In vitro study assessing the response of plasmodium falciparum malaria to chloroquine, sulfadoxine/pyrimethamine, quinine and mefloquine in Wad Medani District, Sudan

Bakri Y. Nour, *Msc, PhD,* ***Ibrahim A. Faragalla,*** *MD,*
Osman K. Saeed, *MBBS, FRCP,* ***A. Abd Allah Mohamadani,*** *DCP, FRCPath.*

ABSTRACT

Objectives: To assess the *in vitro* response of Plasmodium falciparum malaria to chloroquine (CQ), sulfadoxine/pyrimethamine (SDX/PYR), Quinine (QU) and Mefloquine (MQ) and monitoring their resistance.

Methods: In 1999 to 2000, an *in vitro* study was carried out in Wad Medani district in Sudan. The standard protocol of the WHO in vitro micro-test Mark II was used for the selection of the subjects, the collection of blood samples, the culture techniques, the examination of the post-culture blood slides and the interpretation of the results.

Results: *In vitro* micro-test Mark II were performed on 62 *Plasmodium falciparum* isolates. Of these isolates, 42 produced successful schizonts growth. The data obtained showed that 29 of 42 isolates (69%) were CQ resistant with an effective concentrations (EC); EC50 = 399.621 nM, EC90 = 2754.145 nM and EC99 = 13284.967 nM to inhibit the schizont maturation, the values of SDX/PYR showed a flat regression line as an indication of *in vitro* reduced response with an EC50 = 0.262 nM, EC90 = 147.390 nM and EC99 = 25722.296 nM, and the response to the QU indicated only one of the 42 isolates (2.4%) was resistant with an EC50 = 150.085 nM, EC90 = 822.825 nM and EC99 = 3293.667 nM, while all the 42 isolates were sensitive to MQ with an EC50 = 190.763 nM, EC90 = 615.125 nM and EC99 = 1597.504 nM.

Conclusion: The results of this study revealed a high degree of *in vitro* resistance to CQ and reduced *in vitro* response to SDX/PYR and QU, while MQ was fully sensitive. The effective concentrations to inhibit 50, 90 and 99% of the parasite maturation were determined for antimalarial drugs efficacy monitoring surveillance in Sudan.

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In vitro testing to assess sensitivity to antimalarials is an epidemiological tool, useful for monitoring the evolution and spread of antimalarial drugs resistance and susceptibility profiles of *Plasmodium falciparum* isolates obtained from malaria patients. *In vitro* tests avoid many confounding factors, which influence *in vivo* tests by removing the parasites from the host and placing them into controlled experimental

From the Faculty of Medicine, Blue Nile Research and Training Institute, University of Gezira, Wad Medani, *Sudan*.

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Address correspondence and reprint request to: Dr. Bakri Y. Nour, Faculty of Medicine, Blue Nile Research and Training Institute, University of Gezira, PO Box 101, Wad Medani, *Sudan*. Tel. +249 511846183. Fax. +249 511842040. E-mail: bakrinour@hotmail.com

environment.[1] In the most frequently used procedure, the micro-technique, the parasites are obtained from a finger-prick blood sample and exposed in microliter plates to precisely known quantities of the drug under testing and observed for inhibition of maturation into schizonts.[2] Many *in vitro* studies were performed in Africa, South of the Sahara within the antimalarial monitoring programs.

The main aims of this study were for assessing baseline sensitivity and for monitoring the drug response of *Plasmodium falciparum* to 4 antimalarial drugs commonly used.

In Nigeria, an *in vitro* test results revealed 38.5% resistance to CQ, 28.6% to SDX/PYR and 7.7% to QU, while the MQ was fully sensitive.[3] In the south east of Gabon, *in vitro* activity of antimalarial drugs, showed that there was 95%, 10.2% and 47.5% resistance to CQ, QU and MQ.[4] In Madagascar, the results of an *in vitro* study showed that there was 5.8% resistance to CQ and 2% to MQ, and no resistance to QU.[5] In other parts of the world, the results of annual surveys (1986-1998) of *in vitro* sensitivity of isolates from Saudi Arabia showed an increasing frequency of *in vitro* CQ resistance ranging from 5-57% among the *Plasmodium falciparum* isolates.[6] In Brazil, *in vivo* and *in vitro* evaluation indicated that 95.5% of the isolates tested were resistance to CQ and showed low sensitivity to QU and high sensitivity to MQ.[7] In Sudan, many drug resistance evaluations were carried out for different antimalarial drugs used. In 1978, *in vitro* and *in vivo* studies were carried out in Sennar, Central Sudan, which detected less susceptible strains of *Plasmodium falciparum* to CQ.[8] In Eastern Sudan, both *in vivo* and *in vitro* tests were carried out in Gadarif at the end of the rainy season in 1986, the falciparum infections investigated showed 43% resistance to CQ at all levels (RI-RIII).[9] In 1992, *Plasmodium falciparum* isolates from 3 areas of Sudan were recovered from cryopreservation in London and the CQ sensitivity was determined by *in vitro*, CQ resistance was 6/6 in isolates from Khartoum, 1/4 from Sennar and 3/3 from Gadarif while all the isolates were sensitive to MQ.[10] In Gezira, a research was conducted in 1992 on CQ resistance of *Plasmodium falciparum* among Sudanese children (6 month to 16 years) living in Wad Medani, Central Sudan. All RI-III levels of resistance were confirmed, while the *in vitro* sensitivity test showed 25% CQ resistant.[11] The WHO protocol for 14 days follow-up was used in monitoring CQ resistance in 5 states in east, central and west Sudan, indicated that the CQ resistance was >25% in all 5 sentinel sites.[12]

Methods. In the period of September 1999 to March 2000, this study was conducted in Wad Medani town, which is located in the western bank of the Blue Nile river and approximately 187 kilometers south-east Khartoum, where the prevalence of malaria is mesoendemic with an unstable transmission pattern. Sixty-two isolates from uncomplicated *Plasmodium falciparum* malaria cases acquired the infection inside Wad Medani district, were collected for *in vitro* sensitivity to CQ, SDX/PYR, QU and MQ. According to the WHO *in vitro* micro-test,[13] the selected cases had a single infection of *Plasmodium falciparum*, with a parasite density of between 1000 and 80000 trophozoites per μL of blood. All patients did not receive any antimalarial drugs during the last 4 weeks. The urine tests were performed for the detection of antimalarials, using Dill Glazko test[13] for CQ, QU and MQ, and Lignin test[14] for SDX/PYR. Micro *in vitro* tests[15] (Mark II: supported by WHO, Manila, Philippines) were used to assess the response of *Plasmodium falciparum* to these 4 antimalarial drugs. Before starting the treatment, 200 μL of blood were collected from each patient by finger prick using sterile heparinized capillary tubes and added to 1.8 ml of RPMI 1640 culture medium with. L. Glutamine and HEPES buffer. From the blood medium mixture, 50 μL was distributed to each well in the tissue culture micro plates predosed with the tested drugs, starting with the control well (unpredosed) and moving down the respective column wells with increasing concentrations of each antimalarial drug. All micro plates were placed in a candle jar and incubated at 37^{o}C for 40-63 hours. Then, the RBCs layers were harvested on slides as a post culture blood film and stained with 1% solution of Giemsa stain pH 7.2 for 30 minutes and were examined under the microscope. Schizonts containing >3 merozoites for CQ, QU and MQ and 8 or more for SDX/PYR per 200 trophozoites were enumerated, they were considered successful if ≥10% of the parasites in the control well developed into schizonts. The schizonts maturation percentage in each well was calculated by dividing the schizonts count per 200 trophozoites in the control well and multiplied by 100, and the inhibition of schizont maturation percentage were calculated by subtracting the percentage of schizont maturation from 100. Resistance was indicated if schizonts growth appeared in the concentration of 8 pmol well of CQ, and 256 pmol well of QU and 64 pmol of MQ, for SDX/PYR, the break point was established in any well registering inhibition of 90% of schizonts with 8 or more merozoites.

Statistical package for Social Sciences Program (SPSS) version 7.5, California, the WHO (Wernsdorfer

and Wernsdorfer, April 1995) probit 18 for evaluation of grouped isolates were applied for analyzing the *in vitro* data obtained and determining the EC that inhibited 50, 90 and 99% of the parasite maturation and Chi-square test was used to compare between the results of the tested drugs.

This study was approved by the Research Ethical Committee of the Blue Nile Research and Training Institute/University of Gezira. Oral consent was taken from all subjects whose blood was taken for this *in vitro* study. All patients were treated immediately after blood sampling.

Results. Sixty-two males (60%) and females (40%) cases of *Plasmodium falciparum* malaria aged 6-65 years were enrolled in this study and screened for *in vitro* sensitivity to CQ, QU, MQ and SDX/PYR. The parasite count on the blood smears of these cases ranged from 2240 to 76800 per micro liter of blood with a mean of 26020 asexual parasites with variable sizes. Urine tests (Dill-Glazko and Lignin tests) were performed on 52 samples obtained and they were negative for 4-aminoquinoline and SDX/PYR, while 10 subjects did not obtain their urine samples. Of the 62 post-culture blood films, 14 showed no schizont growth, 6 showed inadequate schizont growth and 42 gave successful schizonts growth. These 42 isolates exhibited schizonts count between 20 and 152 per 200 asexual parasites with a mean of 42 schizonts. **Table 1** indicates the relative inhibition in each concentration of the 4 tested drugs, **Table 2** indicates the number and the percentage of the isolates showing complete inhibition, **Table 3** indicates the effective concentrations (EC) that inhibit 50%, 90% and 99% and **Table 4** indicates the comparison of the *in vitro* response of CQ, QU and MQ, and this revealed that the tested isolates were most sensitive to MQ followed by QU, the worst performance was with CQ (p=0.000). **Figure 1** shows the regression line related to the schizont maturation inhibition data obtained by the *in vitro* sensitivity to SDX/PYR among 42 isolates, and it is turned to the right side as an indication of reduced response. The data obtained from this study showed that: 29 of 42 isolates (69%) were CQ resistant with an EC of EC50 = 399.621 nM, EC90 = 2754.145 nM and EC99 = 13284.967 nM, the values for SDX/PYR showing a flat regression line as an indication of *in vitro* reduced response with an EC50 = 0.262 nM, EC90 = 147.390 nM and EC99 = 25722.296 nM, and the break point for SDX/PYR that inhibits 90% was established at 100/1.25 pmol concentration (well D). The response to the QU

Table 1 - Multi-drugs *in vitro* sensitivity data of 42 *Plasmodium falciparum* isolates from Wad Medani district, Gezira, Sudan. Relative inhibition (%).

Sub-heading	CQ	PYR/SDX	QU	MQ
Drug concentrations				
A	0	0	0	0
B	31.70	76.39	31.04	78.91
C	52.72	83.71	55.19	94.57
D	68.02	90.08	72.50	98.93
E	80.11	91.88	84.48	100.00
F	90.58	93.21	92.32	100.00
G	97.56	96.82	98.64	100.00
H	99.26	99.25	99.96	100.00

A - unpredosed well (control), B to H - represent different concentrations of antimalarial drugs (pmol) as follows:
B - CQ = 1, PYR/SDX =10/0.125, QU = 4, MQ = 2;
C - CQ = 2, PYR/SDX =30/o.375, QU = 8, MQ = 4;
D - CQ = 4, PYR/SDX = 100/1.25, QU = 16, MQ = 8;
E - CQ = 8, PYR/SDX = 300/3.75, QU = 32, MQ = 16;
F - CQ = 16, PYR/SDX =1000/12.5, QU = 64, MQ = 32;
G - CQ = 32, PYR/SDX =3000/37.5, QU = 128, MQ = 64
H - CQ = 64, PYR/SDX =10000/125, QU = 256, MQ = 128.
CQ - chloroquine, QU - Quinine, MQ - Mefloquine, SDX/PYR - sulfadoxine/pyrimethamine.

Table 2 - Isolates showing complete inhibition (n = 42).

Drug concentrations (wells)	No. of drugs concentrations (%)							
	Chloroquine		Sulfadoxine/ pyrimethamine		Quinine		Mefloquine	
B	0		3	(7.1)	0		8	(19)
C	2	(4.8)	3	(7.1)	2	(4.8)	17	(40.5)
D	5	(11.9)	13	(31)	2	(4.8)	31	(73.8)
E	13	(31)	15	(35.7)	6	(14.3)	42	(100)
F	15	(35.7)	18	(42.9)	14	(33.3)	42	(100)
G	28	(66.7)	23	(54.8)	31	(71.4)	42	(100)
H	37	(88.4)	35	(83.3)	41	(97.6)	42	(100)

Table 3 - Effective concentrations (EC) in nM.

Drug concentrations (wells)	EC50	EC90	EC99
CQ	399.621	2754.145	13284.967
SDX/PYR	0.262	147.390	25722.296
QU	150.086	822.825	3293.667
MQ	190.764	615.125	1597.504

CQ - chloroquine, QU - Quinine, MQ - Mefloquine, SDX/PYR - sulfadoxine/pyrimethamine

Table 4 - *In vitro* response of 42 *Plasmodium falciparum* isolates to chloroquine (CQ), Quinine (QU) and Mefloquine (MQ).

Tested drugs	No. of response (%)		Total
	Sensitive	Resistant	
CQ	13 (31)	29 (69)	42 (100)
QU	41 (97.6)	1 (2.4)	42 (100)
MQ	42 (100)	0	42 (100)

EC - effective concentrations

indicated one of 42 isolates (2.4%) was resistant with an EC50 = 150.085 nM, EC90 = 822.825 nM and EC99 = 3293.667 nM, while the 42 isolates showed full sensitivity to MQ with an EC50 = 190.763 nM, EC90 = 615.125 nM and EC99 = 1597.504 nM. The schizonts maturation was inhibited by the fourth concentration of MQ (well D-16 pmol concentration) of all the tested isolates, while it was not inhibited by other tested drugs even at the higher concentrations (H) of CQ, SDX/PYR and QU as indicated in **Table 2**.

Discussion. This study revealed a high degree of *in vitro* CQ resistance, similar to the previous reports from Khartoum and Gadarif in Sudan,[10] and higher than those reported from Sennar, Eastern Sudan and Gezira.[8-10] The data obtained from this *in vitro* study, when compared to the results of similar studies in endemic countries, CQ resistance level was higher than that found in Nigeria,[3] Madagascar[5] and Saudi Arabia,[6] and it was lower than that reported from the Gabon[4] and Brazil.[7] The QU sensitivity level was similar to the previous report on QU in Brazil,[7] and it was lower than that found in Nigeria[3] and Gabon.[4] The

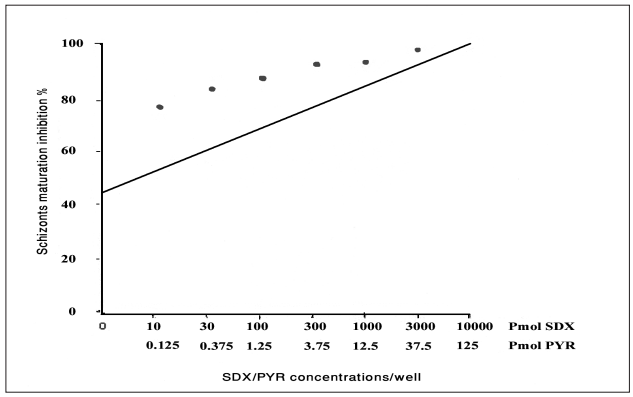

Figure 1 - Shows the regression line of 42 sulfadoxine/pyrimethamine (SDX/PYR) *Plasmodium falciparum* isolates determined by *in vitro* test.

reduced *in vitro* response to SDX/PYR demonstrated by this study was lower than that reported from Nigeria.[3] And the high sensitivity to MQ revealed by this study was similar to that found in Nigeria[2] and Brazil,[7] and it was in contrast to that detected in Gabon[4] and Madagascar.[5] The frequency of *in vitro* CQ resistance indicated in this study was not exactly similar to the result of *in vivo* CQ resistance obtained by another study in the same study area (41.5%),[12] this may be explained by the impact of individual host immunity on the *in vivo* monitoring of CQ resistance. When this research was initiated, blood specimens infected with *Plasmodium falciparum* were adapted to culture conditions and incubated for 24-30 hours as a pilot survey. It was found that the primary isolates failed in ending schizogony within 24-30 hours incubation period as recommended by the WHO,[13] but when incubated for an extended period (36-45 hours), resulted in a successful schizont maturation, which supported the observation and reports on a previous study[16] and noted by researchers in the Solomon Island where they found that there was a variation in the incubation time (26-63 hours) to reach the schizont maturation.[17] This study revealed a high degree of resistance and reduced response to the most affordable antimalarial drugs, CQ and SDX/PYR (**Tables 1 to 4** and **Figure 1**), the CQ with EC90 and EC99 (**Table 3**) was a long way beyond the critical threshold of 1000 nM for EC99. The EC99 for QU falls into the borderline area and this probably the result of liberal use of QU in the study area.

This study concluded that, CQ can no longer be considered an adequately effective therapy for clinical *Plasmodium falciparum* malaria, and the policy makers should be aware about the reduced *in vitro* response to SDX/PYR and QU as an indication for the future *in vivo* resistance.

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