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In vitro Anti-Malarial Drug Susceptibility of Temperate *Plasmodium vivax* from Central China

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Abstract. In the face of recent increase of *Plasmodium vivax* malaria in central China, we conducted a study to evaluate *in vitro* susceptibility of temperate-zone *P. vivax* parasites to antimalarial drugs. During 2005–2006, *in vitro* drug susceptibility was measured for 42 clinical *P. vivax* isolates by using a schizont maturation inhibition technique. Geometric means of 50% inhibitory concentrations (IC_{50}s) and 95% confidence intervals (CIs) were 10.87 (4.50–26.26) ng/mL for chloroquine, 4.21 (1.88–9.42–8) ng/mL for mefloquine, 11.82 (6.20–22.56) ng/mL for quinine, 0.13 (0.09–0.20) ng/mL for artesunate, 18.32 (8.08–41.50) ng/mL for pyrimethamine, and 17.73 (10.29–30.57) ng/mL for piperaquine. The IC_{50} for chloroquine was lower than those obtained from isolates from Thailand and South Korea, suggesting that chloroquine remained effective against *P. vivax* malaria in central China. The results further indicated that temperate-zone *P. vivax* isolates from China were more susceptible to chloroquine, quinine, and mefloquine than isolates from Thailand.

INTRODUCTION

Of the four human *Plasmodium* species, *Plasmodium vivax* has the widest global distribution and accounts for most malaria infections outside Africa.[1] Currently, it is estimated that 130–145 million *P. vivax* malaria cases occur annually.[2] The recent reports of *P. vivax* infections that produce severe clinical manifestations in malaria-endemic areas of Asia and South America have dramatically changed the view of *P. vivax* malaria as benign tertian malaria.[3–7] Furthermore, the ability of the parasite to produce multiple relapses in patients after the initial episode causes increased morbidity.[8]

In spite of its significance in public health, first-line therapies for *P. vivax* malaria, chloroquine (CQ) and primaquine (PQ), have remained unchanged for the past 50 years. The emergence of resistance to CQ and the failing efficacy of PQ regimens in preventing relapses have raised great concerns in the control of *P. vivax* malaria.[9,10] Clinical CQ-resistant (CQR) *P. vivax* was first reported from Papua New Guinea in 1989,[11,12] followed by multiple reports from Indonesia,[13–15] Myanmar,[16,17] India,[18,19] Guyana,[20] Brazil,[21,22] Colombia,[23] and more recently in Ethiopia[24] and South Korea.[25] Continued surveillance of *P. vivax* drug resistance may detect CQR *P. vivax* parasites in most of its geographic range. In view of this critical scenario, it is well justified to consider *P. vivax* malaria as the most neglected, highly prevalent, and potentially dangerous disease.[26,27]

Malaria in China is concentrated in two subtropical provinces Yunnan and Hainan, where *P. vivax* and *P. falciparum* coexist with *P. vivax* as the predominant species. The malaria eradication campaign in the 1950s had significantly reduced the range and prevalence of malaria in China. This reduction is especially evident in the central region, where *P. falciparum* has been largely eradicated. Although malaria is hypoendemic to central China, this region has experienced malaria resurgence since 2000; there have been frequent focal outbreaks in areas along the Huai River. Anhui Province was most seriously affected; malaria incidence increased in 2006 in this province to the highest level of any province in China.[28] Choroquine–primaquine is still the first-line treatment for *P. vivax* malaria in China. Although there was a report of chloroquine treatment failure in four cases of *P. vivax* malaria in Yunnan,[29] no assessment of the CQ-PQ efficacy against temperate-zone *P. vivax* strains has been carried out in central provinces. Therefore, there is an urgent need to monitor drug sensitivity of *P. vivax* for currently used antimalarial drugs in China during the malaria elimination phase.

Most reports of CQR *P. vivax* were based on observations of failures after the standard CQ treatment despite adequate blood levels of CQ and desethylchloroquine (> 100 ng/mL).[9] This finding was largely caused by difficulties involved in culturing *P. vivax* parasites. In recent years, renewed interests in culturing *P. vivax* led to improvement of an *ex vivo* procedure and adaptation of this technique for *in vitro* drug assays.[30–32] These assays, based on schizont maturation, have been used to evaluate drug sensitivity of tropical *P. vivax* isolates from southeast Asia and temperate-zone isolates from South Korea.[33–35] We present the first *in vitro* evaluation of drug sensitivity of *P. vivax* clinical isolates from a temperate malarious region of central China.

MATERIALS AND METHODS

Study site and sample collection criteria. This study was carried out in Bengbu, Anhui Province, China, during the malaria transmission seasons (July–September) of 2005 and 2006. Ethical approval was obtained from the ethical review committees at the National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention and Walter Reed Army Institute of Research. A total of 75 patients ≥ 18 years of age attending county hospitals and diagnosed with *P. vivax* infections were recruited into this study. Informed consent was obtained from all participants before 2 mL of venous blood was obtained from each patient by venipuncture into a heparinized tube. Parasitemia was determined by microscopic examination of Giemsa-stained smears. Blood was stored at 37°C and transported to the laboratory for *in vitro* assays within 8 hours of collection. Only samples from patients with parasitemias ≥ 0.01% and who had not received

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antimalarial treatment in the preceding three weeks were chosen for *in vitro* drug assays.

***In vitro* drug assay.** Parasite *ex vivo* culture was performed essentially as described.[30] Briefly, 2 mL of *P. vivax*-infected blood was centrifuged at 2,000 rpm for 5 minutes, and the pellet was resuspended in two volumes of serum-free RPMI 1640 medium (ICM). Leukocytes were removed by passing the blood through a Plasmodipur® filter (Clonagen, Paris, France). Erythrocytes were washed twice with serum-free RPMI 1640 medium ICM and resuspended in McCoy's 5A medium containing 25% AB^+ serum to make a 2% cell suspension. A 100-μL erythrocyte suspension was dispensed into wells of a pre-dosed antimalarial microtiter plate.

The drugs assayed and their concentration ranges were chloroquine (0.1 ng/mL–100 μg/mL), mefloquine (0.1 ng/mL–100 μg/mL), pyrimethamine (0.1 ng/mL–100 μg/mL), quinine (0.1 ng/mL–100 μg/mL), artesunate (0.006–6 ng/mL), piperaquine (0.6 ng/ml–100 μg/mL), and primaquine (8–500 ng/mL). Each drug concentration was tested in triplicate. Control wells without drugs were included in all tests. The test plates were incubated at 37°C in a gas chamber containing 5% CO_2 for 12–40 hours depending on the stage of the parasites on admission. Parasite development in drug-free wells was monitored every 6 hours. When parasites in control wells developed into mature schizonts, all wells were harvested to prepare thick and thin blood films. Blood films were stained with Giemsa and examined under a microscope. Only schizonts with at least three well-defined chromatin dots were counted. Parasite preparations with > 80% of parasites matured to schizonts in control wells were included in the analysis.

Data analysis. The percentage of parasites matured to schizonts were determined for each drug concentration and compared with those from controls. Data were compared and analyzed by using WinNonlin software version 4.1 (Pharsight Corporation, Mountain View, CA) to determine the 50% inhibitory concentration (IC_{50}) of each drug among different isolates. Geometric mean IC_{50}s and 95% confident intervals (CIs) of each drug were obtained by using SPSS 11.0. (SPSS, Inc., Chicago, IL). Spearman's nonparametric correlation was used to evaluate correlations between IC_{50}s of parasite isolates and different drugs.

RESULTS

In the malaria transmission seasons of 2005–2006, we recruited 75 *P. vivax*-infected patients for *in vivo* drug sensitivity study. Parasitemia on admission varied between 0.01% and 1.8% and showed a geometric mean of 0.08% (95% CI = 0.06–0.10%). Among these clinical isolates tested, 42 successfully developed into schizonts in control wells. All cases had mixed stages of parasites before culture. Twenty-five isolates, with mostly rings, were cultured for 24–35 hours, and the other 17 isolates were cultured less than 24 hours. Seven commonly used antimalarial drugs listed above were assayed in parallel and the IC_{50} for each drug was calculated.

Numbers of isolates that were assayed successfully were 29, 31, 31, 38, 40, and 35 for CQ, mefloquine, pyrimethamine, quinine, artesunate, and piperaquine, respectively. Numbers of isolates that developed under different culture times and drugs are shown in Table 1, and IC_{50}s and 95% CIs are shown in Table 2. Parasites were highly sensitive to mefloquine and artesunate and had IC_{50}s in the lower nanomolar range. For CQ, a recent study showed that *P. vivax* trophozoites were insensitive to CQ.[36,37] Consistent with this finding, we found that most parasite isolates at the trophozoite stage were significantly less sensitive to CQ; four isolates had IC_{50}s > 100 μg/mL. The mean *in vitro* IC_{50}s to pyrimethamine, quinine, and piperaquine were 18.32, 11.82, and 17.73 ng/mL, respectively.

TABLE 1

Number of *Plasmodium vivax* isolates included in drug susceptibility assays for each drug, China

Treatment	No. isolates		
	Culture time < 24 hours	Culture time ≥ 24 hours	Total
Control	17	25	42
Chloroquine	10	19	29
Mefloquine	13	18	31
Pyrimethamine	13	18	31
Quinine	16	22	38
Artesunate	17	23	40
Piperaquine	14	21	35
Primaquine	0	2	2

Primaquine was a poor blood stage schizonticide, and the IC_{50} could be obtained only from two isolates (mean = 69.75 ng/mL). For the remaining isolates, PQ only partially inhibited schizogony at the highest concentration tested. Positive correlations of IC_{50} values were detected between quinine and CQ (r = 0.568, P = 0.002), quinine and mefloquine (r = 0.438, P = 0.016), quinine and artesunate (r = 0.408, P = 0.013), piperaquine and mefloquine (r = 0.426, P = 0.030), artesunate and CQ (r = 0.424, P = 0.027) and artesunate and piperaquine (r = 0.416, P = 0.014).

DISCUSSION

In recent years, the resurgence of *P. vivax* malaria in central China has raised doubts about efficacy of the first-line treatment CQ-PQ. However, no stringent clinical observations on the efficacy of this drug combination have been conducted to date. We tested the sensitivity of *P. vivax* to a number of commonly used antimalarial drugs by using an *in vitro* assay based on schizont maturation. The lower success rate (69%) of the assay in our study may have been caused by the extended transportation time (> 6 hours) for some samples from hospitals to the laboratory and suspected use of antibiotics by some patients before admission to the study. Nevertheless, our success using parasite isolates with parasitemias as low as 0.015% demonstrates applicability of this assay to a wide range of parasitemias.

Although assay conditions may have had some slight variations between different studies and may not be directly comparable with results from other studies,[31,32,34] the same technique has been used to evaluate *in vitro* drug susceptibility of *P. vivax* parasites in Thailand and South Korea.[33,35] These studies detected similar sensitivities to some drugs (artesunate and CQ) but dramatic differences to others (e.g., quinine, pyrimethamine, and mefloquine), which could be explained by difference in malaria epidemiology and antimalarial drug use history in these countries.

Temperate *P. vivax* strains are endemic to central China and South Korea. These strains have a long interval between primary infection and relapse in these two countries. However, tropical *P. vivax* strains have short relapse intervals in Thailand. The intrinsic differences in parasite strains may be partially

TABLE 2
50% inhibitory concentrations and 95% confidence intervals of antimalarial drug susceptibility patterns of *Plasmodium vivax*, China*

Drug	Culture time < 24 hours		Culture time ≥ 24 hours		All cases	
	IC_{50}, ng/mL	95% CI	IC_{50}, ng/mL	95% CI	IC_{50}, ng/mL	95% CI
Chloroquine	28.64	6.27–130.75	6.52	1.98–21.45	10.87	4.50–26.26
Mefloquine	4.15	1.05–16.38	4.25	1.31–13.79	4.21	1.88–9.42
Pyrimethamine	17.26	3.44–86.57	19.13	6.67–54.86	18.32	8.08–41.50
Quinine	20.63	7.53–56.58	7.89	3.14–19.79	11.82	6.20–22.56
Artesunate	0.20	0.12–0.33	0.10	0.05–0.18	0.13	0.09–0.20
Piperaquine	27.40	10.35–72.52	13.27	6.44–27.35	17.73	10.29–30.57
Primaquine	ND	ND	69.75	ND	69.75	ND

* IC_{50} = 50% inhibitory concentration; CI = confidence interval; ND = not done.

responsible for the observed differences in drug sensitivities, but different drug histories in these countries may be the major contributor to the difference. In Thailand, *P. vivax* and *P. falciparum* co-exist, and cryptic infections with one parasite species often occur in clinical malaria.[38] Because drug treatment is primarily dependent on diagnosis by microscopy, which often misses cases of mixed-species infections,[39] drugs used to treat one parasite species will inevitably influence sensitivity of the other species.[40] As a result, *P. vivax* parasites in these countries have been subjected to different drug pressures, even though CQ-PQ is the drug combination used to treat *P. vivax* malaria in all countries. In Thailand, melfoquine has been used extensively for more than 20 years to treat *P. falciparum* malaria,[40] and *P. falciparum* has developed resistance to this drug.[41] *Plasmodium vivax* has been selected for amplification of the *P. vivax* multidrug resistance 1 gene under pressure of the mefloquine treatment for *P. falciparum* malaria in the area that these two species were found.[42,43] Quinine had been used for approximately 5 years in the early 1980s, and is often used to treat severe *P. falciparum* malaria.[40] Although development of resistance in *P. falciparum* to quinine is slow, reduced susceptibility has already been detected in clinical studies.[44] In comparison, mefloquine has not been used in China and South Korea, and quinine has been used for treating severe *P. falciparum* malaria in southern China. Therefore, these different drug histories are consistent with the observed higher sensitivity of the temperate-zone *P. vivax* strains to these two drugs.

We detected reduced *in vitro* sensitivity of *P. vivax* to pyrimethamine than in isolates from Thailand,[33] but greater sensitivity to this drug than in isolates from South Korea.[35] It was suggested the *in vitro* assay performed in the presence of folic acid may not reflect the *in vivo* sensitivity of the parasites,[35] but values obtained in this study may represent the relative sensitivity of the parasites to this antifolate drug. Pyrimethamine, a component of the two combination regimens Maloprim (plus dapsone) and Fansidar (plus sulfadoxine), has been widely used for malaria prophylaxis in China. This use has led to reduced efficacy of pyrimethamine for prophylaxis and clinical resistance in approximately 35% of patients treated for *P. vivax* malaria in temperate-zone provinces in the late 1970s.[45,46] Extensive use of pyrimethamine may have posed substantial selection pressure on *P. vivax* and is probably associated with the reduced *in vitro* sensitivity to pyrimethamine. These *in vitro* studies indicate that parasites of different origins displayed great variations in sensitivity to pyrimethamine, which might be correlated with different prevalences of resistance-conferring dihydrofolate reductase alleles.[47,48] The *in vitro* assay technique we used may be a useful tool for studying relationships between antifolate resistance and mutations in target genes.

The *in vitro* IC_{50}s of the *P. vivax* isolates from China (10.87 ng/mL) for CQ were lower than those of isolates from South Korea (39 ng/mL) and Thailand (50 ng/mL). Because *in vivo* studies showed that the current treatment regimen of CQ-PQ was still effective in Thailand,[49,50] our results suggested that this first-line treatment for *P. vivax* malaria may also be effective in central China. However, although we did not monitor clinical efficacy of CQ in this study, we observed that one of the four patients enrolled in this study who had parasite isolates with higher *in vitro* IC_{50}s had recurrent *P. vivax* infections on day 16, suggesting clinical resistance to CQ in this area.

In addition, the observation of clinical failures after standard CQ treatment in Yunnan Province, where *P. vivax* and *P. falciparum* co-exist, raised further concerns.[29] Therefore, clinical efficacy of CQ in treating temperate-zone *P. vivax* malaria in central China needs to be evaluated. Moreover, studies in other *P. vivax*-endemic regions found great variations in the prevalence of CQR *P. vivax* parasites. Multiple cross-sectional and longitudinal surveys performed in different regions of Indonesia showed considerable geographic variations in the proportion of CQR *P. vivax* parasites.[51–57] In Vietnam, CQR *P. vivax* infection was not found 10 years ago,[58] but a more recent survey found 25% of patients in a study had recurrent *P. vivax* infections after standard treatment, suggesting of emerging CQ resistance.[59] These studies highlight the geographic heterogeneity and temporal dynamics of drug resistance in *P. vivax*, which further emphasizes the importance of continued surveillance efforts.

Currently, mechanism of CQ resistance in *P. vivax* is not well understood, but does not appear to be associated with the ortholog of the *P. falciparum* CQ-resistant transporter gene. Although certain polymorphisms in the *P. vivax* multidrug resistance 1 gene have been shown to be associated with reduced sensitivity to CQ *in vitro*[34] and *in vivo*,[60] this association needs to be further evaluated. Therefore, with the lack of convenient molecular surveillance tools, the *ex vivo* assay is useful for monitoring drug sensitivity of *P. vivax*. Standardization of the assay conditions among investigators is needed so that data from different geographic regions can be compared.

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