PLASMODIUM VIVAX MALARIA IN SOUTHEAST IRAN IN 1999-2001: ESTABLISHING THE RESPONSE TO CHLOROQUINE *IN VITRO* AND *IN VIVO*

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Abstract. Chloroquine-resistant *Plasmodium vivax* is emerging in Oceania, Asia and Latin America. The drug sensitivity of *P. vivax* to chloroquine both *in vivo* and *in vitro* in the southern part of Iran was assessed; chloroquine-resistant *Plasmodium falciparum* has already been documented in this area. The *in vitro* sensitivity of 39 *P. vivax* isolates was assessed: the mean IC50 and IC90 were 189 ng/ml and 698 ng/ml blood respectively; for *in vivo* testing, all 39 vivax malaria patients were treated with a standard regimen of chloroquine and followed-up at 28 days: the mean parasite clearance time was 67.2±22.5 hours. The *in vitro* development of young parasites to mature schizonts in standard test medium was compared with that obtained in McCoy's 5A medium: no significant difference was observed. Synchronization of the blood-stage parasites was performed according to Lambros' method: the method was not suitable because it was detrimental to the parasites. A number of *in vitro* tests were performed using both our own laboratory-predosed microplates and WHO microplates: there was no significant difference between the results.

INTRODUCTION

Plasmodium vivax is the second most common cause of malaria. Chloroquine (CQ), a 4-aminoquinoline antimalarial, has been the drug of choice for the treatment of vivax malaria for more than 40 years in many parts of the world (Collins and Jeffery, 1996). CQ is well absorbed, well tolerated and inexpensive; CQ rapidly and effectively eradicates blood-stage parasites, usually within 36-72 hours. However, in the past decade CQ-resistant *P. vivax* has been found in several countries; the first indication that *P. vivax* might be developing resistance to CQ was the failure of an 8-month-old infant to respond adequately to treatment with chloroquine in Papua New Guinea (Schuurkamp *et al*, 1989); subsequent reports have provided further confirmation of the presence of CQ-resistant vivax malaria in the Southwest Pacific, Indonesia, Brazil, Myanmar and, recently, Colombia (Whitby *et al*, 1989; Baird *et al*, 1991; Canessa *et al*, 1992; Marlar *et al*, 1995; Soto *et al*, 2001).

Malaria is an important infectious disease in Iran; its transmission is prevalent in three of the country's southeastern provinces. According to the records of the Division for Prevention and Control of Diseases, the number of annual cases has fallen in recent years: from 96,340 in 1991 to 22,854 in 1999; this reduction may be attributed to the implementation of malaria control measures and several notably dry seasons. The spread and the intensification of the resistance of malaria parasites to antimalarials are the principal concerns that must be addressed by global malaria control initiatives. Whereas the *in vitro* and *in vivo* resistance of *P. falciparum* to CQ have been

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reported from Iran (Edrissian and Shahabi, 1985), the *in vitro* sensitivity of *P. vivax* to CQ has not been established in Iran. For this reason, this study aimed to determine both the *in vitro* susceptibility of *P. vivax* to CQ and the correlation of *in vitro* responses with *in vivo* and therapeutic outcomes.

MATERIALS AND METHODS

Study site

The study was undertaken between July 1999 and November 2001 at the Tehran University of Medical Sciences Training Center in Bandar Abbas, Hormozgan Province, an area with multidrug-resistant *P. falciparum*. The Subcommittee of Research Affairs of the Tehran University of Medical Sciences approved the research proposal. Hormozgan Province (Lat 25-28ºN; Long 52-59ºE) is hot and humid: during the past decade the average maximum and minimum daily temperatures have been 33ºC and 27.5ºC respectively; the average daily relative humidity during the decade has been 79% (morning) and 55% (afternoon). *P. vivax* is predominant, causing 75% of the cases of malaria. The most important vectors are *Anopheles stephensi* and *An. fluviatilis*, which are believed to be resistant to some insecticides. Active and passive case detection, appropriate treatment, and residual spraying and larviciding are the main malaria control measures applied in the province. Imported cases among Afghan refugees are not uncommon.

Patients

Forty patients were enroled in the study; the age range was 15-59 years; the mean age (±SD) was 27.2 (±9.1) years. There were 35 (87.5%) male and 5 (12.5%) female subjects; the body weight range was 49-85 kg. Prior to admission, the patients signed consent forms. Inclusion criteria were: vivax malaria infection for the first time; parasite density between 2,000-35,000/µl with 90% young trophozoites; no treatment with antimalarials during the 30 days prior to admission. Exclusion criteria were: pregnancy; mixed infection with *P. vivax* and *P. falciparum*. The recent history of antimalarial use was verified by the Dill Glazco urine test (Black *et al*, 1981): this was routinely performed, and patients with urine positive for CQ were excluded from the study.

Treatment

The patients were treated with a standard regimen of CQ (25 mg/kg over 3 days): 600 mg base initially (0 hrs) followed by 600 mg base at 24 hours and then by 300 mg base at 48 hours; in order to achieve a radical cure, primaquine was administered daily (0.25 mg/kg) for 14 days starting on day 14. All medication was administered under supervision and was taken with water. The *in vivo* testing was performed in accordance with World Health Organization guidelines (Payne, 1982; WHO, 1990), with the purpose of determining the parasite clearance time and the probable time of reappearance of the parasite. Parasite clearance time (PCT) was defined as the time from the start of CQ treatment until blood smears became negative for at least 24 hours.

Parasite count

Parasite counts were made at time zero and then once a day until day 7, and then once a week for 3 weeks until day 28. Blood smears were stained with Giemsa's stain and examined with a microscope; asexual parasites were counted against 1,000 RBCs in thin blood films or against 200 WBCs in thick blood films; the smear was reported negative only if at least 200 fields of a thick smear had been examined.

In vitro sensitivity assay

Prior to the commencement of treatment, the *in vitro* susceptibility of *P. vivax* to CQ was determined by using the micro-technique of Brockelman *et al* (1989). Briefly, 1 ml of each patient's venous blood was collected in a test tube containing 1.0 ml of Waymouth medium (Gibco BRL) and 40 IU heparin (Gibco BRL); the sample was centrifuged at 500*g* at 4ºC for 3 minutes, after which 1ml of the plasma-medium mixture was removed; following blood-

agitation, 0.2 ml was mixed with 1.8 ml of test medium (RPMI medium plus Waymouth medium in a ratio of 2:1, supplemented with 10% (v/v) human heat-inactivated AB serum) and dispensed in 50 µl volumes into the wells of microplates in a vertical order from well A to well H, with a duplicate of each sample. The morphological endpoint, which was used as an index of maturation, was schizonts containing 8 or more clearly defined nuclei; this endpoint provided a satisfactory means for the *in vitro* assessment of the maturation of asexual vivax parasites.

All *in vitro* tests were carried out on WHO microplates (Manila-Philippines); McCoy's 5A medium was used separately, in order to allow the comparison of the results obtained *in vitro* using this medium with those obtained using the mixed medium mentioned above. *In vitro* susceptibility of 10 isolates was also assessed using both the WHO and our own laboratory-made predosed microplates (Chloroquine diphosphate salt; Sigma C6628, Lot 95HO355) simultaneously, allowing the comparison of the results of both techniques. The chloroquine concentrations of wells B to H were 1,2,4,8,16,32 and 64 pmol respectively; well A had no drug and served as a control (concentration in pmol may be expressed as ng/ml blood as follows: 64, 128, 256, 512, 1,024, 2,048, and 4,096). In another series of experiments, designed to synchronize the blood-stage parasites, the method of Lambros and Vanderberg (1979) was used: the development of parasites to mature schizonts in sorbitol-treated blood is compared with the development of parasites in untreated blood.

Statistical analysis

Log dose-probit analysis was used to determine the *in vitro* susceptibility of parasites to CQ; the results were expressed as IC_{50} and IC_{90}; a paired *t*-test was used for comparison of the means; the Mann-Whitney U test was used to compare the percentage inhibition of the laboratory-made and the WHO microplates. All statistical tests were considered significant at a p-value of less than 0.05 and were performed with SPSS for Windows version 7.5.

RESULTS

Clinical response

Forty patients were recruited to the study; all the patients responded well to the regimen of CQ, as shown by the complete resolution of parasitemia, usually within 48-72 hours. No reappearance of *P. vivax* parasitemia was observed in any patient at follow-up at 28 days. Parasitemia was cleared in 1 (2.5%), 17 (42.5%), 13 (32.5%), 7 (17.5%) and 2 (5%) patients within the first 24, 48, 72, 96, and 120 hours respectively. The mean PCT was 67.2 hours (SD 22.5); the range of PCT was 24-120 hours (Fig 1). *P. falciparum* was detected in one patient's blood on day 17: this case was treated according to the Iranian national policy for CQ-resistant *P. falciparum* (Edrissian *et al*, 2001) with 10 mg/kg quinine 8 hourly for 3 days and a single dose of sulfadoxine-pyrimethamine (25 mg/kg SDX+1.25 mg/kg PYR) and excluded from the study.

Sensitivity assay *in vitro*

The *in vitro* cultivation of *P. vivax* in the presence and the absence of CQ (control well) showed the development of young-stage parasites to schizonts within 32-40 hours, the in-

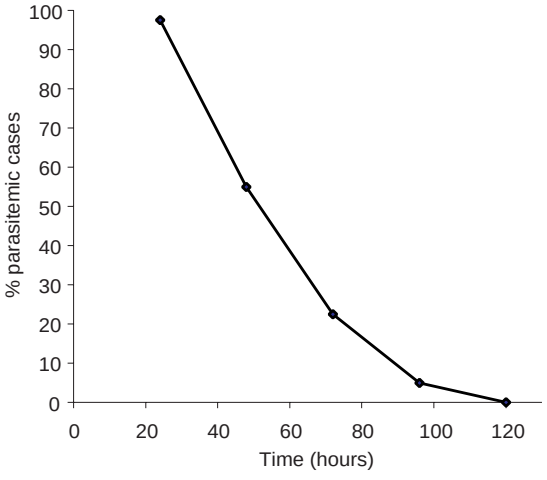

Fig 1–Parasite clearance time of vivax malaria patients from Bandar Abbas, Iran.

cubation period depended on the initial parasite stages; the growth into mature schizonts was inversely related to the level of chloroquine. The CQ susceptibility assay of pretreatment isolates was successful in 31 samples (79.5%) and 8 samples did not develop mature schizonts (20.5%). The mean number of mature schizonts in the control well was 86, whereas the mean numbers in wells B to H were 78, 57, 28.7, 7.5, 4.8, 3.0 and 0.81 respectively. Chloroquine at 256 ng/ml (well D) completely inhibited the development of young stages in 2 of 31 isolates (Fig 2), whereas well E (512 ng/ml) inhibited the development of young stages in 8 of 31 isolates; parasites in the remaining 23 isolates developed to mature schizonts with different rates of success, ranging from 1-34. At concentrations of 2,024 and 4,096 ng/ml (wells G and H) a few parasites differentiated, resulting in an average of 3 and 0.8 mature schizonts respectively.

IC_{50} was 189 ng/ml (95% confidence limit 126-264) and IC90 was 698 ng/ml (95% confidence limit 467-1384). The regression line of logarithmic concentration-response to CQ of the pretreatment isolates is shown in Fig 3.

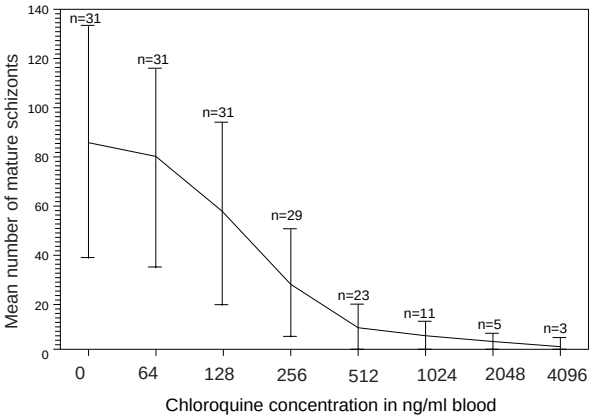

Fig 2–Chloroquine susceptibility of *P. vivax* as shown by the mean number of mature schizonts.

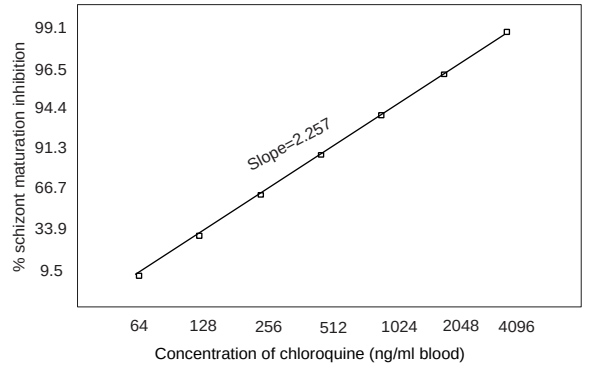

Fig 3–Logarithmic concentration-response curve for the schizont maturation inhibitory effect of chloroquine (CQ) on *P. vivax* isolates before treatment of the patients.

Comparison of microplates and test media

The results obtained using the WHO microplates and the laboratory-predosed plates are summarized in Table 1; although the IC50 and IC90 were somewhat higher for the laboratory-predosed plates, statistical analysis

Table 1
In vitro susceptibility of 10 isolates of *P. vivax* to chloroquine using WHO and laboratory-predosed microplates.

	WHO micropate	Laboratory-predosed microplate
IC_{50}	205.12 ng/ml	220.05 ng/ml[a]
95% confidence limit	65-425.8	122.4-352.8
IC_{90}	720.5 ng/ml	753.8 ng/ml[b]
95% confidence limit	361.4-7,363.1	450.1-2,260.8
Regression coefficient	2.34	2.39

[a]Not significantly different from WHO result; p>0.05 (Mann-Whitney U test)
[b]Not significantly different from WHO result; p>0.05 (Mann-Whitney U test).

Table 2
Paired comparison test of amount of mature schizonts from six isolates of *P. vivax* cultured *in vitro* using two different test media.

	RPMI+Waymouth	McCoy's 5A
	30	35
	101	74
	152	132
	33	57
	21	13
	159	151
X±SD	82.6±25.8	77±22.1

The difference between means of two groups is not significant (p=0.481) by paired *t*-test. Each number represents the number of mature schizonts per 200 asexual parasites.

Table 3
Paired comparison of number of parasites from sorbitol-treated and untreated *P. vivax* originating from same isolates.

Isolate no.	Initial parasite density	Sorbitol-treated blood
Pv 31	68	18
Pv 33	53	19
Pv 34	32	10
Pv 35	168	23
Pv 37	62	15
Pv 42	147	21
Pv 43	129	27
X±SD	94.1±52.8	19±5.5

The difference between the means of the two groups is significant (p=0.006) by paired *t*-test. Each amount represents the number of parasites per 200 a sexual parasite after 32-40 hours *in vitro* cultivation.

showed no significant difference between the two plates. Six isolates of *P. vivax* were cultivated in two different media: a standard test medium and McCoy's 5A. The development of young parasites to schizonts was evaluated and the results are shown in Table 2.

Synchronization

The use of the method of Lambros and Vanderberg (1979) for the synchronization of blood-stage *P. vivax* was not appropriate: sorbitol-treated infected red blood cells did not develop well into mature schizonts; furthermore, the parasite density was clearly diminished (79.8%) (Table 3).

DISCUSSION

The present study was carried out in an area with multidrug-resistant *P. falciparum*, the southern part of Iran. The aim of the study was to establish the *in vitro* susceptibility of *P. vivax* to CQ. The *in vitro* microtest applied in this study was reliable and reproducible and variation in the development of parasites had little influence on the results. This report confirms that the use of this method allows the evaluation of the drug sensitivity of *P. vivax* by microscopy. The presence of different parasite stages in patients' circulation is typical of *P. vivax* and is a major constraint upon those seeking to work with it; this problem was addressed, in this study, by the selection of malarial patients whose blood contained over 90% young parasites. The results of *in vivo* and *in vitro* testing showed that the current CQ regimen is still sufficiently effective: the parasites were cleared usually within 48-72 hours and no parasites had reappeared by follow-up at 28 days. In this study, follow-up at 28 days was chosen because recurrence within 28 days is indicative of chloroquine failure, whereas recurrence after 28 days from initial infection may represent primaquine failure (*ie* relapse) rather than chloroquine failure.

The IC_{90} value obtained in this study is less than that established in Thailand: 698 ng/ml *vs* 947 ng/ml (Tan-ariya *et al*, 1995). The considerable interval between 95% confidence limits of both the IC_{50} and the IC_{90} values shows the heterogeneity of the isolates; while the response of the parasite to CQ in the majority of isolates was good, the susceptibility of some isolates to the drug was reduced substantially

– these isolates developed a great number of mature schizonts, even in wells G and H, resulting in an IC_{50} and an IC_{90} of over 311 and 3,281 ng/ml blood respectively. The variation in response to the drug suggests the presence of different strains of *P. vivax* in the region and the patients may have been infected with strains of *P. vivax* that differed in their sensitivity to CQ (inter- and intra-heterogeneity of isolates). Furthermore, the range of PCT was 24-120 hours: this finding confirms the heterogeneity of the parasite strains. Because there is no sequestration in *P. vivax* malaria, the clearance of circulating parasites can be used as a direct measure of their eradication; as there was no reappearance of parasites within 28 days, it appears that *P. vivax* in Iran is still susceptible to CQ. Because primaquine has some blood schizonticidal efficacy and may mask CQ failure (Pukrittayakamee *et al*, 1994; 2000) primaquine was started on day 14, and therefore any parasitemia during the 14 days after beginning CQ therapy indicated CQ failure.

Although the IC90 in this study was less than that found in Thailand, the mean PCT in Iranian malarial patients is longer than that in Thais: 67.2±22.5 hours *vs* 60.2±18.7 hours (Looareesuwan *et al*, 1999). One explanation for this disagreement may be the timing of blood-smear preparation during treatment: in this study smears were prepared every 24 hours, whereas in Thailand smears had been prepared every 12 hours, which may result in greater accuracy. One interesting finding in this study was that in some instances parasites that had been incubated in the presence of CQ, particularly in well B, were on average more mature than those that had been incubated the in absence of CQ (control well). The interpretation of this phenomenon is challenging: the finding may reflect the fact that the parasites in the control well, uninhibited by CQ, continued to produce new merozoites by schizogony; in turn, newly-formed rings (young stages) were evident on microscopy, leading to a relative reduction in the number of mature schizonts. This effect was marked when samples containing a higher number of mature parasites were used. Parasites in the predosed wells were less able to continue schizogony, leading to a relative increase in the number of mature forms seen on microscopy.

The recrudescence of *P. falciparum* in one patient who had received a standard dose of CQ confirms the presence of CQ-resistant *P. falciparum* in the area (Edrissian *et al*, 2001). The use of the method of Lambros and Vanderberg (1979) for the synchronization of the erythrocytic stages of *P. vivax* was unsuitable because it was detrimental to the parasites: it cannot be applied for the synchronization of *P. vivax* owing to the fact that vivax parasites invade only young erythrocytes, *ie* reticulocytes (Mons *et al*, 1988), whose walls are fragile and therefore easily damaged. This study did not find any difference between the performance of a mixture of RPMI and Waymouth medium and McCoy's 5A medium, in spite of the fact that McCoy's has been used for the successful continuous *in vitro* cultivation of *P. vivax* (Golenda *et al*, 1997).

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