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Short Communication

Comparative assessment of *Plasmodium falciparum* sensitivity to chloroquine and amodiaquine *in vitro*

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The *in vitro* sensitivity of *Plasmodium falciparum* isolates to chloroquine and amodiaquine were assessed in children with symptomatic uncomplicated malaria in Ibadan, Nigeria. The WHO standard *in vitro* micro-test method was employed for the study. A total of one hundred and two children were admitted into the study. Inhibition of schizont maturation at varying concentration of the study drugs was used as an index for drug activity. Effective concentrations by probit analysis of log dose/response for 50, 90 and 99% (EC_{50}, $EC_{90}$and EC_{99}) inhibition were 0.37, 2.38 and 5.76 µmol/l, respectively, for chloroquine and 0.06, 0.26 and 0.59 µmol/l, respectively, for amodiaquine. Forty isolates of *P. falciparum* were tested for chloroquine sensitivity. Eighty percent (32/40) showed schizont maturation at 1.6 µmol/l and were classified as resistant, while 39% (14/36) of isolates tested for amodiaquine matured at 0.4 µmol/l and were also classified as resistant. This shows that amodiaquine is significantly more effective than chloroquine. While this data provides no absolute demonstration of chloroquine resistance, it underlies the need for continuous monitoring of the susceptibility of *P. falciparum* to chloroquine in southwest Nigeria.

Key words: *Plasmodium falciparum*, chloroquine, amodiaquine, *in vitro*, resistance.

INTRODUCTION

Malaria is one of the most common causes of childhood morbidity and mortality in sub-Saharan Africa. Every year, an estimated 1.5-2.8 million people, mostly children, die from *Plasmodium falciparum* malaria (WHO, 1996). The situation is further worsened by the widespread and increasing resistance of *P. falciparum* to chloroquine (CQ), the drug that has been the mainstay of malaria treatment for decades (White et al., 1992). Despite the spread of resistance, most African countries continue to use chloroquine as the first-line drug for uncomplicated malaria as is the case in Nigeria. This is because CQ is relatively affordable, readily available and relatively safe (Sowunmi et al., 1997). Amodiaquine (AQ), a 4-aminoquinoline antimalarial drug similar to CQ is one of the few possible alternatives to chloroquine and it has been shown to be effective and affordable (Brasseur et al., 1999). Although global use of amodiaquine has declined owing to reports of its potential toxicity especially when used prophylactically (Phillips-Howard et al., 1990; WHO, 1990a), evidence are accumulating that supports its use in the treatment of uncomplicated malaria (Olliaro et al., 1996; Staedke et al., 2001) with the provision that monitoring of efficacy and toxicity should continue. In this study, the susceptibility of *P. falciparum* parasites to chloroquine and amodiaquine were evaluated and compared in an *in vitro* assay in order to determine the current level of efficacy of these drugs in the treatment of uncomplicated malaria in southwest

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MATERIALS AND METHODS

Study site and patients

The study was conducted in Ibadan, south-western Nigeria, where *P. falciparum* is the predominant species causing majority of infection (Salako et al., 1990). One hundred and two children were admitted into the study between April and August 2003, at the Children Out Patient Clinic (CHOP) of the University College Hospital Ibadan. Children who were enrolled into the study proper satisfied the following criteria: history of fever in the 24 h preceding presentation or pyrexia at presentation (>37.5°C), pure *P. falciparum* asexual parasitaemia >1,000/µL of blood, no antimalarial drug administration in the two weeks preceding presentation and negative urine tests (Dill-Glazko and Lignin) for antimalarial drugs. Informed (written/verbal) consent of parent or guardian of each child was obtained while ethical approval was given by the local institution.

Collection of *P. falciparum* isolates

1 ml of blood was collected from each child by venepuncture into sterile EDTA tube. Thick and thin blood films were made from the sample for parasitological examinations and were stained with 4% Giemsa's stain for 20 min. Parasitaemia was quantified per 200 white blood cells on a thick film and expressed as parasites/µL. A slide was considered negative if examination of at least 200 oil immersion fields revealed no parasites.

In vitro drug sensitivity assay

Thick and thin blood films were prepared from the blood collected from each subject for parasite identification. Two antimalarial drugs chloroquine (CQ) and amodiaquine (AQ) were used in this assay. Microtitre plates were pre-dosed with 50 µL of varying concentrations of the drugs. Dosing started with the control well (A) and followed an increasing order of drug concentration ending at well H (the highest concentration). For chloroquine, A=0, B=0.1, C=0.2, D=0.4, E=0.8, F=1.6, G=3.2 and H=6.4 µmol/L. For amodiaquine, A=0, B=0.025, C=0.05, D=0.1, E=0.2, F=0.4, G=0.8 and H=1.6 µmol/L. According to WHO standard methods and concentrations (WHO, 1990b), 900 µL of culture medium (RPMI 1640) was added to 100 µL of blood from each patient in a blood medium mixture of ratio 1:9. All the wells of the appropriate column were dosed with 50 µL of the blood-medium mixture. Dosing started with the control well (A) and followed an increasing order of drug concentration ending at well H. The patient's name, the date, the type of drug and the hour of incubation was inscribed on the plate. The pre-dosed microtitre plates were placed into a candle jar (vacuum desiccator) containing a candle. The candle was lit and the lid of the jar was replaced just before the candle flame went out (giving an atmosphere of relatively high CO_2 and low O_2 content). The jar was then incubated at 37°C (±0.5°C) for 24-36 h following the Jensen and Trager method (Jensen et al., 1977; Noedl et al., 2003). After incubation, the supernatant from each microtitre plate was discarded. A thick blood film was made from the sediment at Ihe bottom of each well on a column, on the same microscope slide for each patient and labeled accordingly. The slides were then airdried stained for 20 min and examined by light microscopy. The blood film was examined under x100 oil immersion objective. The number of schizonts per 200 asexual parasites (trophozoites) was used to assess schizont maturation inhibition. Cultures with less than 10 schizonts per 200 trophozoites were excluded from the analysis. The test was considered valid if ≥10% of the parasites in the control well had reached schizont stage within 24-36 h. The IC_{50}, IC_{90} and IC_{99} concentrations producing 50, 90 and 99% inhibition were taken as the measure of the response of the biological systems of the parasite to drugs. Parasite isolates were then classified as sensitive or resistant to a particular drug according to the drug concentration at which schizont maturation was completely inhibited. The degree of resistance of *P. falciparum* was categorized into three: RI, RII and RIII resistance.

Statistical analysis

Drug concentrations inhibiting parasite growth were calculated using the probit regression analysis based on the SPSS software package (Wernsdorfer et al., 1995). Data not conforming to normal distributions were log-transformed.

RESULTS AND DISCUSSION

A total of 102 children were screened for this study. Out of these subjects, only 62 fulfilled the criteria for enrollment. The characteristics of these subjects are shown in Table 1. Sixty two isolates of *P. falciparum* were collected from these subjects for the *in vitro* drug sensitivity assay. The proportions of valid tests were 40/62 for chloroquine and 36/62 for amodiaquine. Other tests were discarded due to lack of satisfactory schizont growth.

The mean IC_{50}, IC_{90} and IC_{99} for chloroquine and amodiaquine, derived using the probit regression analysis are shown in Table 2. This result shows decreased susceptibility of *P. falciparum* isolates to chloroquine compared with amodiaquine. Both drugs however, did not show satisfactory response as growth was observed above the WHO discriminating concentration for satisfactory response which is complete schizont inhibition at ≤ 0.4 µmol/L for chloroquine and at ≤ 0.2 µmol/L for amodiaquine (WHO, 1987). 95% (38/40) of the isolates tested for chloroquine sensitivity matured at 0.8 µmol/L while 39% (14/36) of the isolates tested for amodiaquine sensitivity matured at 0.4 µmol/L, which indicated resistance to these drugs (WHO, 1987).

Chloroquine drug pressure remains high in Nigeria, as it is still the first-line drug in malaria therapy both as self treatment at home and in health care facilities. The spate of chloroquine resistance has therefore necessitated the evaluation of an alternative antimalarial drug that is effective, safe and affordable. Data from our *in vitro* study shows that amodiaquine is more potent blood schizonticide than chloroquine. The minimum drug concentration causing 90% inhibition of schizont maturation shows considerable higher activity of amodiaquine (0.26 µmol/l) when compared with chloroquine (2.38 µmol/l) against sensitive strains of *P. falciparum*. This data lends support to earlier clinical trial

Table 1. Characteristics of subjects who satisfied the inclusion criteria.

Subjects' parameters	Values
Number of subjects	62
Mean age (months)	28.5 (±17.1)[a]
Mean weight (Kg)	11.4 (±3.1)[a]
Mean packed cell volume (PCV)	25.5 (±5.4)[a]
Percentage male	63 (39/62)
Geometric mean parasite density (per µl)	14,674 (468 - 69,474)[b]

[a]Standard deviation in parentheses.
[b]Range in parenthesis.

Table 2. Inhibitory concentrations of chloroquine and amodiaquine on cultures of *P. falciparum* isolates.

Drug	n	Concentration (µmol/L)		
		IC_{50}	IC_{90}	IC_{99}
Chloroquine	40	0.37	2.38	5.76
Amodiaquine	36	0.06	0.26	0.59

which also found amodiaquine to be more effective than chloroquine for the treatment of uncomplicated falciparum malaria (Brasseur et al., 1999; WHO, 1987; Sowunmi et al., 2001). This data therefore suggests that amodiaquine should be investigated more for treatment of chloroquine-resistant *P. falciparum* malaria in Nigeria. At least 80% (32/40) of the isolates tested in the chloroquine group, matured at a concentration above 0.8 µmol/l, which indicate a high level of resistance to the drug. These results also confirm earlier reports that chloroquine resistance is increasing in south-west Nigeria (Spencer et al., 1983; Sowunmi et al., 1997; Salako et al., 1987). However, there seems to be a gradual build up of resistance to amodiaquine as 39% of the isolates tested grew at amodiaquine concentration of 0.4 µmol/l. Such was also observed in a clinical study where resistance was noted in some isolates from subjects in Senegal (Brasseur et al., 1999). Cross-resistance to chloroquine and amodiaquine may rise, since both drugs belong to the same amino-quinoline family. Hence, caution should be exercised with the introduction of amodiaquine as monotherapy for uncomplicated malaria. Studies are underway to assess the potential clinical value of combining amodiaquine with other drugs to delay the occurrence of resistance.

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