IN VITRO SENSITIVITY OF *PLASMODIUM FALCIPARUM* TO EIGHT ANTIMALARIALS IN CHINA-MYANMAR AND CHINA-LAO PDR BORDER AREAS

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Abstract. In 1991-1995 by using the Rieckmann *in vitro* micro-method, susceptibilities of *Plasmodium falciparum* to eight antimalarials in the China-Lao PDR and China-Myanmar border areas were tested. The resistant rates of *P. falciparum* to chloroquinine were 95.0%-100%; IC_{50} 114-240nmol/l. *P. falciparum* resistant rates to amodiaquine resistance accounted for 83.5%-100%, IC_{50} 52-72nmol/l. All cases were sensitive to quinine, IC_{50} 470-608nmol/l. *P. falciparum* isolates from the Lao PDR frontier were highly sensitive to artesunate, dihydroartemisinin, and arteether. Resistant rates from other areas were 0-11%. *P. falciparum* from China-Myanmar and Lao PDR border areas were also sensitive to mefloquine, IC_{50} 68-88nmol/l. A longitudinal survey of the sensitivity of *P. falciparum in vivo* on the China-Lao PDR border showed that the average defervescent time of falciparum malaria was treated by pyronaridine increased from 32.7 ± 16.0 hours during 1984-85 to 56.2 ± 27.4 hours in 1995; the recrudescence rate rose up from 15.2% to 37.5%. The results monitored *in vitro* showed that all cases assessed in 1988 for response to pyronaridine were sensitive, but 36.4% of cases had emerging resistance, IC_{50} increased from 13nmol/l to 40 nmol/l. The above results suggested that *P. falciparum* in these areas has expressed resistance to chloroquine and amodiaquine. However, the parasites are still sensitive to artemisinin, pyronaridine, mefloquine, quinine, but with a declining sensitivities.

INTRODUCTION

Malaria is one of several public health problems in China-Myanmar, China-LaoPDR and China-Vietnam border areas (Zhu *et al*, 1994). Chloroquine-resistant falciparum malaria in China was first discovered in the south of Yunnan in 1973 (Institute of Military Medicine Logistic Department, PLA Kunming, 1978). Then it was confirmed that chloroquine-resistant *Plasmodium falciparum* extended all over the falciparum malaria endemic areas of China at the beginning of the 1980s (Liu *et al*, 1986). Surveys in Yunnan during 1981-1983 (Chen *et al*, 1983; Che *et al*, 1986) established the rates of chloroquine-resistant *P. falciparum* on the China-Myanmar and China-LaoPDR frontiers were as high as 96-100%, while in the middle part of Yunnan province there were approximately 50% of *P. falciparum* isolates resistant to chloroquine. In order to keep abreast of the susceptibilities of *P. falciparum* of the region to chloroquine and other antimalarials, a study in areas near the China-Myanmar, the China-Lao PDR and the China-Vietnam border was conducted in 1991-1995, following the report of Yang *et al* (1995).

MATERIALS AND METHODS

Study area: The study was carried out at Ruili municipality (24° 10' N, 97° 50' E). Mengla county (21° 1' N, 99° 50' E) which are located at the China-Myanmar and the China-LaoPDR borders, respectively.

Cases: 260 patients coming from villages around the China-Myanmar and China-LaoPDR border areas with a monoinfection of *P. falciparum* confirmed by Giemsa stained thick smear were entered into the study. All the patients had no history of taking antimalarials, sulfa-compounds or antibiotics within the previous 2 weeks. Blood and urine samples were collected before treatment. Heparin (20 IU/ml) was added to the venepuncture blood as an anticoagulant. The urine samples were tested for the presence of 4-aminoquinolines by Hasking's test. Malaria parasites were quantified by counting the number of asexual parasites per 50

leukocytes in thick smears and parasite density was calculated on the basis of leukocytes/μl blood. All patients were treated with a standard course of artemether or artesunate after collection of blood samples.

Culture plates and medium: Microplates of artesunate, dihydroartemisinine, arteether, artemether and pyronaridine were prepared according to the methods of Liu *et al* (1983) and Yang *et al* (1983, 1992, 1995). Plates for chloroquine, mefloquine, quinine and amodiaquine were supplied by WHO, made in 1992 and 1993. The culture medium used in the *in vitro* assay was prepared in accordance with the method of Liu *et al* (1989). 0.2ml of parasitized blood was added to 1.8ml of culture medium. Then 50 μl aliquots were dispensed into wells predosed with varying concentrations (picomole) of artesunate, dihydroartemisinin (0.125, 0.25, 0.5, 1, 2, 4, 8, 16), arteether (1, 2, 4, 8, 16, 32, 64, 128), pyronaridine (0.25, 0.5, 1, 2, 4, 8, 16, 32). No. 1 and No. 10 wells were undosed, set up as controls. Predosed wells with chloroquine were (1, 2, 4, 8, 16, 32, 64), mefloquine (2, 4, 8, 16, 32, 64, 128), quinine (4, 8, 16, 32, 64, 128, 256), amodiaquine (0.25, 0.5, 1, 2, 4, 8, 16), with No. 1 well undosed used as control.

***In vitro* assay and criteria for resistance:** The *in vitro* microtechnique used to detect parasite resistance was that described by Rieckmann *et al* (1978). Maturation rate of rings to schizonts (three or more nuclei present) in control wells of 20% was considered as a success, conversely as a failure. Based on the criteria recomended by WHO and those adopted in Yang *et al* (1988, 1993, 1995) previous studies, resistance is defined as schizonts developing in the presence of 8 pmoles of chloroquine, 4 pmoles of amodiaquine, 64 pmoles of mefloquine and 256 pmoles of quinine, 4 pmoles of artesunate, 8 pmoles of dihydroartemisinine, 64 pmoles of arteether, 8 pmoles of pyronaridine. Counting the number of schizonts among 200 asexual parasites in each well, taking the sum of schizont in control wells of all isolates tested as the base, the inhibition rate of each well was evaluated by dividing this base by the sum of schizonts in each well. Effective drug concentration for 50% inhibition (IC_{50}) and 95% inhibition were calculated (IC_{95}) by linear regression analysis. Mean concentrations completely inhibiting schizont fomation (CIMC) were computed by geometric mean.

The WHO (1973) standard 4 weeks method of assessing falciparum malaria sensitivity *in vivo* to pyronaridine was performed in 1984-85 and 1995. A patient was asked to take pyronaridine 5mg/kg at 0, 4, 24, 48 hours, total dosage 200mg/kg. *In vitro* sensitivity of *P. falciparum* to pyronaridine was assayed in Mengla county of Yunnan Province in 1988-1995.

RESULTS

The initial parasitemia level of the 260 patients ranged from 2,000-80,000/μl blood. All the pretreatment urines tested negative for 4-aminoquinolines, of 156 primary isolates in the *in vitro* susceptibility test for chloroquine, 125 isolates produced schizonts, a successful rate of 80.1%. The successful rates for artesunate, dihydroartemisinine, arteether, and pyronaridine were 85.6% (95/111), 86.5% (64/74), 79.7% (59/74), and 77.1% (145/188), respectively. The succesful rates for mefloquine, amodiaquine and quinine were in the range 75.0%-80.0%.

The resistance rates of *P. falciparum* to the above-mentioned antimalarials, IC_{50}, IC_{95} and mean concentrations for complete inhibition schizont (CIMC) are shown in Tables 1,2. The resistance rate of *P. falciparum* to chloroquine in the China-Myanmar border area was similar to that in the China-Lao PDR frontier, but the resistance degree in the former was higher than in the latter. All *P. falciparum* isolates from Lao PDR were sensitive to artemisinine; IC_{50}, IC_{95} and CIMC were higher than in other areas. The cross-resistance rate of chloroquine-resistance falciparum malaria was 93.8% (30/32) to amodiaquine in the study.

In 1984-1985 and in 1995, the sensitivity of 75 patients with acute uncomplicated falciparum malaria was observed to pyronaridine using *in vivo* measures in Mengla county of Yunnan province; the results are shown in Table 3. The *in vitro* sensitivity of *P. falciparum* to pyronaridine was assayed during 1988-1995 in the region; the results are shown in Table 4.

DISCUSSION

The cases of our study came from China-

Table 1

In vitro sensitivity of *P. falciparum* from the China-Myanmar border against 7 antimalarials.

Drug	Case from China border					Case from Myanmar border				
	No. tested	Resistance rate (%)	CIMC* (nmol/l)	IC_{50} (nmol/l)	IC_{95} (nmol/l)	No. tested	Resistance rate (%)	CIMC* (nmol/l)	IC_{50} (nmol/l)	IC_{95} (nmol/l)
Artesunate	24	16.5	32	6	34	29	13.8	22	6	34
Dihydroarte-misinin	9	22.2	46	13	80	6	16.7	50	14	80
Arteether	9	11.1	344	118	650	6	16.7	404	120	650
Pyronaridine	24	20.8	50	16	88	28	21.4	56	18	95
Chloroquine	23	95.7	840	176	830	29	100	1,220	240	1,408
Amodiaquine	9	88.9	320	72	512	6	83.3	202	52	384
Quinine	9	0	2,022	608	2,560	6	0	2,874	470	1,690

* Mean concentration completely inhibition schizont formation.

Table 2

In vitro sensitivity of *P. falciparum* from the China-Lao PDR border to 8 antimalarials.

Drug	Case from China border					Case from Lao PDR border				
	No. tested	Resistance rate (%)	CIMC* (nmol/l)	IC_{50} (nmol/l)	IC_{95} (nmol/l)	No. tested	Resistance rate (%)	CIMC* (nmol/l)	IC_{50} (nmol/l)	IC_{95} (nmol/l)
Artesunate	33	12.1	46	7	42	9	0	22	5	23
Dihydroarte-misinine	32	12.5	26	5	37	10	0	16	4	11
Arteether	32	6.3	376	74	371	10	0	232	57	221
Pyronaridine	29	34.5	74	32	325	5	10.0	28	16	108
Chloroquine	63	96.4	672	122	625	10	90.0	524	114	570
Amodiaquine	30	100.0	306	52	292	-	-	-	-	-
Mefloquine	21	0	88	68	160	-	-	-	-	-
Quinine	30	0	1,896	480	1,536	-	-	-	-	-

* Mean concentration completely inhibition schizont formation.

Table 3

In vivo sensitivity of *P. falciparum* to pyronaridine in Mengla county of Yunnan.

Year	No. cases observed	Average deferves-cence time (h)	Mean asexual parasite clearance time (h)	Recrudescence rate (%)
1984-1985	36	32.7 ± 16.0	64.2 ± 22.9	15.2 (5/33)
1995	39	56.2 ± 27.4	55.3 ± 11.8	37.5 (9/24)

Table 4

In vitro sensitivity of *P. falciparum* to pyronaridine in Mengla county of Yunnan.

Year	No. cases tested	Resistant rate (%)	ID (nmol/l)	ID_{95} (nmol/l)	MIC* (nmol/l)
1988	24	0	13	48	32.6
1990	34	8.8	20	136	99.5
1992	29	13.8	32	325	148.2
1995	11	36.4	40	330	190.1

* Mean concentration completely inhibition schizont formation.

Lao PDR, and China-Myanmar border areas which are typical and representative frontiers. The results represent the situation of sensitivity of *P. falciparum* generally in the border areas. It is important for improving falciparum malaria control, directing reasonable drug-utilization, and preventing or delaying resistance of *P. falciparum* to multiple antimalarials.

The results confirmed that *P. falciparum* in border areas have commonly developed resistance to chloroquine and amodiaquine, but the degrees of resistance are different: those in the China-Myanmar areas were much higher than others. All strains of *P. falciparum* were sensitive to mefloquine and quinine. Most strains of *P. falciparum* were also sensitive to artemisinine and its derivatives, and to pyronaridine. These data suggested that at present chloroquine and amodiaquine can not be used in malaria treatment in these areas. However, artemisinine, pyronaridine and mefloquinine should replace 4-aminoquinolines for malaria therapy, but monitoring of parasite sensitivity should be strengthened.

The sensitivity of *P. falciparum* isolates to artemisinine and its derivatives in this study was evidently higher than that of *P. falciparum* isolates obtained from patients living the southern part of Yunnan adjacent to the China-Lao PDR border; however, it was lower than in isolates obtained from patients who acquired the infection across the border in Lao PDR (Yang *et al*, 1995). Possibly this was related to differences of the drug pressure. Artemisinine was put into use as early as in the middle of the 1980s in southern Yunnan, while it was only towards the end of the 1980s that artemisinine came into use extensively on the China-Vietnam border, and this drug was rarely used in Lao PDR. As to pyronaridine, clinical studies have been rather limited (Che *et al*, 1987; Yang *et al*, 1989b) and the prescription of this drug for malaria treatment has never been wide-ranging in Yunnan. The results *in vivo* (Table 3) and *in vitro* (Table 4) showed that the sensitivity dropped remarkably. Although a few local strains of *P. falciparum* may possess innate resistance to pyronaridine, the emergence of pyronaridine-resistant *P. falciparum* strains was chiefly associated with the presence of artesunate-resistant strains cross-resistant to pyronaridine (Yang *et al* 1989a; 1994).

Amodiaquine has never been used for malaria control in this region. Resistance to amodiaquine was probably related to the existence of chloroquine-resistant strains which were cross-resistant to amodiaquine. Quinine has been used in the treatment of acute attacks of malaria in this locality for many years. However, the result of this survey demonstrated that all the isolates of *P. falciparum* were sensitive to quinine, a finding similar to that on the China-Vietnam border (Yang *et al*, 1995).

The data on these two separate *in vitro* assays point out that *P. falciparum* seemed not to develop resistance to quinine. In this connection it is regarded that lack of compliance rather than drug resistance was the major cause of treatment failure in the clinical use of quinine to cure falciparum malaria. Mefloquine has never been used at the places in question. Although no mefloquine-resistant strain of *P. falciparum* was found in the *in vitro* study, nearly 20% of isolates showed a level of sensitivity to mefloquine approaching resistance, a matter that should be taken seriously.

The microtitration plates used in the study were prepared by ourselves. These preparations have the

same effectiveness as those provided by WHO. The successful rates for these drugs is also similar. Growth of *P. falciparum* went down in proper order with increasing concentrations of each drug. The result by microtitration method *in vitro* is in agreement with the results *in vivo*, suggesting that drugs in these plates are stable, that testing results are reliable.

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