

In-Vitro Susceptibility of Plasmodium falciparum to Antimalarial Drugs in Abuja, Nigeria

I Peletiri, B Matur, N Ibecheozor, J Ihongbe, T Wakama

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Abstract

The susceptibility of Plasmodium falciparum

to Chloroquine, Quinine, Dihydroartemisinin and Monodesethylamodiaquine was investigated in the Federal Capital Territory (FCT), Abuja. The standard WHO in vitro micro test methodology was used in the study. Of 18 isolates evaluated for susceptibility to chloroquine, 16 (88.9%) were resistant with median IC_{50} of 0.06 μ mol/l of blood (range 0.36 – 1.32 μ mol/l). The other 2 (11.1%) were however susceptible with median IC_{50} of 0.27 μ mol/l (range 0.24 – 0.30 μ mol/l) of blood. With quinine, 13 (72.2%) were susceptible with median IC_{50} of 1.8 μ mol/l of BMM (range 1.4 – 2.3 μ mol/l). For dihydroartemisinin, an IC_{50} of 13.5nmol/l of BMM (range 7.8 – 26 nmol/l of BMM) was indicated from the isolates. With monodesethylamodiaquine, 10 (55.6%) were susceptible with median IC_{50} of 0.09 μ mol/l (range 0.08 – 0.10 μ mol/l of blood). The results of this study revealed a high level and degree of chloroquine resistance, a dwindling susceptibility to quinine, and a moderate resistance to monodesethylamodiaquine. With the implementation of the National Antimalarial Treatment Policy as regards diagnosis of malaria in the home and disease management at the home (presumptive treatment), development of resistance to the ACTs – Artemisinin based combined therapy may not be too far as a result of selective pressure on the circulating population.

INTRODUCTION

Malaria remains an important public health concern in countries where transmission occurs regularly, as well as in areas where transmission has been largely controlled, or eliminated. Malaria is a complex disease that varies widely in epidemiology and clinical manifestation in different parts of the world. This variability is the result of factors such as the species of malaria parasites that occur in a given area, their susceptibility to commonly used or available antimalarial drugs, the distribution and efficiency of mosquito vectors, climate and other environmental conditions and the behaviour and level of acquired immunity of the exposed human populations. In particular, young children, pregnant women, and non-immune visitors to malarious areas are at greatest risk of severe or fatal illness⁽¹⁾.

The world's most important infectious diseases include malaria, tuberculosis and HIV/AIDS. Together, these diseases threaten the lives and well – being of millions of people, particularly in the tropics. They place enormous burdens on national health services and impair the development of many emerging nations⁽²⁾. Antimalarial drug resistance has emerged as one of the greatest challenges

facing malaria control today.

The WHO⁽³⁾ estimated that more than 90% of the 1.5 to 2.7 million deaths attributed to malaria each year occur in African children. This study is therefore justified owing to the fact that in-vitro susceptibility testing as an epidemiological tool for assessing baseline sensitivity and for monitoring the drug response of Plasmodium falciparum over time and place can provide background information for the development and evaluation of drug policies.

The aim of this research work is to ascertain the degree of chloroquine resistant strains of Plasmodium falciparum in our environment. The specific objective on the other hand is to generate data on in-vitro susceptibility of Plasmodium falciparum to Chloroquine, Quinine, Dihydroartemisinin, and Monodesethylamodiaquine.

MATERIALS AND METHODS

STUDY AREA

The Federal Capital Territory (FCT), Abuja is the study area.

Abuja has a daily temperature range between 20.4 – 34.7°C with an average of 27.2°C/81°F in January; and between 21.9

– 29.1°C with an average of 25.6°C/78°F in July⁽⁴⁾.

SAMPLE SITES

Three representative hospitals within the FCT – National Hospital, Abuja, Wuse

General Hospital, and the Asokoro General Hospital, was the study sites.

Collection and Processing of Pre-culture Blood Specimens

Fresh blood samples already collected from patients for the diagnosis of malaria from the three representative hospitals were used following permission by the respective medical laboratory authorities. Pre-culture thick and thin films were prepared for each blood sample.

SUBJECT SELECTION

This was based on exclusion and inclusion criteria set for the research. Results obtained from the processed pre-culture thick and thin films formed the basis for the criteria.

EXCLUSION CRITERIA

Patients' blood samples were considered inadequate for the study if microscopy results of Giemsa stained films had a falciparum parasitaemia (in a thick film) of less than two trophozoites in every high power microscope field (< 1000 parasites/μl of blood). Patients with mixed infections were also excluded from the study.

INCLUSION CRITERIA

The patients' blood samples were considered adequate for the study if microscopy results of Giemsa stained thick films had mono-infections with *Plasmodium falciparum* and asexual parasitaemias in excess of 1000 parasites, but less than 80,000 parasites per μl of blood (more than one trophozoite in every high power microscope field - > / = +++).

COUNTING PARASITE NUMBERS

Parasites were counted by estimating the parasite numbers/μl of blood from the thick film. This was carried out by multiplying the average number of parasites per high power field (100 x objective) by 500. Between 10-50 fields (depending on parasitaemia) were examined to determine the average number of trophozoites per high power field (HPF). Ten fields are sufficient when the parasite density is high.

The factor of 500 was proposed by Greenwood and Armstrong⁽⁵⁾. They calculated that 5 – 8 μl is the volume of blood required to make a satisfactory thick film and that the

volume of blood in one HPF (100 x objective) of a well-prepared thick film is about 0.002μl. Therefore the number of parasites per HPF multiplied by 500 gives the estimated number of parasites/μl of blood. This method, Greenwood and Armstrong found to be more accurate and quicker than counting the parasites against white cells.

IN-VITRO SUSCEPTIBILITY TEST PROCEDURE MATERIALS

1. WHO pre-dosed micro – test plates for chloroquine, quinine, dihydroartemisinin, and mododesethylamodiaquine. These were supplied by the Vector Control Research Unit, Universiti Sains Malaysia (USM) under the auspices of the Communicable Disease Surveillance and Response (CDR), WHO, 1211 Geneva, 27 Switzerland.

2. Positive patients' sample having mono-infections with *Plasmodium falciparum* and asexual parasitaemias in excess of 1000 parasites, but less than 80,000 parasites per μl of blood.

RPMI – 1640 medium (product number R6504), purchased from and supplied by the Vector Control Research Unit, Universiti Sains Malaysia (USM) under the auspices of the Communicable Disease Surveillance and Response (CDR), WHO, 1211, Geneva, 27, Switzerland.

Methanol

Giemsa stain

Microscope slide with frosted end.

IN-VITRO MICROTTEST TECHNIQUE

The drug susceptibility of *Plasmodium falciparum* was determined using the World Health Organization (WHO) standardized in-vitro microtest system⁽⁶⁾ developed by Rieckmann et al.,⁽⁷⁾; adapting the methodology used for cultivation of *Plasmodium falciparum*⁽⁸⁾. The technique involves quantitating schizont maturation following cultivation of infected erythrocytes in plates charged with defined quantities of drug.

DRUG PREPARATION

Pre-dosed plates of chloroquine, quinine, dihydroartemisinin, and monodesethylamodiaquine were obtained from WHO in Sains, Malaysia.

IN-VITRO MICROTTEST

Following centrifugation of infected blood samples at low speed (200 rpm) the plasma was discarded and the

erythrocytes (packed cells) were washed three times in culture medium (RPMI 1640 with L-glutamine, without sodium bicarbonate) at 2000 rpm for 5 minutes. 100µl of washed infected erythrocytes was then mixed aseptically with 900µl of complete culture medium (1:10) and 50µl of the blood-medium mixture (BMM) was transferred into each well of the pre-dosed plates.

The plates were incubated at 37°C for 24-30 hours (depending on schizont maturation) using the candle jar method (5% CO₂)⁽⁸⁾. All the susceptibility assays for each drug were done in duplicate.

After incubation, the plates were allowed to stand for 30 minutes in a semi-vertical position (about 45 ° inclined). The supernatant was then removed, erythrocytes re-suspended in the remaining fluid, and a thick blood film was made from each well. The blood smears were air-dried for 24 hours and stained with 3% Giemsa stain for 30 minutes. The stained thick films were examined with the oil immersion objective (100x). The number of schizonts with three or more nuclei out of 200 asexual parasites (i.e., schizonts and trophozoites) was counted. For an acceptable test, schizont maturation in the control (well A) must be 10% or more (i.e., 20 schizonts with three or more nuclei per 200 asexual parasites). The counts read in the drug wells were then expressed as a percentage of the control.

INTERPRETATION AND REPORTING OF TEST REPORTS

For data analysis, a simple evaluation from a table showing the original data and those derived after standardizing for control was used.

Schizont growth (formation) at 8 pmol/well (1.6 µmol/l) or more for chloroquine, 256 pmol (51.2µmol/l) or more for quinine, 4 pmol (0.8µmol/l) or more for monodesethylamodiaquine (amodiaquine) were considered as threshold for in vitro Plasmodium falciparum resistance⁽¹⁾. In other words, complete inhibition of schizont growth at 4 pmol or less for chloroquine, 128 pmol or less for quinine and 2 pmol or less for momodesethylamodiaquine were considered as threshold for in-vitro Plasmodium falciparum susceptibility.

Calculation of IC₅₀

The IC₅₀ (50% inhibitory concentration) represents the concentration at which 50% of the isolates were inhibited from maturing to schizonts. The IC₅₀s for the individual

isolates were determined by the linear extrapolation method described by Freese et al.,⁽⁹⁾.

RESULTS

A total of 22 falciparum isolates were collected and cultured, but only 18 isolates were evaluated. The reasons for discarding the other tests were contamination and failure of schizonts to mature satisfactorily.

CHLOROQUINE

Of the 18 isolates evaluated, 16 (88.9%) were resistant with a median IC₅₀ of 0.60 µmol/l of blood (range 0.36 – 1.32 µmol/l of blood). The 2 (11.1%) other isolates were however susceptible with a median IC₅₀ of 0.27 µmol/l of blood. The concentrations are expressed in µmol/l of blood as the malaria parasite shows selective uptake of chloroquine.

Figure 1

Results of in-vitro microtests with Chloroquine

Drug Dose pmol/well	Drug Conc. µmol/l	No.(%)of isolates totally inhibited	% inhibition schizont maturation
1.0	0.20	0 (0%)	16.4%
2.0	0.40	0 (0%)	42.5%
4.0	0.80	2 (11%)	67.8%
8.0	1.60	2 (11%)	84.7%
16.0	3.20	6 (33%)	95.2%
32.0	6.40	14 (77%)	98.8%
64.0	12.80	18(100%)	100.0%

NB:

pmol/well = (p=pico=10⁻¹²)pmol/well is the dose of drug in each well before inoculation of parasitized blood cells.

µmol/l = (µ=micro = 10⁻⁶)µmol/l is the concentration of drug in a litre of blood realized after the inoculation of 50 µl of Blood Medium Mixture (BMM).

Both the doses/concentrations were given by the manufacturers as engraved on the WHO pre-dosed microtitre plates.

Figure 2

Chloroquine Drug Susceptibility profile of isolates of Plasmodium falciparum

Chloroquine Susceptible isolates			Chloroquine Resistant isolates		
No.	Media	Range	No.	Median	Range
	IC ₅₀	(μmol/l)		IC ₅₀	(μmol/l)
2	0.27	0.24–0.30	16	0.60	0.36–1.32

QUININE

Of the 18 isolates evaluated, 13 (72.2%) were susceptible to quinine with a median IC₅₀ of 1.8μmol/l of blood-medium mixture (BMM) (range 1.4–2.3μmol/l). 5 (27.8%) of isolates were however resistant with a median IC₅₀ of 4.7μmol/l (range 3.2 – 6.4 μmol/l). The concentrations are expressed in μmol/l BMM as the malaria parasite does not show selective uptake of quinine.

Figure 3

Results of in-vitro microtests with Quinine

Drug dose	Drug Conc.	No. (%) of isolates	% inhibition of
pmol/well	μmol/l	totally inhibited	schizont maturation
4.0	0.8	0 (0%)	8.95%
8.0	1.6	0 (0%)	40.95%
16.0	3.2	0 (0%)	64.78%
32.0	6.4	0 (0%)	82.50%
64.0	12.8	10 (55.5%)	93.37%
128.0	25.6	13 (72.2%)	96.99%
256.0	51.2	13 (72.2%)	98.70%

NB

pmol/well = (p=pico=10⁻¹²)mol/well is the dose of drug in each well before inoculation of parasitized blood cells.

μmol/l = (μ=micro = 10⁻⁶)μmol/l is the concentration of drug in a litre of blood realized after the inoculation of 50μl of Blood Medium Mixture (BMM).

Both the doses/concentrations were given by the manufacturers as engraved on the WHO pre-dosed microtitre plates.

Figure 4

Quinine Drug susceptibility profile of isolates of Plasmodium falciparum

Quinine susceptible isolates			Quinine resistant isolates		
No.	Median	Range	No.	Median	Range
	IC ₅₀	μmol/l		IC ₅₀	μmol/l
13	1.8	1.4–2.3	5	4.7	3.2–6.4

DIHYDROARTEMISININ

The cutoff concentrations for the determination of in-vitro resistance for artemisinin compounds have yet been established. This would be realized based on comparative in vivo and in vitro tests. However, a median IC₅₀ of 13.5nmol/l of BMM (range of 7.8 - 26 nmol/l) was realized. The concentrations are expressed in nmol/l of BMM as the malaria parasite does not show selective uptake of artemisinin.

Figure 5

Results of In-vitro microtests with Dihydroartemisinin

Drug dose	Drug Conc.	No. (%) of isolates	% inhibition of
pmol/well	nmol/l	totally inhibited	schizont maturation
0.15	3	0 (0%)	11.6%
0.5	10	0 (0%)	48.9%
1.5	30	0 (0%)	75.4%
5	100	8 (44%)	91.2%
15	300	11 (61%)	97.3%
50	1000	16 (88.8%)	99.5%
150	3000	18 (100%)	100%

NB

pmol/well = (p=pico=10⁻¹²)pmol/well is the dose of drug in each well before inoculation of parasitized blood cells.

nmol/l = (n=micro = 10⁻⁹)nmol/l is the concentration of drug in a litre of blood realized after the inoculation of 50μl of Blood Medium Mixture (BMM).

Both the doses/concentrations were given by the manufacturers as engraved on the WHO pre-dosed microtitre plates.

MONODESETHYLAMODIAQUINE

10 (55.6%) of the 18 isolates were susceptible with a median IC₅₀ of 0.09μmol/l of blood (range 0.08–0.10μmol/l). 2 (11.1%) of the isolates with median IC₅₀ of 0.096μmol/l had intermediate susceptibility. The remaining 6 (33.3%) isolates with a median IC₅₀ of 0.14μmol/l (range 0.11–0.19μmol/l)

were however resistant. The concentrations are expressed in $\mu\text{mol/l}$ of blood as the malaria parasites shows selective uptake of monodesethylamodiaquine.

Figure 6

Results of in-vitro microtests with Monodesethylamodiaquine

Drug Dose $\mu\text{mol/l}$	Drug Conc. $\mu\text{mol/l}$	No. (%) of isolates totally inhibited	% inhibition of schizont maturation
0.25	0.05	0 (0%)	13.67%
0.5	0.1	0 (0%)	48.88%
1.0	0.2	0 (0%)	77.74%
2.0	0.4	10 (55.5%)	93.29%
4.0	0.8	12 (66.6%)	98.00%
8.0	1.6	18 (100%)	100.00%
16.0	3.2	18 (100%)	100.00%

NB

$\mu\text{mol/well} = (\text{p=pico}=10^{-12})\text{mol/well}$ is the dose of drug in each well before inoculation of parasitized blood cells.

$\mu\text{mol/l} = (\mu=\text{micro} = 10^{-6})\mu\text{mol/l}$ is the concentration of drug in a litre of blood realized after the inoculation of 50 μl of blood.

Both the doses/concentrations were given by the manufacturers as engraved on the WHO pre-dosed microtitre plates.

Figure 7

Monodesethylamodiaquine Drug susceptibility profile of isolates of Plasmodium falciparum

Monodesethylamodiaquine susceptible isolates			Monodesethylamodiaquine resistant isolates		
No.	Median IC_{50}	Range $\mu\text{mol/l}$	No.	Median IC_{50}	Range $\mu\text{mol/l}$
10	0.09	0.08-0.10	6	0.14	0.11-0.19

Intermediate Susceptibility		
No.	Median IC_{50}	Range $\mu\text{mol/l}$
2	0.096	0.093-0.10

DISCUSSION

The resistance of Plasmodium falciparum to various antimalarial drugs has become a big concern as it poses a major obstacle to the control of malaria. This has been linked to recent increases in malaria morbidity and mortality⁽¹⁰⁾.

In this study, the level of chloroquine resistance was 88.9 %. In 2003, Abdullahi et al.⁽¹¹⁾ reported a 100% chloroquine resistance in Sokoto, while Sowunmi et al.,⁽¹²⁾ reported 81.4% chloroquine resistant strains from Ibadan. Elsewhere in Papua, Indonesia Maguire et al.,⁽¹³⁾ reported 74% chloroquine resistant Plasmodium falciparum from that part of the world. In Cameroon, a lower but moderate chloroquine resistant rate of 60% was reported by Bascol and Ringwald⁽¹⁴⁾.

Quinine, with a 72.2% susceptibility rate and moderate resistant rate of 27.8% suggests that the usefulness of the drug will not last for a long time. In 1998, Hambhanje⁽¹⁵⁾ reported a 10% quinine resistance strains from Papua New Guinea.

As for dihydroartemisinin, the significance of the broad variation observed in the IC_{50} values (7.8 – 26 nmol/l) cannot be adequately understood because discriminative values for resistance are not well – established. In their work, Cerutti et al.,⁽¹⁶⁾ reported a range of 0.3 – 2.63 ng/ml for arteether (artemisinin) but could not analyze it because of the reason proffered above. In the laboratory, artemisinin resistant forms have since been demonstrated⁽¹⁷⁾.

In conclusion, the results of this study revealed a high level and degree of chloroquine resistance, a dwindling susceptibility of quinine, and a moderate resistance to monodesethylamodiaquine.

Drug resistance will certainly be a problem for the future, and it is unlikely that the drug manufacturers will ever produce a drug which can be described as a flawless antimalarial with no possibility of resistance. Plasmodium falciparum has demonstrated that it is extremely good at adapting to any drugs we may use against it – and there is no reason to suspect that this would be different for the future if such drugs are not to become as clinically compromised as chloroquine.

Finally, the in vitro assays for assessing antimalarial drug sensitivity have become indispensable tools for the surveillance of drug resistance and the planning of therapeutic guidelines. However, the in vivo implications of these in vitro findings are not immediately known. It therefore becomes imperative for the FMOH to periodically check the antimalarial status of Plasmodium falciparum to various drugs in our environment by concurrently using both in vivo and in vitro techniques.

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Author Information

I.C. Peletiri

Department of Medical Laboratory Science, Ambrose Alli University

B.M. Matur

Department of Biological Sciences, University of Abuja

N.K.O. Ibecheozor

Department of Medical Microbiology & Parasitology, National Hospital

J.C. Ihongbe

Department of Medical Laboratory Science, Ambrose Alli University

T.T. Wakama

Department of Haematology, National Hospital