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The prevalence and degree of resistance of *Plasmodium falciparum* to first-line antimalarial drugs: an in vitro study from a malaria endemic region in Yemen

Hassan Al-Shamahy, Abdulilah Hussein Al-Harazy, Nabil S. Harmal, Abdulgodos M. Al-Kabsi

From the Department of Medical Microbiology, University of Sana'a, Sana'a, Yemen

Correspondence and reprint requests: Hassan A. Al-Shamahy, MD · Department of Medical Microbiology · University of Sana'a · P.O. Box 775 · Sana'a · Yemen · T: +967-1-239-551 · shmahe@yemen.net.ye · Accepted for publication May 2007

BACKGROUND: Unpublished studies on antimalarial drug efficacy have found low levels of chloroquine resistance in Yemen. This study was carried out to determine the current prevalence of drug resistance in *Plasmodium falciparum* in Yemen to the main anti-malarial drugs and to determine the effective concentration (EC) values.

METHODS: The WHO standard protocol was used for the selection of subjects, collection of blood samples, culture techniques, examination of post-culture blood slides and interpretation of results. The in vitro micro-test Mark III was used for assessing susceptibility of *P. falciparum* isolates.

RESULTS: The criteria for blood parasite density was met by 219 *P. falciparum* malaria patients. Chloroquine resistance was found in 47% of isolated *P. falciparum* schizonts. Mefloquine resistance was found in 5.2%. In addition, the EC50 and EC95 values in blood that inhibited schizont maturation in resistant isolates were higher than the normal therapeutic level for mefloquine. No resistance occurred against quinine or artemisinin, with no growth at the cut-off level for quinine and inhibition at low concentrations of artemisinin.

CONCLUSION: Our study confirmed the occurrence of chloroquine-resistant *P. falciparum* and a slow increase in the rate of this resistance; it is likely that resistance will increase further and spread over all the foci of malaria in Yemen. The low rate of mefloquine-resistant *P. falciparum,* was lower than that reported in Africa or Southeast Asia, but it is the first report of mefloquine resistance in Yemen. Finally, the isolates were sensitive to low concentrations of quinine and artemisinin.

Malaria is the most important parasitic disease of man. The human disease is a protozoan infection of red blood cells transmitted by the bite of the blood-feeding female anopheline mosquito. Approximately 270 million people suffer from malaria, and there are between one and 2.5 million deaths each year, mostly among African children.[1]

Antimalarial drugs fall into three broad groups: the quinoline-related compounds (quinine, quinidine, chloroquine, amodiaquine, halofantrine, primaquine), the antifols (pyrimethamine, proguanil, chlorproguanil, trimethoprim), and artemisinin compounds (artemisinin, artemether, artesunate). Of these, the artemisinin drugs have the broadest time window of action on the asexual malaria parasites, from the medium-sized rings to early schizonts, and produce the most rapid therapeutic responses.[2,3]

The problem of drug resistance is most important with *Plasmodium falciparum.* There is widespread but patchy resistance to proguanil and pyrimethamine wherever *Plasmodium falciparum* occurs. Chloroquine resistance has been known for years in parts of Southeast Asia, South America and Oceania. In the past decade, the prevalence and degree of chloroquine resistance have increased greatly in sub-Saharan Africa, at first in the east and later in the west of the continent.[4,5] In Yemen, several unpublished studies on antimalarial drug efficacy were carried out in the past two decades in the southern parts of the country by WHO consultants for malaria control. Their results showed a low level of chloroquine resistance, and although it appears to be not totally resistant, chloroquine remains a useful first-line therapy in Yemen.[6,7]

The main purpose of this study was to describe the state of drug-resistant malaria and to outline current thinking regarding strategies to limit the advent, spread

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and intensification of drug-resistant malaria in Yemen.

SUBJECTS AND METHODS

This study was conducted between August 2002 and March 2003. Patients of both sexes between the ages of 3.5 and 45 years living in Tihamah (Bajil city and surrounding villages) in the Republic of Yemen suffering from fever and/or other malaria symptoms were screened at field hospitals and malaria control centers. Rainfalls during 2002-2003 were recorded between May and October.

No selectivity parameters were taken except those required for the Mark III in vitro test technique.[8] Informed consent was obtained from patients or their parents. Fever was defined as a body temperature of 37.5°C or above. Patients were asked for any history of diarrhea and vomiting.

Any history of antimalarial drug ingestion within the past 2-4 weeks was an exclusion criteria. Only patients with negative urine tests to four aminoquinolines and sulphonamides were enrolled. Patients with complicated disease such as cerebral malaria, anemia, jaundice and bronchopneumonia were excluded. Enrolled patients were registered and given special codes. The diagnosis for all the patients was confirmed by the presence of asexual forms (trophozoite) of *P. falciparum* in the peripheral blood smear (thick film) and by the immunochromatography (HRP2) method as confirmation. Patients with mixed infections (*P. falciparum, P. malariae* or *P. ovale*) were excluded from the study.

Patients whose parasite count was not less than 1 000 and not more than 80 000 asexual parasites per mm³ of blood were selected. The WHO in vitro antimalarial drugs susceptibility test (Mark III) technique[8] was used to determine the antimalarial drugs susceptibility of the *P. falciparum* isolates. The first choice drugs for malaria, which include chloroquine, quinine and artemisinin, were tested.

Data analysis was carried out using test-record sheets and the porbit analyzing program (WHO analyzing program) for interpretation of results.

RESULTS

During the 12 months of the study period, 219 Plasmodium falciparum malaria patients met our study criteria for parasite density in their blood. They comprised 156 males and 63 females; 183 passed the urine test for antimalarial drug absence (Table 1). For the 602 tests, the rate of successful schizont growth for the four drugs was 42% (Table 2). The frequency and percentage of *P. falciparum* schizont maturation for the different concentrations of the antimalarial drugs is shown

Table 1. Urine test results and patients selected for study.

	Tested	Positive urine test for anti-malarial drugs	Patients included in the study
Male	156	27	129
Female	63	9	54
Total	219	36	183

Table 2. Successful schizont maturation (positive culture), negative cultures and bacterial contamination.

Drug	Positive cultures	Negative cultures	Contaminated cultures	Total
Chloroquine	100	25	58	183
Mefloquine	58	36	71	165
Quinine	51	20	67	138
Artemisinin	47	12	57	116
Total	256	93	253	602

Number of cultures.

Table 3. The frequency and percentage of *P. falciparum* schizont maturation in the presence of different concentrations of the four antimalarial drugs.

Wells	Chloroquine*		Mefloquine**		Quinine***		Artemisinin****	
	n	%	n	%	n	%	n	%
B	4	4	3	5.2	6	11.8	3	6.4
C	9	9	25	43.1	15	29.4	13	27.7
D	40	40	19	32.8	17	33.3	17	36.2
E	31	31	8	13.8	10	19.6	8	17.0
F	9	9	3	5.2	1	2.0	6	12.8
G	5	5	0	0.0	2	3.9	0	0.0
H	2	2	0	0.0	0	0.0	0	0.0
Total	100	100	58	100.0	51	100.0	47	100.0

*The chloroquine dilution was from 1.0 to 64.0 pmol/per well; the cut-off value indicating resistance is 8.0 pmol per well (well E).
**The mefloquine dilution was from 2.0 to 128.0 pmol/per well; the cut-off value indicating resistance is 32 pmol per well. (Well F)
***The quinine dilution was from 4.0 to 256.0 pmol/per well; the cut-off value indicating resistance is 256 pmol per well. (Well H)
****The artemisinin dilution was from 0.15 to 150 pmol/per well, the cut-off value indicating resistance is not yet established.

in Table 3. The level of chloroquine resistance (cut-off indicating resistance) was calculated to be 8 pmol/well. At this cut-off, 47% of isolates showed resistance to chloroquine. The chloroquine values show the growth of five different schizonts at five different concentrations of chloroquine, which suggests the occurrence of

Table 4. In vitro response of *Plasmodium falciparum* isolates to chloroquine, mefloquine, quinine and artemisinin.

Tested drugs	Sensitive		Resistant		Total
	n	%	n	%	
Chloroquine	53	53	47	47	100
Mefloquine	54	94.8	3	5.2	58
Quinine	51	100	0	0	51
Artemisinin	47	100	0	0	47

Table 5. Effective concentration (EC) in µmol/L blood for all isolates and for sensitive (S) and resistant (R) isolates.

	EC50			EC90			EC95			EC99		
	S+R	S	R	S+R	S	R	S+R	S	R	S+R	S	R
Chloroquine	1.9	1.6	3.0	8.1	5.0	14.8	12.2	6.9	23.3	26.4	12.6	54.4
Mefloquine	1.5	1.5	0.6	6.3	6.2	13.7	9.4	9.2	33.1	20.2	19.4	71.8
Quinine	4.5	-	-	15.0	-	-	21.1	-	-	40.0	-	-
Artemisinin	0.1	-	-	0.9	-	-	1.6	-	-	5.1	-	-

five different strains of *P. falciparum* among our study patients. The cut-off for mefloquine was calculated to be 32 pmol/well with 5.2% of tested schizonts showing resistance to mefloquine. No resistance against quinine or artemisinin occurred in our study as indicated by no growth at the cut-off level (Table 3). Table 4 shows the schizont maturation rate for all drugs. Effective concentrations (EC) in blood for sensitive and resistant isolates are shown in Table 5. The EC50 and EC95 values for mefloquine that inhibited schizont maturation in resistant isolates were higher than the normal therapeutic level for mefloquine.

DISCUSSION

In our study, the first reason for selecting in vitro testing was that the alternative in vivo test used for the same purpose by WHO may be influenced by host factors such as malabsorption, immunity and other unpredictable factors, whereas our tool is not affected. In addition, the interpretations of the WHO in vivo test may be complicated in the case of resistance type I by re-infection during the 28-day follow-up period. The second reason is that in vitro testing can be done to test several types of drugs and drug combinations with different concentrations, while in vivo tests cannot. The third reason is that in vivo testing may lead to severe progression in a patient if the *P. falciparum* resists the tested drug, which may lead to complication and life-threatening infection, while with in vitro testing the patient

can be treated with suitable drugs after collection of the specimen, and the failure of inhibition of *P. falciparum* in the well would not effect the patient's situation. In vitro testing was performed 183 times for chloroquine, 165 times for mefloquine, 138 times for quinine and 116 times for artemisinin. In total, 42.5% of in vitro testing of schizont maturation was successfully recorded, while 57.5% were affected by bacterial contamination or failure of schizont growth. Bacterial contamination occurs easily in field studies where the tests are carried out in remote areas without enough facilities to make the place of work sterile enough to prevent contamination of the culture wells. Cross-contamination during plate disposing and/or during harvesting procedures also can be a factor. In addition, the original contamination of the plates during preparation by the producer could not be excluded. Similar problems of high contamination have faced other researchers in malaria foci in Africa and Southeast Asia.[9]

The second problem was the failure of growing schizonts when there was no bacterial contamination. This might be due to the presence of antimalarial drugs in patient plasma in spite of negative results for the presence of antimalarial drugs in urine. Several recent reports confirm this suggestion and refer these phenomena to the long half-life of antimalarial drugs in plasma despite disappearance from urine. In addition, this failure may be related to technical errors (which are also quite common) such as pH change of the medium,

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direct contact of the BMM with ice during transfer and/or incubation interruption.[10]

Chloroquine

In Yemen, the occurrence of chloroquine-resistant *P. falciparum* strains were noticed in the early 1990s. In the last year, WHO and the National Malaria Control Program conducted an in vivo study to estimate chloroquine resistance in Tihamah, and their results showed that in more than 40% of the sample there was treatment failure in either early or late stages.[6,7] The WHO and our study confirmed the occurrence of chloroquine-resistant *P. falciparum* and indicated that the rate was rising slowly, but eventually was expected to increase and spread over all the foci of malaria in Yemen due to several reasons, the main reason being that chloroquine is one of the most widely consumed drugs in Yemen.[6,7] Others reasons are the high frequency of self-medication, inadequate dosing, and sub-therapeutic levels in the blood. These factors are believed to be predominant factors that contribute to chloroquine resistance in P. *falciparum*, not only in Yemen but also in most third world countries.[11,12]

One of the main aims of our study was to determinate the EC95. This result was needed to estimate the effective therapeutic drug level in the human body. Our results showed the EC95 for chloroquine was 12.26 µmol/L of blood. This result indicates a high resistance of *P. falciparum* for chloroquine among the study cases, and this is confirmed by the EC95 which was equal to the 1.5 times (8 µmol/L blood) the therapeutic level required for treating cases of *P. falciparum* worldwide by chloroquine.[2,11,13]

Mefloquine

Mefloquine is a quinoline methanol compound available only for oral administration. It has a terminal elimination half-life of 2 to 3 weeks in patients with malaria.[14,15] *P. falciparum* mefloquine resistance has been reported from Southeast Asia and Africa.[16,17] In Yemen, no research has been done to study mefloquine resistance, so our study is the first in vitro test of mefloquine and the first to report the presence of mefloquine-resistant *P. falciparum* in Yemen. The resistance rate in our study was 5.6%. Our prevalence rate was lower than those reported from Africa (range from 20% to 65%) and from Southeast Asia (range, 30 to 80%).[18,19] Of the 11 isolates of mefloquine-resistant strains, 8 exhibited successful growth in 16 pmol/well (equivalent to 3.2×10^{-6} mol/L blood), which is the therapeutic level, and three isolates show a high degree of resistance growing in the 32 pmol/well (equivalent to 6.4×10^{-6} mol/L blood), which

demonstrates that the mefloquine-resistant strains can adapt up to double the therapeutic level. In addition, the EC50 (1.4971 µmol/L blood) and EC95 (4.4971 µmol/L blood) confirmed resistance since these values are higher than the normal therapeutic level for mefloquine.[20]

Quinine

Reports of quinine resistance are still rare in Middle East countries, but a high rate of true resistance has been reported from Thailand (50%) and clinical failure of the quinine test has been reported from Africa.[21,22] In our study, no quinine resistance occurred and isolates of *P. falciparum* are still sensitive to quinine even at low concentrations of the drug, since the data show that the (SMI) schizont maturation index percentage of 97.6 occurred at 0.64 µmol/L blood. The high efficiency of quinine in Yemen is different from that reported in East Africa and other Middle East countries where there is a steady increase in the time parasites take to disappear from the blood after treatment with quinine.[21,22]

Artemisinin

The most important development in recent years has been the discovery and development of drugs related to artemisinin in China.[23] This drug is the best drug available for treatment of severe malaria in malaria drug-resistant *P. falciparum* areas. No resistance occurred in our study to artemisinin and the tested isolates were still very sensitive to low doses of the drug. Our result is similar to results reported from malaria foci worldwide where no artemisinin primary resistance was reported for the SMI (96%) of the isolates at 5.0 pmol/well and the EC95 was 1.64 pmol/well.

Our study confirmed both the occurrence of chloroquine-resistant *P. falciparum* and a slow increase in the rate of this resistance. It is likely that resistance will increase further and spread over all the foci of malaria in Yemen. It is recommended that the policy of treatment of malaria infection in the foci of chloroquine resistance be modified. The use of the combination of pyrimethamine/sulfadoxine as the first choice of treatment is recommended to avoid the spread of chloroquine-resistant *P. falciparum*. Investigation of the prevalence and degree of resistance of *P. falciparum* in malaria endemic foci in Yemen, and continual monitoring of the increasing resistance rate, is recommended as well. A molecular study to find out the drug-resistant *P. falciparum* strains in Yemen, which this study suggests to be five in number, is also needed. A low rate of mefloquine resistance in *P. falciparum* is present in Yemen, and it is lower than rates

reported from Africa and Southeast Asia. To prevent the further development of mefloquine-resistance in Yemen, the drug should never be used in isolation and a combination with sulfadoxine and pyrimethamine should be used. Finally, no quinine or artemisinin resistance occurred and isolates of *P. falciparum* are still sensitive to quinine and artemisinin even at low concentrations of both drugs. The SMI percentage, EC90, and EC95 of both drugs should be evaluated from time to time and correlated with previous results. This will provide good information about the situation of these drugs in Yemen as well as for other drugs and combinations.

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