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Plasmodium falciparum chloroquine and quinine sensitivity in asymptomatic and symptomatic children in São Tomé Island

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Summary *Plasmodium falciparum* sensitivity to quinine in São Tomé was determined by *in vivo* and *in vitro* tests in 56 children with mild or cerebral malaria. Chloroquine sensitivity was assessed by *in vitro* tests in 105 parasitaemic asymptomatic children from the same community as the cases. The WHO standard methodology was used. No resistance to quinine was found by *in vivo* or *in vitro* tests in either group of patients or in asymptomatic children, although some degree of chloroquine resistance was found with the *in vitro* test. This was more common in patients than in asymptomatic children. Chloroquine resistance may be explained by the recent history of malaria in São Tomé Island, which caused an important decrease of immunity among the population and consequently the emergence of resistant strains. Implications of the use of *in vivo*/*in vitro* tests for determining the antimalarial drug policy within the primary health care system are discussed.

keywords malaria, *Plasmodium falciparum*, chloroquine, quinine, sensitivity, São Tomé

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Introduction

Appropriate case management is one of the methods to reduce the malaria burden in Africa. The impact of the emergence and spread of chloroquine resistance on case management and consequently on malaria morbidity and mortality remains difficult to assess, although chloroquine resistance is not necessarily associated with a higher malaria-related mortality (Hoffman *et al.* 1984; Blandling-Bennett *et al.* 1988).

Evaluation of chloroquine resistance is normally performed by two standard methods (*in vivo* and *in vitro*) (Bruce-Chwatt *et al.* 1986; Rieckman 1990; Prasad *et al.* 1990). The *in vitro* test assesses the parasitological response while the simplified *in vivo* test assesses the clinical response to therapy during the 7 days after the start of the treatment. *In vitro* tests are usually performed in asymptomatic children by isolating circulating parasites, which may be a mixture of different isolates with different degrees of drug sensitivity (Wernsdorfer 1994). *In vitro* resistant parasites may be found to be susceptible *in vivo* because of the interaction of the host's immune system with the treatment (Koella 1993). Selection of less susceptible parasites has been associated with permanent drug exposure and with the immunological properties of malaria parasites, and there is some evidence that resistant strains are more pathogenic than susceptible ones (Thaithong *et al.* 1984; Coosemans *et al.* 1985).

We determined the efficacy of quinine (*in vivo*/*in vitro*) and compared the *in vitro* sensitivity to

chloroquine in children of the same community with asymptomatic *P. falciparum* parasitaemia with that found in children with mild or cerebral malaria admitted to the local health centre. The results of this study are reported below.

Patients and methods

Study area

This study was conducted in 5 villages and in the health centre of Guadalupe, all located in Lobata District (14 173 people living in small rural communities) (DEESTP 1991), in São Tomé (Democratic Republic of São Tomé e Príncipe), an island situated in the Gulf of Guinea, about 240 km west of Gabon. In São Tomé Island, where the climate is tropical with a well-defined rainy and dry season, malaria is mesoendemic. In 1947, São Tomé authorities initiated a chemoprophylaxis programme based on free distribution of chloroquine (Baptista 1947; Rendas *et al.* 1985). In the 1980s, effective control measures (DDT spraying and sanitation) reduced malaria transmission (Ceita 1979; 1983), and caused a significant decrease in malaria morbidity with consequent loss of anti-malaria immunity by the population. Clinical cases of malaria remained scanty throughout this period and eventually disappeared completely between 1981 and 1983. However, all control measures were stopped in 1984 and this caused a large malaria outbreak in 1985–86. Since then, *P. falciparum* transmission has become stable (Baptista, in press). Until recently, chloroquine has been a highly effective antimalarial drug in São Tomé (Bakri & Haliri 1977; Benthain & Buisson 1978; Ceita 1979). Chloroquine resistance was first reported in 1991 (Martet *et al.* 1991).

Test *in vivo*

Subjects

From November 1992 to November 1993, children aged 4–14 with mild or cerebral malaria according to the World Health Organization's criteria (Warrel *et al.* 1990) were recruited at the Guadalupe Health Centre (Lobata District). Informed consent was obtained from parents or guardians before enrolment.

Cerebral malaria was defined as coma (no directional response to painful stimulus at least 6 hours after the last convulsion, if one had occurred), presence of a detectable *P. falciparum* parasitaemia (asexual forms) and no other evident cause of illness. Mild malaria was defined as an axillary temperature >38 °C and/or high fever during the last 2 days and *P. falciparum* parasitaemia. Patients who had taken antimalarials were excluded from the study. Patients with mixed infections or those positive for the Lelijveld and Kortmann urine test for 4-aminoquinolines (Bruce-Chwatt *et al.* 1986), for sickle-cell test (Lévy-Lambert 1973), for HIV I and II (HIV I–ELISA, Wellcome; HIV II–ELISA, Quilaban) and for hepatitis B surface antigen (Hbs Ag–ELISA–Abbot), were also excluded. Lumbar punctures were performed on all children at the time of admission. The cerebrospinal fluid was examined immediately and a cell count performed with a standard counting chamber. Patients with more than 10 leucocytes/mm^3 in the cerebrospinal fluid or those with evidence of other severe infections were excluded from the study.

Study procedure

All patients were admitted for parenteral therapy. A loading dose of quinine dihydrochloride (20 mg salt/kg in a 4-hour perfusion containing 5% dextrose) was given, followed by perfusions of 10 mg salt/kg every 8 hours until oral tablets could be given. Patients were treated for 7 days and then discharged. A quinine sensitivity 28-day *in vivo* standard test was performed (Bruce-Chwatt *et al.* 1986).

At the time of hospitalization, every 4 hours, thick blood smears, later stained with Giemsa, were collected. The parasite clearance time was defined as the time between drug administration and the time when at least 2 consecutive blood slides were negative. Parasite density was expressed as the number of asexual forms of *P. falciparum* per microlitre of blood (parasites/µl) and was computed from the number of asexual parasites over the number of leucocytes in 100 high-power fields, assuming a leucocyte count of 8000/µl.

During the 3 weeks following discharge, children were visited at home once a week for a brief clinical examination and collection of a blood slide. Parasites were considered sensitive if the child's thick

blood film became negative at least by day 7 after admission and remained parasite-free during follow-up. Low-grade resistance (RI) was defined as disappearance of parasites from peripheral blood in the first 7 days, followed by recrudescence/reappearance by days 14, 21 or 28. High-grade resistance (RII/RIII) was defined as the presence of asexual parasites at day 7.

Test *in vitro*

The WHO *in vitro* microtest (mark II) (WHO 1990) was used to assess the susceptibility of *P. falciparum* isolates to chloroquine and quinine. Standard tests were obtained from the World Health Organization, Geneva, Switzerland.

In October 1993, all asymptomatic children aged 4–14 years from 5 villages (Caldeiras, Canavial, Boa Entrada, Fernão Dias, St Clara) located less then 10 km from Lobata District Health Centre were screened for parasitaemia. Those with a *P. falciparum* parasite density of $\geq$10 parasites per high power field (HPF) and no other malaria parasite species were selected for the study. Before treatment with chloroquine (25 mg/kg of body weight base given orally over 3 days), blood samples were collected in heparinized 100 µl tubes and the *in vitro* test was performed. The same microtitration WHO standard plates, culture medium, HEPES and bicarbonate bulk were used throughout the study.

Growth inhibition percentage for each drug concentration and for each sample was calculated on 200 parasites as follows: number of schizonts with chloroquine or quinine/number of schizonts in the control × 100.

Resistance was measured as the amount of inhibition observed in function of the schizonticide concentration expressed as µmol/well of blood. Schizont growth ($\geq$3 nuclei) at 8 pmol/chloroquine/well ($\geq$1.6 µmol/l of blood) and 256 pmol/quinine/well ($\geq$51.2 µmol/l of blood) were assumed to indicate resistance (WHO 1990). Drug resistance of *P. falciparum* was determined by the inhibitory concentrations preventing 50% of the parasites to mature to schizonts (IC_{50}) and was quantified by the drug concentration added to an *in vitro* culture of parasites that reduces their density by 50% compared to controls grown without any drug.

Statistical analysis

Probit analysis was used to quantify the inhibitory effect of the drug on the different isolates. This method usually gives a precise estimate of the mean drug resistance in a population (Grab & Wernsdorfer 1983; Huber & Koella 1993). A computer probit inhibition-dose–response regression programme (Bureau voor Biometrie, Lektie–Gent, IWONL) was used to determine IC_{50}. Different regression lines (patients and asymptomatic carriers) for different susceptibility to chloroquine and quinine were compared. Non-parametric tests were used for the analysis. The level of significance was $P<0.05$.

Results

Test *in vivo*

Fifty-six children with mild (n=44: 16 males and 28 females; median age: 9.0 years; range 4–14) or cerebral (n=12: 7 males and 5 females; median age: 5.0 years; range 4–9) malaria were recruited. Three cerebral malaria patients died within the first 3 days of treatment, 2 of them probably of severe anaemia (level of haemoglobin of 4.5 and 5.5 g/dl, respectively) and the other of bronchopneumonia. Thus, 53 (95%) children completed the 28-day test.

On day 0 the median parasite density was 34 720 parasites/µl (q1–q3: 26 100–48 740) for mild malaria patients and 62 100 parasites/µl (q1–q3: 53 480–103 280) for cerebral malaria patients ($P<0.05$).

All children became aparasitaemic between days 1 and 4 (maximum parasite clearance time: 96 hours), and no difference in the clearance of parasitaemia between mild and cerebral malaria cases was found. The percentage of parasitaemic children among malaria cases (mild and cerebral) declined progressively from day 1 to day 4 (D1 86.4%; D2 50.0%; D3 3.3%; D4 0.0%), although on day 1 all children with cerebral malaria were still positive. All children remained negative until the end of the test, indicating that the parasites were sensitive to quinine.

All mild malaria cases and the surviving cerebral malaria cases responded promptly to quinine treatment and all symptoms suggesting malaria disappeared by day 7. The mean fever clearance time was

Table 1 Minimum inhibitory concentrations of chloroquine *in vitro* for *Plasmodium falciparum* isolates collected in Lobata District (São Tomé Island) from various parasite carriers, including patients with cerebral and mild malaria

Chloroquine concentration (μmol/l)	Number of isolates in each group of children		
	Asymptomatic children (n=18)	Symptomatic children (n=11)	Total (n=29)
≤0.8	11	3	14
1.6	5	—	5
3.2	1	1	2
6.4	—	3	3
12.8	1	4	5
Total resistant	7 (38.8%)	8 (72.7%)	15 (51.7%)

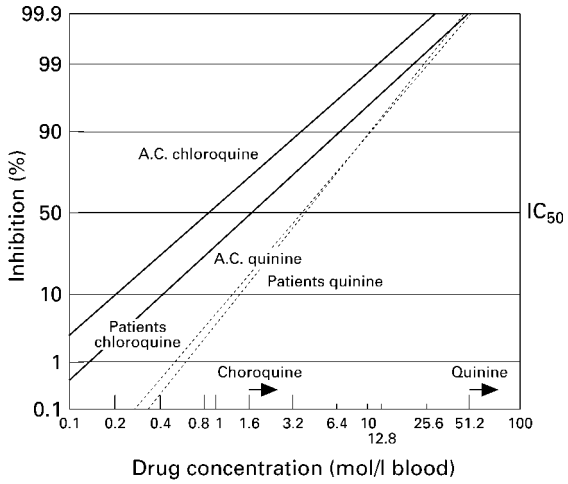

Figure 1 Activity probits analysis in function of the dosis-log of chloroquine and quinine. Regression lines compare patients (n=11) and asymptomatic children (A.C.) (n=18) of the same district in S. Tomé Island.

1.3 ± 0.4 days in the mild cases and 1.3 ± 0.6 days in the surviving cerebral malaria cases. No major adverse events were observed.

Test *in vitro*

Among the examined 508 healthy children, 203 (39.9%) had *P. falciparum* parasitaemia and 65 (12.8%) mixed infections (*P. malariae* with *P. falciparum* or *P. vivax*). Among those having *P. falciparum* parasitaemia alone, 98 had less than 10 parasites per high power field (HPF) and were excluded. Thus, *in vitro* sensitivity tests were performed on the remaining 105 children and these were successful in 18 (17.1%). A similar proportion of successes was observed among the symptomatic malaria cases (11/53, 19.6%).

All isolates tested were quinine-sensitive: 6.4 μmol/l of blood inhibited growth in ≈50% and 100% growth inhibition was achieved at a concentration of 25.6 μmol/l of blood (IC_{50}=3.4 μmol/l in patients and 2.4 μmol/l in asymptomatic children).

Fourteen isolates (48.3%) were sensitive to chloroquine, the majority of them (11/14, 78.6%) from asymptomatic children. Of the 15 resistant isolates (51.7%), 5 underwent schizogony at the highest drug concentration (12.8 μmol/l of blood). Seven (39%) of the 18 isolates from asymptomatic children were resistant to chloroquine (Table 1). Figure 1 shows the difference in sensitivity to chloroquine (IC_{50}) between isolates from cases and healthy children (1.6 μmol/l vs. 0.7 μmol/l).

Discussion

Quinine cleared parasitaemia in all our patients by day 4 and none of them had any recrudescence during the following 28 days, confirming that, in Lobata District in São Tomé, *P. falciparum* is still sensitive to this drug. Similar results were obtained with the *in vitro* test.

Chloroquine resistance was determined only by *in vitro* tests; previous reports on the level of resistance in São Tomé were confirmed (Martet *et al.* 1991). We also found a high percentage of resistant isolates (72% at ⩾1.6 μmol/l) among patients. This could be explained by the different geographical origin of the isolates or by assuming that resistant parasites are more pathogenic than sensitive ones. The former hypothesis can be ruled out as most of the patients

and healthy controls came from the same villages. It is more likely that the risk for an episode of clinical malaria is higher in children infected with resistant strains.

The discrepancy between the 2 groups of children poses serious problems of interpretation for such a test. Furthermore, the performance of the *in vitro* test in the field is not an easy task, shown by the low number of successes which may be unrepresentative of the parasite strains circulating in the community. The *in vitro* test reflects the intrinsic properties of the parasite and its response to the drug, while the *in vivo* test is influenced by the resistance itself and the human immune system (Koella 1993). It is for this reason that WHO developed the simplified *in vivo* 7 day-test (Prasad *et al.* 1990; Rieckman 1990), which has been used for the definition of the malaria drug policy in several regions in Africa (Khoromana *et al.* 1986; Sexton *et al.* 1988; Paluku *et al.* 1988; Prasad *et al.* 1990). However, the *in vitro* and the *in vivo* simplified tests also have limitations: the former assesses only the initial parasitological response, while the latter assesses the clinical response just during the first 7 days immediately following treatment (Bloland *et al.* 1993).

In view of other reports (Martet *et al.* 1991), our results seems to indicate an increase in the prevalence of chloroquine resistance in São Tomé, although this should be taken with caution as different methods of sampling were used. The selection of resistant strains and their transmission to new hosts depend on the drug pressure (Wernsdorfer 1994). It has been suggested that chloroquine-resistant strains were introduced in São Tomé in 1984 by migrant workers from Angola, Congo and Gabon (Loureiro *et al.* 1996). However, the present situation could be interpreted in a different way. Immunity against malaria is probably strain-specific and resistance to chloroquine is associated with the parasite's immunological properties (Koella 1993): when resistant parasites encounter little immunity, they spread rapidly until they are slowed by the increased level of immunity among the population. In São Tomé, as a result of the malaria eradication program implemented at the beginning of the 1980s, population immunity decreased. But when this program was stopped in 1983/84, a large outbreak ensued in 1985/86. Thus resistant strains already present in the population may have spread when population immunity was at its lowest level.

Reports from other endemic regions (Khoromana *et al.* 1986; Brandling-Bennett *et al.* 1988; Sexton *et al.* 1988) indicate that the majority of children with clinical malaria treated with chloroquine do not clear their parasitaemia, although they improve clinically, becoming afebrile and returning to normal activity. Changes of the current antimalarial drug policy should be supported by the results of drug resistance studies. However, the question of how results of *in vitro* tests influence the choice of the first-line antimalarial drug remains. This decision should take into account the consequences, such as anaemia, of the malaria infection/disease; there may be chloroquine resistance but the treatment may still be able to decrease the prevalence of anaemia (Greenwood 1987). This kind of information cannot be obtained just by performing *in vitro* or *in vivo* simplified tests (Hoffman *et al.* 1984; Guiguemdé *et al.* 1996).

We found that resistant isolates were more common among patients attending the health centre than among healthy children sampled from the same community. Therefore, in order to know whether there are chloroquine-resistant strains in a particular region, *in vitro* sensitivity tests should not be performed on school children or asymptomatic carriers of *P. falciparum* because most of the resistant strains will probably be found among patients attending health centres.

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