Pôle 2

Comparison of *in vivo* and *in vitro* tests of resistance in patients treated with chloroquine in Yaoundé, Cameroon

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The usefulness of an isotopic *in vitro* assay in the field was evaluated by comparing its results with the therapeutic response determined by the simplified WHO *in vivo* test in symptomatic Cameroonian patients treated with chloroquine. Of the 117 enrolled patients, 102 (87%) completed the 14-day follow-up, and 95 isolates obtained from these patients (46 children, 49 adults) yielded an interpretable *in vitro* test. A total of 57 of 95 patients (60%; 28 children and 29 adults) had an adequate clinical response with negative smears (n = 46) or with an asymptomatic parasitaemia (n = 11) on day 7 and/or day 14. The geometric mean 50% inhibitory concentration of the isolates obtained from these patients was 63.3 nmol/l. Late and early treatment failure was observed in 29 (30.5%) and 9 (9.5%) patients, respectively. The geometric mean 50% inhibitory concentrations of the corresponding isolates were 173 nmol/l and 302 nmol/l. Among the patients responding with late and early treatment failure, five isolates and one isolate, respectively, yielded a discordant result (*in vivo* resistance and *in vitro* sensitivity). The sensitivity, specificity, and predictive value of the *in vitro* test to detect chloroquine-sensitive cases was 67%, 84% and 86%, respectively. There was moderate concordance between the *in vitro* and *in vivo* tests (kappa value = 0.48). The *in vitro* assay agrees relatively well with the therapeutic response and excludes several host factors that influence the results of the *in vivo* test. However, in view of some discordant results, the *in vitro* test cannot substitute for *in vivo* data on therapeutic efficacy. The only reliable definition of "resistance" in malaria parasites is based on clinical and parasitological response in symptomatic patients, and the *in vivo* test provides the standard method to determine drug sensitivity or resistance as well as to guide national drug policies.

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Introduction

Chloroquine-resistant *Plasmodium falciparum* has been reported in all sub-Saharan African countries (*1*). The extent of such resistance is still limited in many areas of Africa, and most reported cases of resistance are at the RI level (*2–4*). These findings, together with economic considerations and a generally high level of acquired immunity, justify the recommendation to use chloroquine for the first-line treatment of acute uncomplicated falciparum malaria infections in indigenous patients in most sub-Saharan Africa where acceptable clinical cure rates can be obtained (*5*). However, the possibility of an increasingly ineffective chloroquine therapy, as confirmed in other endemic areas, necessitates regular assessment of the therapeutic efficacy of chloroquine to guide drug policies in Africa.

There are two general methods to assess drug efficacy in the field: *in vivo* and *in vitro* tests (*6*). In the past, both WHO standard tests were largely applied in the field but accumulated experience has shown that neither of these tests has been adopted widely for making decisions on drug policies (*7*). Clinical evaluation of therapeutic efficacy is based on the determination of the proportion of treatment failure in a patient population at a particular study site. The aim of *in vivo* tests is to detect drug resistance, defined as "the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug in doses equal to or higher that those usually recommended but within the limits of tolerance of the subject" (*8*). The standard *in vivo* tests described in 1973 allowed enrolment of either symptomatic patients or asymptomatic parasite carriers and required either a daily 7-day ("7-day test") or a 28-day follow-up (daily for the first 7 days, weekly thereafter; "extended test") in a malaria-free zone (*8*). Such a test is hardly practical in Africa, especially from the social and economic viewpoint if a 1-month hospitalization period is required. In addition, drug efficacy determined for symptomatic patients cannot be extrapolated from studies conducted on asymptomatic parasite carriers. A considerable improvement was introduced recently with the development of a more practical and simplified 14-day *in vivo* test that is performed on symptomatic patients with acute uncomplicated falciparum malaria (*7*). The simplified *in vivo* test is performed by a regular measurement of body temperature and

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microscopic examination of blood films. The standard or simplified *in vivo* test is the reference method to detect drug resistance.

In vitro assays are based on culturing *P. falciparum* isolates in the presence of a range of concentrations of an antimalarial drug for one life cycle or part of a life cycle. Drug efficacy is assessed by counting the number of parasites developing into schizonts (WHO *in vitro* test) or by measuring the quantity of radiolabelled hypoxanthine, a DNA precursor, incorporated into the parasites (isotopic microtest) (*6, 9–13*). Chloroquine resistance is deduced when the parasite growth is not inhibited below a threshold concentration.

It has been argued that the results of *in vitro* tests of resistance do not always coincide with those of *in vivo* tests and may thus be irrelevant for clinical studies (*7*). Part of the problem is associated with the type of *in vitro* assay as well as insufficient data on *in vitro* assays performed in parallel with *in vivo* tests in individual patients. A comparison of *in vivo* and *in vitro* tests of resistance has not been extensively investigated for chloroquine because this drug had lost its efficacy in many endemic areas before *in vitro* culture techniques were developed and its use is no longer recommended to treat *P. falciparum* infections in these areas.

In the present retrospective analysis, we compared the results of isotopic *in vitro* assays and clinical response (measured using the simplified WHO *in vivo* test) of Cameroonian patients treated with a standard regimen of chloroquine. The aim was to evaluate whether *in vitro* assays from a given patient actually measure *in vivo* chloroquine sensitivity or resistance and to assess to what extent *in vitro* assays may be complementary to the *in vivo* test.

Patients and methods

Patients

A total of 117 Cameroonian adults and children aged >5 years (range: 5–50 years) residing in Yaoundé were recruited at the Nlongkak Catholic missionary dispensary, Yaoundé, Cameroon, between 1994 and 1996, with their free and informed consent. The following inclusion criteria were used: signs and symptoms of acute uncomplicated falciparum malaria (fever >37.5 °C on enrolment or a history of fever within the previous 24 h), monoinfection with *P. falciparum*, initial parasitaemia >5000 asexual parasites per µl blood, and negative Saker–Solomons urine test for antimalarial drugs (*14*). Pregnant women, patients with signs and symptoms of severe and complicated malaria, as defined by WHO (*15*), and patients with severe anaemia (haemoglobin <6 g/dl) were excluded. The study was approved by the Cameroonian National Ethics Committee.

In vivo test

Chloroquine (total dose, 25 mg/kg body weight in three divided doses: 10 mg/kg on days 0 and 1; 5 mg/kg on day 2) was administered under supervision. The patients were followed on an outpatient basis on days 1, 2, 3, 4, 7 and 14. The clinical condition, body temperature, and parasite density were assessed at each visit. Parasite density was determined by counting the number of infected red blood cells against 20 000 red blood cells in Giemsa-stained thin blood films (on day 0) or the number of asexual parasites against 1000 white blood cells in Giemsa-stained thick blood films (from day 1 onwards) and expressed as the number of asexual parasites per µl of blood.

In the revised 1996 WHO classification of *in vivo* response to antimalarial treatment, patients are followed on days 3, 7 and 14, and both clinical and parasitological responses are monitored (*7*). The clinical and parasitological responses are classified as early treatment failure (ETF), late treatment failure (LTF), or adequate clinical response (ACR). ETF is defined by one of the following four criteria: positive smear and signs and symptoms of severe malaria on days 1, 2 or 3; positive smear (parasite density > day 0 density) and fever on day 2; positive smear and fever on day 3; and positive smear on day 3 (parasite density ≥ 25% of pre-treatment density). LTF is defined as positive smear and signs and symptoms of severe malaria between day 4 and day 14; or positive smear and fever between day 4 and day 14. ACR refers to patients who have completed the 14-day follow-up and have a negative smear on day 14, with or without fever; or positive or negative smear and apyrexia during the follow-up, without previously meeting the criteria of ETF or LTF. Patients who responded with either ETF or LTF were treated with oral halofantrine (total dose = 1500 mg for adults or 24 mg base/kg body weight for children in three divided doses at 6-hour intervals) or oral quinine (25 mg base per kg body weight per day for 5 days).

The parasitological response was also interpreted using the classical S–RI–RII–RIII classification system (*8*). The interpretation of the parasitological response using the WHO standard tests requires daily monitoring of the parasite density between day 0 and day 7 for the "7-day test", followed by a weekly blood film examination until day 28 for the "extended test". Since the 1996 simplified *in vivo* test does not require daily monitoring of blood films and is terminated at day 14, some parasitological responses cannot be clearly distinguished. For this reason, the definitions of parasitological responses were modified as described below.

- Sensitive (S)/late resistant grade I (RI) response. Asexual parasite clearance on or before day 6 and negative smears on day 7 and day 14. As in the "7-day test", an S response and a late RI response cannot be distinguished since the difference between the two responses depends on the presence (late RI) or absence (S) of recrudescence between day 15 and day 28.
- Early resistant grade I (RI) response. Asexual parasite clearance on or before day 6, followed by recrudescence between day 7 and day 14, inclusive.

- Early RI/resistant grade II (RII) response. Marked reduction of asexual parasitaemia (<25% of the pre-treatment parasite density) within 48 h of initiation of treatment, and persistent asexual parasitaemia until day 7 (and on day 14 if left untreated between day 7 and day 13). Some cases that do not fulfil the criteria of "early RI response" or "RII" are classified under "early RI/RII response" because asexual parasite clearance was not observed between days 4 and 6 due to the absence of follow-up smears. Furthermore, seven cases of "early treatment failure" that were unclassifiable because of the absence of follow-up blood smears beyond day 3 (and had <25% of the pre-treatment parasitaemia on day 2) after an alternative treatment were added to the early RI/RII group.
- Resistant grade III (RIII). Slight reduction (>25% of the pre-treatment parasite density), no change, or increase of asexual parasitaemia during the first 48 h of treatment, and no subsequent clearance of parasitaemia (until day 7). Patients whose parasite density was >25% of the pre-treatment density on day 2 and who required alternative treatment due to deteriorating clinical conditions before day 7 were also classified as RIII response (*16*).

In vitro assay

Samples of venous blood (5–10 ml) were obtained before treatment for *in vitro* assay. Clinical isolates were tested for *in vitro* sensitivity on day 0 without prior culture adaptation. The procedures for the isotopic *in vitro* assay have been described previously (*17*). Briefly, blood samples were washed three times in the culture medium RPMI 1640. The infected red blood cells (1.5% haematocrit, 0.1–1.0% parasitaemia) were suspended in RPMI 1640 supplemented with 10% human serum and buffered with 25 mmol/l sodium bicarbonate solution and 25 mmol/l HEPES. The mixture was then distributed (700 µl per well) in 24-well test plates that had been pre-coated with chloroquine (final concentration range: 12.5–1600 nmol/l in triplicate), except for three drug-free control wells. Culture plates were incubated for a total of 42 h at 37 °C in 5% CO_2. Tritium-labelled hypoxanthine (40 µCi/ml) was added 18 h after the initial incubation period. At the end of the incubation period, the plates were frozen at –20 °C and thawed to lyse the cells. After collection on glass-fibre filter-paper using a cell harvester, incorporation of [^{3}H]hypoxanthine was quantified using a liquid scintillation counter. The results of the *in vitro* assay were expressed as 50% inhibitory concentration (IC_{50}), defined as the concentration at which 50% of the incorporation of [^{3}H]hypoxanthine was inhibited, as compared with the result for the drug-free control wells. Based on our previous *in vitro* studies, *in vitro* chloroquine resistance was defined as IC_{50} > 100 nmol/l (*17*). Some of the *in vitro* and *in vivo* results have been described previously (*17–19*).

Table 1. **Clinical and parasitological outcome of the simplified *in vivo* test in Cameroonian patients treated with 25 mg/kg body weight of chloroquine**

	No. of patients	
Response[a]	Children (*n* = 46)	Adults (*n* = 49)
Adequate clinical response		
S/ late RI	23	23
Early RI	5	6
Early RI/RII	0	0
RIII	0	0
Late treatment failure		
S/late RI	0	0
Early RI	4	5
Early RI/RII	8	10
RIII	1	1
Early treatment failure		
S/ late RI	0	0
Early RI	0	0
Early RI/RII	3	4
RIII	2	0

[a] Therapeutic response (adequate clinical response, late or early treatment failure) was defined according to the 1996 revised WHO classification (*7*). The grading system of parasitological responses (S/late RI, early RI, early RI/RII and RIII) was adapted and modified from the 1973 WHO classification (*8*). See text for the definitions.

Statistical analysis

The clinical, haematological and biochemical parameters were compared between patients with an adequate clinical response and patients with either early or late treatment failure, using the Mann–Whitney U test. The level of significance (*P*) was fixed at 0.05. Since the *in vivo* test is the reference method for determining chloroquine resistance, the validity of the *in vitro* assay was gauged against the therapeutic response by calculating the sensitivity, specificity and predictive value. The therapeutic response of each patient was compared separately with the IC_{50} values for chloroquine obtained from the corresponding *P. falciparum* isolate. The results of the *in vivo* and *in vitro* tests of resistance were also compared using kappa statistics to calculate the index of agreement (*20, 21*). A kappa value represents the degree of agreement between two methods (or observers) beyond chance, 1 denoting perfect agreement. Kappa coefficients in the range 0–1 are arbitrarily interpreted as follows: 0–0.20, slight agreement; 0.21–0.40, fair agreement; 0.41–0.60, moderate agreement; 0.61–0.80, good agreement; and >0.81, very good agreement.

Results

A total of 117 patients (65 adults and 52 children) were enrolled in the study. Of these patients, 102 (87%; 55 adults and 47 children) completed the 14-day follow-up. Of the 15 patients lost to follow-up, three did not complete the 3-day chloroquine treatment (lost to follow-up before day 2), while two, five, and three patients were lost to follow-up on day 3, day 7 and day 14, respectively. Two patients were excluded because of concomitant self-medication with oral quinine. Clinical isolates obtained from 107 of 117 patients (91%) were successfully cultured to determine the IC_{50} value for chloroquine. Of the 15 isolates obtained from patients lost to follow-up, four were chloroquine-sensitive *in vitro*, eight were chloroquine-resistant *in vitro*, and three results were not interpretable. Seven isolates from patients who completed the 14-day follow-up (5 ACR and 2 ETF) did not yield interpretable *in vitro* results. Patients who did not complete the 14-day follow-up (n = 15) and/or whose clinical isolates failed to grow or were lost due to bacterial contamination (n = 10) were excluded from further analysis.

Of the 95 patients who completed the 14-day follow-up and whose isolates were successfully cultured for *in vitro* drug assay, 46 were children under the age of 15 years and 49 were adults (Table 1). Of these patients, 57 (60%; 28 children, 29 adults) had an ACR (Table 1); 46 of these were apyretic on or before day 3 and remained afebrile until day 14 and had either a negative thick blood smear or a positive smear (density < 25% of day 0 density) on day 3 and negative smears on days 7 and 14 (S/late RI); 8 had a negative smear on day 3 and a positive smear on days 7 (n = 2) and/or 14 but remained afebrile between days 3 and 14 (early RI); and 3 had a positive smear on day 3 (density <25% of day 0 density), a negative smear on day 7, and a positive smear on day 14 but were afebrile between days 3 and 14 (early RI). Thus, there were a total of 11 patients in the ACR group with an asymptomatic recrudescent parasitaemia on day 7 and/or day 14 (early RI).

Treatment failure with chloroquine was observed in a total of 38 patients (40%). Late treatment failure was exhibited by 29 (30.5%) patients and several subgroups can be distinguished. A total of 20 of these did not clear their parasitaemia during the 14-day follow-up and presented fever between day 7 and day 14 (early RI/RII or RIII); 6 patients cleared their parasitaemia on day 2 and/or day 3 but returned between day 7 and day 14 with fever and positive smear (early RI); and 3 patients had positive smears until day 3, a negative smear on day 7, and a positive smear with fever on day 14 (early RI). Early treatment failure was observed for 9 patients (9.5%); fever and a positive smear on day 3 were presented by 6 of these patients; and clinical deterioration in the presence of parasitaemia was observed in 3 of them on day 2 or day 3. The clinical and laboratory parameters of patients are summarized in Table 2. Although the initial parasitaemia tended to be slightly higher in patients who failed to respond to chloroquine (76 400 vs. 90 800 asexual parasites per µl blood; $P > 0.05$), both groups of patients had similar clinical parameters and haematological and biochemical values ($P > 0.05$) before chloroquine treatment.

The *in vitro* geometric mean IC_{50} for chloroquine for isolates obtained from 57 patients with an ACR was 63.3 nmol/l (range, 8.9–486 nmol/l) (Fig. 1). A total of 38 of these isolates (35 from S/late RI cases and 3 from early RI cases) were sensitive *in vitro* to chloroquine, while 19 of them (11 from S/late RI cases and 8 from early RI cases) were resistant *in vitro* to chloroquine. Among the isolates obtained from patients with LTF, the geometric mean IC_{50} was 173 nmol/l (range, 23–690 nmol/l; n = 29). A total of 5 of 29 isolates (3 from early RI and 2 from early RI/RII) were chloroquine-sensitive *in vitro*, while 24 of 29 isolates (6 early RI, 16 early RI/RII, and 2 RIII) were chloroquine-resistant *in vitro*. Among the 9 isolates originating from patients with ETF, the geometric mean IC_{50} was 302 nmol/l (range, 53–641 nmol/l). Only one isolate (early RI/RII) was sensitive *in vitro* to chloroquine in this group.

Table 2. **Pre-treatment clinical and laboratory parameters of patients who cleared or failed to clear malaria infections with chloroquine therapy**

Parameter[a]	Therapeutic response[b]	
	ACR	Treatment failure
No. of patients	57	38
No. of children (aged 5–15 years)	28	18
Mean age (years)	17.7 ± 9.8 (5–47)[c]	19.1 ± 12.0 (5–50)
Mean weight (kg)	50.7 ± 21.8 (20–99)	48.2 ± 18.2 (20–88)
Sex ratio (male: female)	28:29	17:21
Symptoms before treatment (mean days)	5.7 ± 6.2 (1–30)	4.6 ± 4.9 (1–30)
Geometric mean parasitaemia (asexual parasites/µl)	76 400 (9 420–508 700)	90 800 (19 920–579 400)
Mean temperature (°C)	37.9 ± 1.3	38.2 ± 1.3
Mean haemoglobin level (g/dl)	11.4 ± 2.6 (6.9–17)	11.3 ± 2.2 (6.3–14.7)
Mean white blood cell count (x 10^6/l)	5 640 ± 1 820 (2 900–11 000)	5 800 ± 1 890 (3 300–11 700)
Mean platelet count (x10^9/l)	145 ± 68 (22–295)	152 ± 83 (34–362)
Mean serum ASAT (IU/l)	31.0 ± 27.3 (10–180)	35.3 ± 33.6 (8–150)
Mean serum ALAT (IU/l)	19.7 ± 20.1 (2–84)	19.9 ± 22.4 (4–84)
Mean creatinine (µmol/l)	68.2 ± 25.0 (30–138)	69.0 ± 29.0 (20–143)

[a] The mean values of ACR and treatment failure groups do not differ significantly ($P > 0.05$; Mann–Whitney U test). ASAT = aspartate aminotransferase; ALAT = alanine aminotransferase.

[b] Therapeutic responses as defined in the 1996 revised WHO classification (*7*). ACR = adequate clinical response; "treatment failure" group includes both late treatment failure (LTF) and early treatment failure (ETF).

[c] Figures in parentheses are the range.

The validity of the *in vitro* results, determined using the threshold IC_{50} value for chloroquine resistance of 100 nmol/l, as compared with the therapeutic response, is shown in Table 3. The sensitivity, specificity, and predictive or diagnostic value of the *in vitro* test for distinguishing between chloroquine-sensitive and chloroquine-resistant cases were 67%, 84% and 86%, respectively. The *in vitro* and *in vivo* results were further compared using the kappa statistics; the kappa coefficient between the two tests of resistance was 0.48 (moderate agreement).

Fig. 1. **Distribution of IC_{50} for chloroquine, determined by the isotopic *in vitro* assay, compared with the therapeutic response to chloroquine among the study patients.** (Clinical response was graded as adequate clinical response (ACR), late treatment failure (LTF), and early treatment failure (ETF), according to the 1996 WHO classification scheme (*7*)).

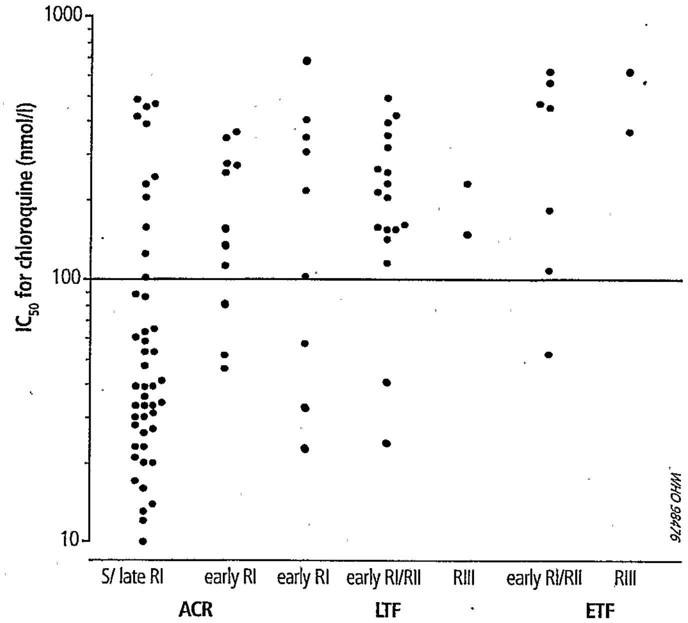

Table 3. **Validity of isotopic *in vitro* test of chloroquine resistance to detect *in vivo* drug efficacy based on therapeutic response**

	Therapeutic response[b]		
		Treatment failure	
In vitro test result[a]	ACR	LTF/ETF	Total
IC_{50} < 100 nmol/l	38	6(5/1)[c]	44
IC_{50} ≥100 nmol/l	19	32 (24/8)	51
Total	57	38 (29/9)	95

[a] *In vitro* chloroquine sensitivity was defined as IC_{50} < 100 nmol/l and *in vitro* chloroquine resistance as IC_{50} ≥100 nmol/l. Based on the therapeutic response, sensitivity of the *in vitro* assay = 67%, specificity = 84% and predictive value = 86%.

[b] Therapeutic response as defined in the 1996 revised WHO classification (*7*). ACR = adequate clinical response; LTF = late treatment failure; ETF = early treatment failure. Patients responding with either late or early treatment failure were grouped together for statistical analysis.

[c] Figures in parentheses are number of subjects with LTF/ETF.

Discussion

Several previous studies have been conducted to assess the correspondence between *in vivo* and *in vitro* responses to antifolate drugs (*22–24*). The results of these studies are not comparable because of differences in the *in vitro* techniques used and in the interpretation of results and are not significant due to the small sample size of field isolates. In addition, antifolate drugs are administered in combination *in vivo*, and the *in vitro* activity of the two drugs in fixed concentrations may not accurately reflect the *in vivo* conditions. Most other previous studies have performed *in vivo* and *in vitro* evaluation of drug efficacy separately (*25–27*). The real implications of these studies, especially those that were based exclusively on *in vitro* assays or *in vivo* tests on asymptomatic patients, are not clear clinically or epidemiologically.

The present study is the first to compare simultaneously the *in vitro* and *in vivo* responses to chloroquine of a large number of individual *P. falciparum* field isolates that were obtained from symptomatic patients. Comparison of pre-treatment clinical and laboratory parameters between patients with ACR and those responding with treatment failure showed that there were no significant risk factors for therapeutic failure. Compared with the simplified *in vivo* test for chloroquine resistance, the predictive value of the isotopic *in vitro* assay suggests that 86% of the patients for whom the *in vitro* test indicated the presence of chloroquine-sensitive isolates actually responded adequately to chloroquine therapy. Thus, *in vitro* assay seems to reflect relatively well the *in vivo* response of chloroquine-treated patients evaluated by clinical and parasitological examination.

All patients with ACR cleared their fever on or before day 3, with no recurrent fever until day 14, and either negative or positive (<25% of initial parasite density) smears on day 3. Among patients with an ACR, three subgroups can be distinguished on the basis of parasitological responses on day 7 and/or day 14: type 1 or S/late RI (n = 46) refers to those patients with negative smears on day 7 and day 14; type 2 or early RI (n = 8) refers to patients with a negative smear on day 3 but with an asymptomatic parasitaemia on days 7 and/or 14; and type 3 or early RI (n = 3) refers to patients with a positive smear on day 3, negative smear on day 7, and an asymptomatic parasitaemia on day 14. The importance of this subclassification of the ACR group lies in the improvement of the sensitivity of the *in vitro* test to determine the presence of chloroquine-sensitive isolates in aparasitaemic ACR patients. Thus, if asymptomatic, persistent or recurrent parasitaemia on day 7 and/or day 14 is considered to be a criterion of treatment failure (instead of ACR, according to the 1996 WHO classification), the *in vitro* test would have a higher sensitivity (67% vs. 76%) and slightly lower specificity (84% vs. 82%) and predictive value (86% vs. 80%) relative to the *in* *vivo* test. The kappa coefficient between the two tests would also increase from 0.48 to 0.58. All three type 3 (early RI) ACR patients had chloroquine-resistant isolates, as determined by the *in vitro* assay (chloroquine IC_{50} > 100 nmol/l; 137, 272, and 348 nmol/l). Five type 2 (early RI) ACR patients were infected with chloroquine-resistant isolates (IC_{50} = 114, 158, 258, 278, and 368 nmol/l), while three type 2 patients harboured chloroquine-sensitive isolates (IC_{50} = 46, 51 and 81 nmol/l). Of the 46 type 1 (S/late RI) ACR-patients, 35 chloroquine-sensitive isolates and 11 chloroquine-resistant isolates were obtained.

If we suppose that types 2 and 3 (early RI) ACR responses should be chloroquine-resistant, discordant results between the therapeutic response and *in vitro* response are observed in three type 2 ACR-patients and 11 type 1 ACR-patients. Of the type 2 patients, two were children and one was adult. One child had a chloroquine-sensitive isolate with diminished *in vitro* sensitivity (81 nmol/l). In the other patients, there seem to be no obvious reasons for the failure to clear parasitaemia, other than possible individual variations in the chloroquine pharmacokinetics, unreported vomiting, and, most importantly, reappearance of asexual parasites due to reinfection. Type 1 patients were clinically and parasitologically cured on day 14. A total of 35 patients with chloroquine-sensitive isolates were cured, as expected, while 11 patients were cured despite the presence of chloroquine-resistant parasites. The discordance in type 1 ACR-patients may also be related to age. Of the 11 patients with discordant results (range, 125–486 nmol/l), one was aged 6 years, four were aged 11–14 years, and six were adults. Although we have no biological proof, we hypothesize that discordance may be related to a high level of acquired immunity that enhances the clearance of parasites, independently of drug sensitivity.

In contrast, with the ACR group, which consisted of several subgroups, fewer discordant cases between the therapeutic response and *in vitro* response were observed in the treatment failure groups. A total of 24 LTF patients had chloroquine-resistant isolates, while five discordant cases of LTF patients (3 children and 2 adults; 3 early RI and 2 early RI/RII) were infected with chloroquine-sensitive isolates. The situation for three of five LTF patients may be analogous to that of three type 2 (early RI) ACR patients who responded with asymptomatic parasitaemia on day 7 and/or day 14, despite the presence of chloroquine-sensitive isolates. Two of five discordant LTF patients (a 5-year-old and a 7-year-old) had persistent parasitaemia during the 14-day follow-up period despite the presence of chloroquine-sensitive isolates.

Patients responding with ETF were not easily classifiable on the basis of the definitions of RI, RII and RIII applied to the standard "7-day test" or the "28-day extended test". Two types of parasitological responses were observed in patients with ETF. The first subset of patients (two children) had a modest diminution of parasitaemia on day 2 (>25% of the pre-treatment parasitaemia) which was classified as an RIII response according to the criteria set by Rieckmann (*16*), even though follow-up until day 7 was not possible due to a requirement for an alternative treatment on day 3 or day 4. Retrospectively, this classification appeared justified on the basis of high levels of IC_{50} values for chloroquine obtained from the isolates from these patients (373 nmol/l and 641 nmol/l). The second subset of patients (3 children and 4 adults) in the ETF group were unclassifiable in the S–RI–RII–RIII system because an alternative treatment was administered on day 2 or day 3 and their parasitological response did not fulfil the criteria for RIII. The response of these seven patients was considered to be late RI/RII based on two observations. First, compared with the pre-treatment levels the mean parasitaemias on day 2 were 1.6%, 2.4% (excluding one patient with 180%), 3.7%, 7.3% and 56% in the S/late RI, early RI, early RI/RII, 7 unclassifiable patients, and RIII groups, respectively. Second, analysis of the distribution of the day 2 parasitaemia compared with the initial levels showed that the 50th percentiles were, in the same order, 0.4%, 1.3%, 1.7%, 3.4% and 51%. Thus, the parasitological response of the seven unclassifiable patients on day 2 was closest to that of those who exhibited an early RI/RII response.

Six of seven isolates from ETF patients displayed high levels of IC_{50} for chloroquine (110, 187, 461, 477, 575 and 635 nmol/l). Only one case of *in vivo/in vitro* discordance was observed in the ETF group. This patient was a 5-year-old child presenting with a low-grade fever (37.8 °C) and a positive smear on day 3 (131 760 asexual parasites per µl on day 0 and 9 asexual parasites per µl on day 3). This particular patient may have been treated too hastily with an alternative drug on day 3 and probably should have been re-examined on day 4 without an alternative treatment on day 3. These cases of ETF illustrate the importance and responsibility of the attending clinicians to administer an alternative treatment as soon as early clinical deterioration or fever associated with a positive smear is observed on or before day 3. Although only a small number of ETF cases were observed in our study, the subjective criterion of ETF (aggravated clinical condition) does seem to be supported by parasitological criteria in the majority of cases and potentially represents most severe cases of drug failure.

In vivo testing is an accurate and valid measure of therapeutic efficacy and is the most reliable means for detecting drug resistance. Compared with *in vitro* assays, the *in vivo* test of resistance may be conducted in remote areas by qualified personnel with minimal training. It also permits working directly with malaria-infected patients, deriving clinical data, monitoring clinical response over a short time frame, and modifying treatment in case of a therapeutic failure. However, the test is not entirely free of potential problems of bias and precision. When therapeutic failure is observed using an *in vivo* test, establishment of a causal relationship between treatment failure and *in vivo* drug resistance requires further investigations since various parasite and host factors contribute to a therapeutic failure. For example, factors related to the characteristics and dynamics of parasite transmission include the presence of intraerythrocytic parasites with a drug-resistant phenotype on day 0 of the treatment, late emergence of secondary or tertiary "broods" of parasites from the liver after a subtherapeutic level of the drug is attained in the host, and reinfection of the host with new populations of parasites after drug treatment. Host factors that play an important role in therapeutic failure include variability in pharmacodynamics and pharmacokinetics and the level of acquired immunity. There may be other factors that contribute to or delay parasite and fever clearance, such as intrinsic virulence of parasite strains, host genetic factors unrelated to immunity, concomitant diseases that were undiagnosed at the time of patient enrolment, and social behaviour of the host (for example, concomitant self-medication with other classical antimalarial drugs or traditional herbal medicine). These considerations show that, unless such factors are excluded, a case of therapeutic failure cannot be attributed to *in vivo* drug resistance with certitude. These limitations may diminish the precision of the *in vivo* test and need to be taken into consideration in assessing our findings since they lead to decreased measures of validity of the *in vitro* test. Furthermore, unless some of the above factors can account for *in vivo*/*in vitro* discordant cases, the *in vitro* threshold for chloroquine resistance can not be redefined on the basis of our *in vivo* data.

In addition to the difficulties in establishing a causal relationship between therapeutic failure and *in vivo* drug resistance, the revised WHO criteria themselves may not be appropriate in all cases. One of the criteria for treatment failure (aggravation of clinical conditions requiring an alternative treatment) is based on a subjective clinical evaluation that may introduce a bias towards an increased proportion of "resistant" cases. In our experience, the criterion of persistent or recurrent fever on day 3 sometimes leads to a wrong classification of patients. When left untreated, some patients with fever and positive thick film on day 3 ("treatment failure" according to the WHO classification) cleared the parasites and became afebrile on day 4 and remained so and aparasitaemic until day 14 (ACR). These cases illustrate the limits of the *in vivo* test of resistance. Despite these potential shortcomings, the *in vivo* test ("7-day test", "28-day extended test", or simplified test) must be considered to be the only currently available, valid measure of drug resistance that may be used to guide national antimalarial drug policy.

In vitro assays may be a more objective method to detect drug resistance since *in vitro* tests eliminate several host factors that interfere with the clear interpretation of results, including reinfections, immunity, pharamacodynamics, and pharmacokinetics. *In vitro* assays are complementary to *in vivo* tests, and their results are theoretically more directly associated with drug resistance (*28*). However, most specialized laboratories that conduct *in vitro* assays as a routine procedure are located far from clinical study sites and require a high level of training and technical capability, transport of blood samples from the field and sophisticated equipment to perform isotopic assays. As a result, *in vitro* assays have been used to describe the epidemiology of drug resistance independently of clinical studies and to screen new compounds (*10, 25–29*). Although these two applications of *in vitro* assays have provided important information, the results of the present study suggest the usefulness of *in vitro* assays as a complementary diagnostic tool for drug resistance but do not suggest that the *in vitro* test can replace the *in vivo* test in the field.

Another major problem with the *in vitro* test is the selection of threshold values to classify results in terms of sensitivity or resistance. Use of the therapeutic plasma level as the threshold value is theoretically plausible but disregards the technical constraints of the *in vitro* culture method (*30*). The optimal conditions for *in vitro* culture are markedly different from those for *in vivo* conditions, and include the composition of culture medium, haematocrit (1–2.5% for *in vitro*, 35–45% for *in vivo* tests) and proportion of serum (10% for *in vitro* culture, 55–65% for *in vivo*). Thus therapeutic plasma levels may not be appropriate for *in vitro* parasite growth conditions. Comparison of different clones or laboratory-adapted strains of parasites and determination of the limiting drug concentration that produces a response in these reference strains has also been used to estimate the threshold value. Even if the drug response of the original isolate from which a clone or strain was derived is known, adaptation of parasites to *in vitro* conditions alters the original phenotype and may not reflect the characteristics of the original isolate (*31*). In addition, threshold values determined using this method may not be clear-cut for some isolates obtained in the field because of the presence of mixed populations of parasites with different phenotypes. Thus, although a clone or strain of *P. falciparum* with well-defined phenotype and genotype may be useful in laboratory experiments, various *in vivo* factors preclude direct comparison between *in vitro* and *in vivo* conditions; consequently, any threshold value for differentiating sensitive and resistant isolates may largely remain arbitrary unless large-scale trials are conducted under various epidemiological conditions to define simultaneously *in vitro* drug sensitivity pattern, genotype, pharmacokinetic parameters and immune response.

At present, chloroquine is still the rational choice for the first-line treatment of the majority of cases of acute, uncomplicated malaria in indigenous patients in Africa because it is cheap, safe, well-tolerated, widely available, and highly effective against *P. vivax*, *P. ovale*, *P. malariae*, and sensitive strains of *P. falciparum*. In some endemic areas, however, concern is increasing about *in vivo* chloroquine-resistant cases. The extent of chloroquine resistance therefore needs to be monitored to guide the rational use of antimalarial drugs in Africa. Both *in vitro* and *in vivo* tests of resistance have their limitations and in any case do not measure the same biological phenomena. Our results show that the *in vitro* test of resistance is a complementary tool that is moderately concordant with the simplified *in vivo* test. The use of *in vitro* tests should be limited to research purposes to provide baseline data on drug response and monitor cross-resistance patterns. The *in vitro* test cannot replace the *in vivo* test for therapeutic efficacy and should not play any role in guiding antimalarial drug policy. Although it may be difficult to define exactly the criteria for *in vivo* chloroquine resistance and to fulfil them, especially in the field, a standardized *in vivo* test based on all available clinical and epidemiological information is still the best available means for defining drug resistance within a given epidemiological context. ■

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Résumé

Comparaison des tests *in vivo* et *in vitro* de résistance chez des malades traités à la chloroquine à Yaoundé (Cameroun)

La résistance de *Plasmodium falciparum* à la chloroquine a été décrite dans tous les pays de l'Afrique subsaharienne. Néanmoins, la chloroquine reste le médicament de première intention pour le traitement de l'accès palustre simple dans la plupart des pays africains. L'extension de la résistance à la chloroquine nécessite une surveillance permanente soit par des tests *in vivo*, soit par des tests *in vitro*. Afin de rechercher une concordance entre ces deux types de test, nous avons comparé les résultats du nouveau test d'efficacité thérapeutique introduit par l'OMS en 1996 à ceux du semi-microtest isotopique. Ce nouveau test *in vivo* est basé sur l'évolution, après un traitement standard par la chloroquine à 25 mg/kg sur 3 jours, de l'état clinique et de la parasitémie chez des malades atteints d'un accès palustre simple à *P. falciparum*. Les résultats sont exprimés en réponse clinique adéquate ou en échec thérapeutique précoce ou tardif en fonction de la disparition, de l'aggravation, de la persistance ou de la réapparition des signes cliniques, en particulier la fièvre, et en fonction de l'évolution de la parasitémie. Le semi-microtest consiste à étudier la croissance *in vitro* des parasites en présence de concentrations croissantes de chloroquine (25 à 1600 nmol/l). La croissance est mesurée par l'incorporation d'hypoxanthine tritiée. Les résultats sont exprimés en concentration inhibitrice 50% (CI_{50}) correspondant à la concentration inhibant la croissance de 50% des parasites par rapport à un témoin. Le seuil de résistance pour la chloroquine est fixé à 100 nmol/l. Parmi les 117 malades inclus, 102 (87%) ont été suivis pendant 14 jours, et 95 tests in vitro réalisés avec les isolats des malades (46 enfants et 49 adultes) ont pu être interprétés. Cinquante-sept (60%; 28 enfants et 29 adultes) malades ont présenté une réponse clinique adéquate après le traitement à la chloroquine avec une goutte épaisse négative à J14 (n = 46) ou avec une parasitémie asymptomatique à J7 et/ou à J14 (n = 11). La moyenne géométrique des CI_{50} des isolats correspondants était de 63,3 nmol/l. Les échecs thérapeutiques tardifs et précoces ont été observés chez 29 (30,5%) et 9 (9,5%) patients, respectivement. La moyenne géométrique des CI_{50} des isolats correspondants était de 173 nmol/l pour les échecs thérapeutiques tardifs et de 302 nmol/l pour les échecs thérapeutiques précoces. En considérant le test *in vivo* comme test de référence, la sensibilité, la spécificité et la valeur prédictive positive du test *in vitro* étaient de 67%, 84% et 86%, respectivement. Le coefficient de kappa mesurant la concordance entre les deux tests était de 0,48, indiquant un degré de concordance modéré. Contrairement au test *in vitro*, le test *in vivo* est plus le reflet de l'efficacité thérapeutique de la chloroquine qu'un test mesurant la résistance des parasites. En effet, l'efficacité thérapeutique est fonction de nombreux facteurs liés à l'hôte et aux parasites. Ces différents facteurs peuvent expliquer les 20% de discordance observés entre les tests *in vivo* et *in vitro*. Néanmoins, nos résultats montrent que le test *in vitro* et le test *in vivo* sont des méthodes d'analyse de la résistance concordantes et complémentaires, mais seul le test *in vivo* pratiqué chez des malades permet de juger de l'efficacité d'un médicament antipaludique et d'orienter les traitements antipaludiques dans le cadre de la politique nationale de lutte.

Resumen

Comparación de pruebas de resistencia *in vivo* e *in vitro* en pacientes tratados con cloroquina en Yaundé (Camerún)

La resistencia de *Plasmodium falciparum* a la cloroquina se ha observado en todos los países del África subsahariana. Sin embargo, la cloroquina sigue siendo el medicamento más socorrido para el tratamiento de la infección palúdica no complicada en la mayor parte de los países africanos. Se requiere una vigilancia per-

manente de la magnitud de la resistencia a la cloroquina por medio de pruebas *in vivo* o *in vitro*. Con objeto de establecer una concordancia entre estos dos tipos de análisis, hemos comparado los resultados del nuevo método de valoración de la eficacia terapéutica adoptada por la OMS en 1996 con el semimicroanálisis isotópico. Esta nueva prueba *in vivo* se basa en la evolución del estado clínico y la parasitemia de los enfermos afectados por un acceso de paludismo simple causado por *P. falciparum*, al cabo de un tratamiento estándar con cloroquina a razón de 25 mg/kg durante tres días. Los resultados se expresan como respuesta clínica adecuada o fracaso terapéutico precoz o tardío en función de la desaparición, la agravación, la persistencia o la reaparición de las manifestaciones clínicas, en particular la fiebre, y en función de la evolución de la parasitemia. El análisis consiste en estudiar el crecimiento *in vitro* de los parásitos en presencia de concentraciones crecientes de cloroquina (25 a 1600 nmol/l). El crecimiento se mide por la incorporación de hipoxantina tritiada. Los resultados se expresan en concentración inhibitoria 50% (CI_{50}), correspondiente a la concentración que inhibe el crecimiento del 50% de los parásitos en relación con un testigo. El umbral de resistencia respecto de la cloroquina está fijado en 100 nmol/l. De los 117 enfermos participantes, 102 (87%) fueron objeto de seguimiento durante 14 días y se pudieron interpretar 95 pruebas *in vitro* realizadas con los aislados de los enfermos (46 niños y 49 adultos). Cincuenta y siete enfermos (60%; 28 niños y 29 adultos) presentaron una respuesta clínica adecuada después del tratamiento con cloroquina, con gota gruesa negativa el día 14 (n = 46) o con una parasitemia asintomática los días 7 y/o 14 (n = 11). La media geométrica de las CI_{50} de los aislados correspondientes fue de 63,3 nmol/l. Se observaron fracasos terapéuticos tardíos y precoces en 29 (30,5%) y 9 (9,5%) pacientes, respectivamente. La media geométrica de los CI_{50} de los aislados correspondientes fue de 173 nmol/l para los fracasos terapéuticos tardíos y de 302 nmol/l para los precoces. Considerando la prueba *in vivo* como valoración de referencia, la sensibilidad, la especificidad y el valor predictivo positivo de la prueba *in vitro* fueron del 67%, el 84% y el 86%, respectivamente. El coeficiente kappa, que mide la concordancia entre las dos pruebas, fue de 0,48, lo que indica un grado de concordancia moderado. Contrariamente a la prueba *in vitro*, la prueba *in vivo* es más el reflejo de la eficacia terapéutica de la cloroquina que un análisis que permita medir la resistencia de los parásitos. En efecto, la eficacia terapéutica depende de muchos factores vinculados al huésped y a los parásitos. Esos diferentes factores pueden explicar el 20% de discordancia observado entre las pruebas *in vivo* e *in vitro*. No obstante, nuestros resultados muestran que la prueba *in vitro* y la prueba *in vivo* son métodos de análisis de la resistencia concordantes y complementarios, aunque sólo la prueba *in vivo* realizada en enfermos permite juzgar la eficacia de un medicamento antipalúdico y orientar los tratamientos en el marco de la política nacional antipalúdica.

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