Full Length Research Paper

Sensitivity patterns of isolates of *Plasmodium falciparum* to amodiaquine (AQ) as resistance reversing compounds.

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In vitro assays still remain a vital stage of antimalarial drug development process and resistance monitoring. This study assessed the sensitivity patterns of isolates of *Plasmodium falciparum* to amodiaquine (AQ) alone or in combination with verapamil (VER), chlorpheniramine (CP) and promethazine (PRO) as resistance reversing compounds. The test involves monitoring the ability of antimalarial drugs to prevent parasite transition from trophozoites to schizont stages over a 24-48 h incubation period *in vitro* by World Health Organization (WHO) schizont inhibition assay. The MIC of AQ alone ranged from 6.2-500.0 ng/ml while those of its reversing agents ranged from 2.1-500.0 ng/ml. Mean MIC for AQ=120.51±15.10. Based on the cut-off value for AQ *in vitro* susceptibility, 73% (76/104) of the *P. falciparum* isolates were sensitive to AQ while 27% (28/104) were resistant. The mean MIC values for AQ + VER, AQ + CP and AQ + PRO were 83.08 ± 9.39, 106.93 ± 13.28 and 111.09 ± 14.82 respectively. Based on the reversal phenomenon, 75% (78/104) of the isolates were classified sensitive to amodiaquine, while 25% (26/104) were classified resistant with verapamil as reversing agent (P<0.05). Furthermore, 85% (88/104) were sensitive to amodiaquine while 15% (16/104) were resistant with chlorpheniramine as reversing agent (P<0.05). In the same vein, 78% (81/104) were sensitive to amodiaquine while 22% (23/104) were resistant with promethazine as reversing agent (P<0.05). The present results demonstrated high sensitivity pattern to the drug combinations. However, the very low levels of *in vitro P. falciparum* resistance against chlorpheniramine may demonstrate its pharmacological advantage as a better reversing agent over others. Subsequent surveillance should, in addition, integrate both *in vivo* and molecular surveillance to characterize the true nature of *P. falciparum* isolates in this area.

Key words: World health organization (WHO) schizont inhibition assay, amodiaquine, verapamil, chlorpheniramine, promethazine.

INTRODUCTION

Worldwide, drug resistance constitutes an impediment to the control of malaria (Barnes et al., 2007). This has led to the need for constant surveillance and monitoring for changes in the sensitivity of malaria parasites to different antimalarial drugs (Ikpa et al., 2009). As a result of widespread resistance, malaria therapy now consists of combination of two or more drugs that attack different

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biochemical processes in the *Plasmodium* species (Le Bras and Durand, 2003). Amodiaquine (AQ), a Mannich base, is frequently used in many parts of Africa or in combination with other antimalarial drugs. Resistance to AQ and CQ may have the same genetic basis (Ochong et al., 2003).

It was observed that drug resistance in *Plasmodium falciparum* could be modulated by verapamil (Masseno et al., 2009). In addition, poor sensitivity of *P. falciparum* to some antimalarial drugs have been reversed both *in vitro* and *in vivo* by the concomitant administration of antihistamine type 1 (H1) receptor, notably chlorpheniramine and promethazine (Kyle et al., 2002). *In vitro* drug susceptibility testing provides a complementary surveillance method to clinical studies in malaria endemic areas, where drug susceptibility of parasite isolates can be obscured by different levels of acquired immunity in patients treated with antimalarial drugs (Russell et al., 2003). In Nigeria, data on the susceptibility profile of *P. falciparum* isolates to antimalarial drugs are sparse. The effect of an antimalarial drug is generally characterized by the inhibition of parasite growth and consequently their multiplication. There are various standard *in vitro* techniques developed to evaluate parasite susceptibility pattern. The most commonly used methods for the antimalarial *in vitro* testing are the *in vitro* micro-test Mark III, the isotopic test, the drug sensitivity assay based on the measurement of HRP2 / or PLDH in an enzyme-linked immunosorbent assay (ELISA) and SYBER green test (Abiodun et al., 2010).

In this paper, a modified WHO schizont inhibition assay (Rieckmann et al., 1978) was used to determine the *in vitro* susceptibility profile of 104 patient isolates of *P. falciparum* to amodiaquine by the determination of the minimum inhibitory concentration (MIC) of the drug in isolation and also in combination with verapamil, chlorpheniramine and promethazine as markers to define drug resistance in Ibadan, South Western Nigeria.

MATERIALS AND METHODS

The WHO schizont inhibition assay has been useful in epidemiological evaluation of *in vitro* sensitivity profiles of strains of *P. falciparum* to amodiaquine and other antimalarial drugs (Kyle et al., 2002). The test involves monitoring the ability of antimalarial drugs to prevent parasite transition from trophozoites to schizont stages over a 24 to 48 h incubation period *in vitro*. This procedure has proven useful in identification of drug resistant parasites (Kyle et al., 2002).

Patient selection and sample collection

Isolates of *P. falciparum* were obtained from patients presenting with acute uncomplicated malaria at malaria clinics situated at three locations: Institute for Advanced Medical Research and Training; University College Hospital Ibadan; and Adeoyo State Hospital, Ibadan, Nigeria. Patients with clinical symptoms and microscopically confirmed *P. falciparum* infections were enrolled for the studies during the transmission (rainy) and dry seasons. Oral informed consent was also obtained from each patient or guardian.

Drug preparation

Five milligram of each of the drugs used for the assays was weighed. Amodiaquine was initially dissolved in 1.5 ml distilled water. The solution was sonicated and 3.5 ml of absolute alcohol was later added. Verapamil, chlorpheniramine and promethazine were dissolved in 5 ml of 70% alcohol. Each drug suspension was sonicated for one hour to obtain total dissolution of the drugs.

Preparation of culture media

Powdered RPMI 1640 culture medium (10.4 g/litre) (Sigma Chemical Company, St Louis O) contained HEPES (N-2-hydroxyethylpiperazine-N-2-ethane sulphonic acid) (25 mM) (Gibco Laboratories, Grand Island, NY). The culture medium was filtered using a 0.22 micron filter and stored at 4°C. Buffered RPMI 1640 culture medium (CM) containing 30 Mm $NaHCO_3$ (Gibco Laboratories, Grand Island, NY) was prepared weekly in 45 ml aliquots. Buffered media was used for washing red blood cells obtained from malaria patients and for screening of non-infected blood donors. Complete culture media with plasma (CMP) was prepared by the addition of 10% (v\v) fresh frozen human plasma to buffered RPMI 1640 and stored at 4°C.

Parasite preparation

Five milliliter of venous blood was obtained from each child enrolled into the study for *in vitro* sensitivity to antimalarial drugs and cryopreservation.

Parasite preparation for drug susceptibility testing

Two milliliter parasitized blood was diluted (1:10) with buffered culture medium (RPMI 1640 culture medium supplemented with 25 mM HEPES and 32 nM $NaHCO_3$). Two-hundred microliters of the diluted parasitized red blood cell suspension was added into each of the wells in the test plate containing AQ alone, AQ combined with either VER, CP or PRO. The test plates containing suspension of parasitized RBC were incubated at 37°C in a candle jar.

Preparation of working solution

After the initial preparation of stock solutions for all the drugs, working solution for each drug was prepared. Fixed volume of each solution was diluted with buffered

Table 1. Summary of the *in vitro* susceptibility profile of patient isolates of *Plasmodium falciparum* to amodiaquine and in combination with verapamil, chlorpheniramine and promethazine at the Malaria Clinic, Institute for Advanced Medical Research and Training, College of Medicine, Ibadan.

Drug/Drug combinations	MIC Range (ng/ml)	Mean ± SEM MIC	% *in vitro* sensitive to AQ	% *in vitro* resistance to AQ	T values
AQ alone	6.2-500.0	120.51 ± 15.10	73% (76)	27% (28)	
AQ + VER	2.1-500.0	83.08 ± 9.39	75% (78)	25% (26)	2.105*
AQ + CP	2.1-500.0	106.93 ± 13.28	85% (88)	15% (16)	0.500
AQ + PRO	2.1-500.0	111.09 ± 14.82	78% (81)	22% (23)	0.657

culture media (RPMI 1640 supplemented with 25 mM HEPES and 32 mM NaHCO$_3$) in order to obtain a working concentration of 5000 ng/ml for AQ and VER, 3984 ng/ml for chlorpheniramine (CP), and 2801 ng/ml for promethazine (PRO).

Preparation of serial drug dilution templates (standard microtest plate)

A 96-well microtitre plate consisting of a matrix of 8 rows (A-H) and 12 columns was used. A template containing the antimalarial drug (AQ) was prepared using columns (1-8) in 3-fold serial dilution of the working solution. The drug template for AQ was aseptically prepared using multi channel pipette under a laminar flow hood.

Three hundred microliters of the working concentration of AQ was transferred into wells 1-4 in row A of the 96-microtiter plate. Two hundred microliters of complete buffered culture media (CM) was transferred into all the wells in rows B to H. A three-fold dilution of amodiaquine was prepared in wells of rows A to G by transferring 100 μl of the content of each well in one row to the next starting from row A. No transfer was made to wells in row H. Row H served as control containing buffered culture media alone. At the end of the dilution, concentrations of amodiaquine in rows B to G, wells 1 to 4, ranged from 5000 - 6.86 ng/ml.

Seven identical test plates were prepared from the template described above. 25 μl of working solution of the reversing compounds {verapamil (5000 ng/ml), chlorpheniramine (3984 ng/ml), promethazine (2801 ng/ml)} were added to the wells in columns 2, 3 and 4 of rows B to G respectively, with the exclusion of Row H (control). 25 μl of buffered culture media was also added into the wells with no reversing compounds and wells in row H.

Monitoring of schizont inhibition assay

Thick blood films were prepared from the control well to monitor the experiment starting from 18 h after incubation depending on the stage of the parasite at the beginning of the assay. The thick blood films were Giemsa stained and examined under oil immersion objective lens of a light microscope. Experiments were considered successful

and terminated when 60% of parasites in the control wells have matured to schizonts within 48 h of incubation. After termination of successful experiments,

150 μl of the culture supernatant was removed from all the wells in the test plate. Thick films (5 μl) were prepared from parasitized packed cells of each well. These were microscopically evaluated by counting the number of schizont, per 200 white blood cells.

Interpretation of *in vitro* assays

MIC of amodiaquine alone was compared with MIC of the amodiaquine combined with its respective reversing compounds. Parasites were categorized into sensitive and resistant; a resistant isolate is considered to have been reversed if there is a reduction in the MIC of amodiaquine combined with their respective reversing compound when compared with amodiaquine alone.

Statistical analysis

In vitro antiplasmodial activities of the different drugs used were analysed quantitatively using the student T-test. Values were considered significantly different at $p < 0.05$.

RESULTS

In vitro susceptibility testing for amodiaquine (AQ) and the reversing agents were successful in 104 of 108 (96%) of *P. falciparum* isolates analyzed. The MIC of AQ alone ranged from 6.2-500.0 ng/ml, while those of its reversing agents ranged from 2.1-500.0 ng/ml. Mean MIC for AQ=120.51±15.10. Based on the cut-off value for AQ *in vitro* susceptibility, 73% (76) of the *P. falciparum* isolates were sensitive to AQ, while 27% (28) were resistant. The mean MIC values for AQ + VER, AQ + CP and AQ + PRO were 83.08 ± 9.39, 106.93 ± 13.28 and 111.09 ± 14.82 respectively (Table 1).

Based on the reversal phenomenon, there was a reduction in the MIC of amodiaquine occurring in 25% (26/104) of the patient isolates when verapamil was combined with amodiaquine (P<0.05). The MIC of amodiaquine remains unchanged in 75% (78/104) of the patient isolates (Table 1). Therefore, 25% of the isolates

were classified as resistant to amodiaquine while 75% were sensitive to amodiaquine with verapamil as reversing agent (Table 1).

Furthermore, there was a reduction in the MIC of amodiaquine occurring in 15% (16/104) of the patient isolates when chlorpheniramine was combined with amodiaquine (P<0.05). The MIC of amodiaquine remains unchanged in 85% (88/104) of the patient isolates as shown in Table 1. Therefore, 15% of the isolates were classified as resistant to amodiaquine, while 85% were sensitive to amodiaquine with chlorpheniramine as reversing agent. In the same vein, there was a reduction in the MIC of amodiaquine occurring in 22% (23/104) of the patient isolates when promethazine was combined with amodiaquine (P<0.05). The MIC of amodiaquine remains unchanged in 78% (81/104) of the patient isolates. Therefore, 22% of the isolates were classified as resistant to amodiaquine while 78% were sensitive to amodiaquine with promethazine as reversing agent (Table 1).

DISCUSSION

In vitro assay has become a welcome tool for evaluating the responses of patient isolates of *P. falciparum* to antimalarial drugs and also to monitor the spread of drug resistant malaria in the region. It provides an alternative to clinical studies in malaria endemic areas, where drug susceptibility of parasite isolates can be obscured by different levels of acquired immunity in patients treated with antimalarial drugs (Russell et al., 2003).

In Nigeria, data on the susceptibility profile of *P. falciparum* isolates to antimalarial drugs are sparse. However, it is imaginable that resistant *P. falciparum* may have contributed reasonably to the malaria burden in the country (Ikpa et al., 2009).

The present results demonstrated that there was a high sensitivity pattern to the drug combinations. It was observed that 75% *in vitro* sensitivity of *P. falciparum* fresh parasite isolates to amodiaquine were combined with verapamil, 78% *in vitro* sensitivity to amodiaquine were combined with promethazine, and 85% sensitivity to amodiaquine were combined with chlorpheniramine. However, moderately low sensitivity pattern to the drug combinations was also observed, where 25% *in vitro* parasite resistance against amodiaquine was combined with verapamil, 22% *in vitro* parasite resistance against amodiaquine was combined with promethazine, and 15% *in vitro* parasite resistance against amodiaquine was combined with chlorpheniramine. The reversing abilities follow the order chlorpheniramine> promethazine> verapamil. The results of this study demonstrated that chlorpheniramine mostly potentiates *in vitro* antimalarial action of amodiaquine of all the screened reversing agents. In addition, chlorpheniramine is a safe therapeutic drug; the clinical use of chlorpheniramine as a cheap and highly effective combination with quinoline-

containing antimalarial drugs holds great promise against multidrug-resistant falciparum malaria (Sowunmi et al., 2007). However, subsequent surveillance should in addition to the present procedure integrate both *in vivo* and molecular surveillance to characterize the true nature of *P. falciparum* isolates in this area.

The very low levels of *in vitro P. falciparum* resistance against the latter drug may demonstrate the pharmacological advantage of chlorpheniramine as a better reversing agent over others. In addition, the levels of resistance reported by *in vitro* findings could sometimes overestimate the degree of resistance *in vivo.* This therefore calls for the need to compare in *vivo* treatment outcome with *in vitro* susceptibility findings. This would go a long way in enhancing new treatment modalities and antimalarial drug policies in Nigeria. The findings, regarding the sensitivities profile of amodiaquine, represent a sharp contrast from the results obtained by Oyedeji et al. (2005) in South western Nigeria, in which 39% of 36 *P. falciparum* isolates were reported to be *in vitro* resistant to amodiaquine as compared to 11% *in vivo* amodiaquine insensitivity as reported by Graupner et al. (2005).

All the resistance reversing agents employed in this study enhanced intrinsic *in vitro* antimalarial activity of amodiaquine against the resistant parasites at different varying levels. Generally, *in vitro* resistance findings cannot be extrapolated directly to *in vivo* studies. The possible understanding of lack of accurate correlation between *in vivo* and *in vitro* response to both drugs may be attributed to sensitivity pattern of the infecting parasites, acquired immune factors, host pharmacokinetics and drug-drug interactions. Molecular studies involving the DNA analysis using PCR techniques to determine accurately the molecular profile of both the initial infecting parasites and the recrudescence parasites would definitely go a long way and shed more light on the correlation between *in vivo* and *in vitro* response. It is also clear that host factors including pharmacodynamics coupled with the half-life of the drug and complexity of infections most especially in high transmission areas may also play vital roles in determining the outcome of therapy in patients (Happi et al., 2003). Determination of blood levels of antimalarial drugs in samples from each patient after failure will be an additional concept and would be valuable in defining drug resistant infections and also for revalidating the reversal techniques (Barnes et al., 2007).

Precautionary measures employed in this study involved careful selection of patient to include those who have not ingested antimalarial drug in the preceding two weeks. This requirement is necessary because the presence of residual amount of antimalarial drug in patient blood could also affect the sensitivity patterns of parasites, although this does not necessarily alter the relative potentiation of the antimalarial activity of the drug being investigated. Drug susceptibility testing using culture adapted parasites (Kyle et al., 2002), provides an

alternative, result-oriented valuable method of monitoring susceptibility profiles of *P. falciparum* to standard antimalarial to define drug resistance. However, the test requires the use of cloned strains of *P. falciparum* for comparative identification of resistant parasites. Generally, the *in vitro* technique appears to be a more objective method of detecting drug resistant infections because several host factors, which interfere with clear interpretation of test results, are eliminated in the *in vitro* tests. However, both *in vivo* and *in vitro* tests should complement one another and adequate standardization of both tests would be vital in making them more reliable and effective surveillance tools. Nevertheless, the *in vitro* data still represent an important indicator for monitoring trends in antimalarial drug resistance.

Conclusion

From this study, it is hoped that chemotherapeutic approach utilizing the reversal phenomenon may influence strongly the institution of evidenced-based policy towards the management of drug resistant malaria in Ibadan, South-west Nigeria. However, subsequent surveillance should in addition to the present procedure integrate both *in vivo* and molecular surveillance to characterize the true nature of *P. falciparum* isolates in this area. The results in this study indicate that antihistaminic drugs may be promising candidates for potentiating antimalarial drug action against drug resistant malarial parasites.

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