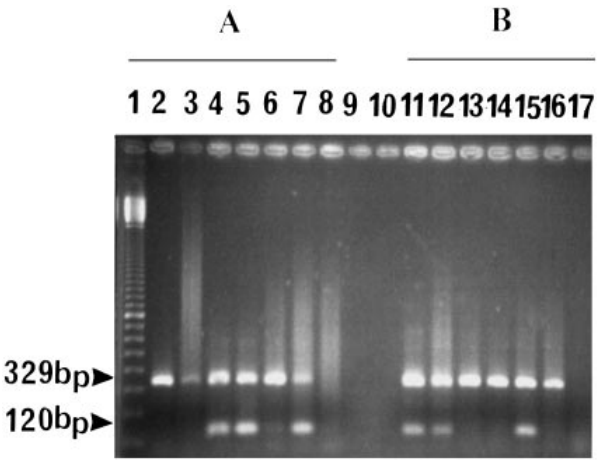

Fig. 2. The semi-nested allele specific PCR (A) mutant (B) wild-type.

threshold value, only 4 (27 %) of the 15 isolates were sensitive to chloroquine. The remaining 11 (73 %) were chloroquine resistant (Table 2 and Fig. 1).

Allele-specific polymerase chain reaction

The semi-nested allele specific PCR (AS–PCR) generated 2 products for each allele (Fig. 2A and B). The outer primer ISA-2 and DCW-2 directed amplification of the 329 bp product. DCW-3 or DCW-4 and DCW-2 directed amplification of the 120 bp product. An annealing temperature of 50 °C was optimal for the amplification of the 329 bp product. The genomic DNA was subjected to 8 rounds of amplification and the product (329 bp) served as template for the next 32 rounds of amplification during which the annealing temperature was stepped down to 45 °C – optimal for the 120 bp product. The higher molecular weight product, 329 bp, which could be seen in all cases (Fig. 2) also serves as a positive internal control for DNA template suitability. The 120 bp product is indicative of either mutant codon [86^{TAT}] (if amplification involved DCW-3, Fig. 2A) or wild-type codon [86^{AAT}] (when amplification involved DCW-4, Fig. 2B). Except for isolates containing MT and WT clones (e.g. lanes 6 and 15 Fig. 2A and B respectively), those carrying 86^{TAT} (Fig. 2A) did not yield the 120 bp product with DCW-4 (Fig. 2B). Likewise, isolates in Fig. 2B producing the 120 bp fragment with DCW-4 failed to produce this band when the other allele-specific oligonucleotide DCW-3 was used (compare Fig. 2A and B). In all, 15/15 (100 %) isolates characterized for sensitivity to chloroquine carry the codon 86^{TAT} polymorphism.

Eleven (73 %) of the 15 isolates were chloroquine resistant while the remaining 4/15 (27 %) were sensitive to chloroquine. Two isolates found to carry the WT codon also have the MT codon. These are probably cases where there are at least 2 clones in the population. Examination of another 73 isolates (not characterized for sensitivity to chloroquine) revealed that 58 (79·5 %) of these had the 86^{TAT} codon. Two (3·4 %) of these 58 also had the 86^{AAT} codon suggesting the presence of MT/WT lines. The remaining 15 (20·5 %) had the WT codon only. Overall, in a total of 88 samples, 69 (78·4 %) carry the MT codon only, 15 (17 %) carry WT codon only while 4 (4·5 %) carry both MT and WT codons.

PCR/RFLP

As reported elsewhere (Frean *et al*. 1992), PCR amplification of *Pfmdr1* gene region containing codon 86 and 1246 polymorphisms produced 355 and 500 bp products respectively (not shown). Restriction digestion of the 355 bp products gave a restriction pattern of 255 and 100 bp in mutant isolates. Similar restriction patterns (not shown) were obtained for *Afl*III and *Apo*1, except that one is a direct opposite of the other, *Apo*1 digestion was carried out to confirm the *Afl*III results. The AS–PCR and PCR/RFLP gave identical results with the 15 isolates which have been examined *in vitro*. Restriction pattern (2 × 250 bp fragments which co-migrate on gel electrophoresis as 1 band (not shown) indicative of the presence of codon 1246 polymorphism was produced only by the positive control. None of the 15 field isolates displays this restriction pattern – suggesting absence of the codon 1246 ($^{Asp}1246^{Tyr}$) polymorphism.

DISCUSSION

This study presents *in vitro* susceptibility of Gabonese *P. falciparum* to chloroquine and prevalence of $^{Asn}86^{Tyr}$ in these isolates. The number of isolates (15) assayed for sensitivity to chloroquine, though small, is more than 10, the WHO recommended minimum (WHO, 1990). The WHO *in vitro*

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test procedure was strictly applied; it is, however, not clear why some fresh field isolates would not adapt to culture, a limitation which led to successful cultivation and characterization (sensitivity to chloroquine) of only 15 of the 36 isolates put to culture. The 73 % chloroquine resistance observed here is indicative of a high prevalence of resistance in the Lambarene area of Gabon. This is consistent with previous findings (Kremsner *et al*. 1994; Winkler *et al*. 1994; Philipps *et al*. 1997) indicating a high level of chloroquine resistance in the Moyen Ogooue province around Lambarene. Three of the 4 chloroquine-sensitive isolates (MR009, MR056 and MA059) have remarkably low IC_{95} values (35·6, 44·3 and 68·5 nM, Table 2). Values obtained for the resistant isolates are well above the cut-off value of 114 nM. More than 10-fold this critical value (114 nM) was obtained for 2 isolates (MU092 and MA102) suggesting a very high resistance status. Maturation of isolates TA007, MJ080 and AS105 to schizonts was inhibited only by 32 pmol chloroquine/well, while isolates BP070, NS078, MU092, MA102 and BG102 were still growing even in the presence of the highest drug concentration (64 pmol chloroquine/well).

We report here a semi-nested, 1 tube, allele-specific PCR for the detection of *Pfmdr1* 86^{Tyr} polymorphism. Although allele-specific PCR techniques have been reported before for the detection of 108^{Asn} mutation in the *dhfr* gene (Peterson *et al*. 1991), normal or sickle cell β-globin allele (Wu *et al*. 1992) and have been made more specific by introducing additional mismatches in detecting sequence variation in the *dhps* gene (Wang *et al*. 1995), the present semi-nested technique combines high sensitivity shown by the former method with the specificity of the latter. Both mutation-specific and restriction PCR techniques enabled us to classify our samples into MT, WT and MT/WT, however, the allele specific method was quicker. There could be little or no competition between the allelic forms for primers DCW-3 or 4 since they are designed to be specific and are used in separate tubes. Ten parasites/μl of blood is the lowest parasitaemia we examined by AS–PCR and this gave a readable result. Based on the sector (1/8th) of a 20 μl blood spot used in PCR, 25 parasites/PCR would be the minimum number of parasites detected.

The genetic studies reported here show that both chloroquine-sensitive and resistant isolates carry the codon 86 polymorphism. In agreement with previous observations, results presented here show a high level of chloroquine resistance in our study area. Since the association between Tyr-86 and resistance is not absolute, it is difficult to recommend the *Pfmdr1* PCR technique for detection of individual chloroquine-resistant infections. However, we have here as in other parts of West Africa a more than coincidental association of the Tyr-86 and resistance which is suggestive. In other areas where resistance prevalence is less it is possible to prove the association statistically. Based on this, the epidemiological use of codon 86^{Tyr} may be feasible in some areas.

AS–PCR revealed that 58 (79·5 %) of the 73 isolates that were not assayed for sensitivity to chloroquine carry 86^{Tyr} polymorphism. Two of these 58 isolates carry both MT and WT codons. Interestingly, none of the 15 isolates examined show codon 1246 polymorphism ($^{Asp}1246^{Tyr}$), a point mutation which to date is almost entirely associated with South American isolates (Foote *et al*. 1990; Adagu *et al*. 1997; Povoa *et al*. 1997). Although the number of isolates examined for codon 1246 polymorphism is relatively small, studies on 41 Nigerian isolates (Adagu *et al*. 1995*a*, *b*, 1997) did not detect this mutation. Indeed as revealed by our results, this polymorphism may also be scarce in Central Africa, at least in Gabon.

There is growing evidence associating *Pfmdr1* $^{Asn}86^{Tyr}$ polymorphism with the chloroquine-resistant phenotype particularly in West African *P. falciparum* isolates. This study has once more demonstrated an agreement between this polymorphism and the chloroquine-resistant phenotype of *P. falciparum* isolates from Lambarene, Gabon. Chloroquine resistance appears to be multigenic in nature (see Wellems *et al*. 1990, 1991); it is therefore not surprising that 3 of our 4 chloroquine-sensitive isolates carry 86^{Tyr} polymorphism while the remaining 1 carries both the MT and WT codons. The likely explanation will be that the yet unknown genetic factor(s) which perhaps could be the changes in the 400 kb region of chromosome 7 may be required in addition to 86^{Tyr} polymorphism for parasites to show the chloroquine-resistant phenotype. It appears therefore that our chloroquine-sensitive isolates, at the time of isolation, probably lacked this unknown factor(s).

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