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Antimalarial Drug Susceptibility of *Plasmodium vivax* in the Republic of Korea

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Abstract. The antimalarial susceptibility of ring stage (> 80%) *Plasmodium vivax* from the Republic of Korea, where long incubation–period strains are prevalent, was evaluated using the schizont maturation inhibition technique. During 2005–2007, susceptibility to seven antimalarial drugs was evaluated with 24 fresh isolates. The geometric mean (95% confidence interval) 50% inhibition concentration (IC_{50}) were quinine 60 (54–75) ng/mL, chloroquine 39 (22–282) ng/mL, piperaquine 27 (17–58) ng/mL, mefloquine 39 (35–67) ng/mL, pyrimethamine 138 (89–280) ng/mL, artesunate 0.6 (0.5–0.8) ng/mL, and primaquine 122 (98–232) ng/mL. Positive correlations were found between quinine and mefloquine ($r = 0.6$, $P = 0.004$), piperaquine and chloroquine ($r = 0.6$, $P = 0.008$), and piperaquine and primaquine IC_{50} values ($r = 0.5$, $P = 0.01$). Compared with *P. vivax* in Thailand, *P. vivax* in the Republic of Korea was more sensitive to quinine and mefloquine, but equally sensitive to chloroquine and artesunate.

INTRODUCTION

Plasmodium vivax is the major cause of human malaria in many parts of Central America, South America, and Asia. The Republic of Korea was declared as a malaria-free country by World Heath Organization in 1979.[1] Susequently, a few cases were reported as imported malaria.[2] *Plasmodium vivax* has re-emerged in the Republic of Korea since 1993 in areas adjacent to the demilitarized zone, with more than 1,700 cases in 1997 and an estimated 4,000 cases in 1998.[2–4] During the entire period from 1993 through 2005, 937,634 cases were reported in the Korean peninsula.[5] *Plasmodium vivax* malaria in the Republic of Korea typically has a long interval between primary infection and relapse, and many strains exhibit a long incubation period (lasting ≥ 6–12 months)[6–8] and is therefore similar to the infection (*P. vivax hibernans*) that was once prevalent across northern Asia and northern Europe.

Although severe complications of *P. vivax* malaria are observed rarely in Asia, in China a similar parasite named *P. vivax multinucleatum* has been associated with increased virulence.[6,8] *Plasmodium vivax* may cause multiple relapses and is a cause of considerable morbidity, particularly in childhood. *Plasmodium vivax* malaria in pregnancy is associated with low birth weight.[9,10] Choroquine has been the drug of choice for treatment of patients with *P. vivax* malaria for many years. In recent years, resistance to chloroquine in *P. vivax* has been demonstrated conclusively *in vivo*, on the island of New Guinea,[11,12] in different regions of Indonesia,[13–15] South America,[16] and more recently in Central America.[17] There have also been reports of this resistance from India,[18] Myanmar,[19,20] and several other countries. Chloroquine-resistant *P. vivax* is still susceptible to mefloquine and piperaquine, and to some extent to amodiaquine.[21]

The susceptibility of *P. vivax* to antimalarial drugs has not been monitored widely *in vitro* because of difficulties in cultivating in *P. vivax ex vivo*. These difficulties are related to differences in nutrient requirements compared with *P. falciparum*; the conditions in standard malaria culture medium, which induce premature rupture of the infected erythrocytes,[22] inhibition by leukocytes, and the limited provision of young erythrocytes or reticulocytes, which *P. vivax* invades preferentially. *Plasmodium vivax* invades erythrocytes only in the first two weeks after their emergence from the bone marrow.[23] In the Republic of Korea, different antimalarial drugs are available and used for treatment. Antimalarial susceptibility of *P. vivax in vitro* in this country has not been assessed previously. In this study, the susceptibility of *P. vivax* in the Republic of Korea to different antimalarial drugs was assessed in short-term cultures.

MATERIALS AND METHODS

The susceptibility of 24 isolates of *P. vivax* to 7 antimalarial drugs was evaluated using the schizont maturation inhibition technique as described below.

Study site. The studies were carried out at public health centers in Paju and Ansan provinces and at the National Institute in the Republic of Korea during 2004–2005. This study was a part of *P. vivax* study in the Republic of Korea sponsored by the U.S. Military Infectious Diseases Research Program. The study was reviewed and approved by the Ethical and Scientific Review Committees of Korean Institute of Health and Social Affairs and the Walter Reed Army Institute of Research Human Use Research Committee.

Parasites. Five milliliters of blood were collected in heparin tubes from patients attending an outpatient clinic. Thick and thin blood films were prepared by using standard procedures. Parasite species, morphology, and parasitemia were assessed by microscopic examination. Only patients with > 80% ring forms on initial examination were enrolled. Species identification was confirmed by nested polymerase chain reaction.[24] Blood samples were taken only from patients with no history of antimalarial drug treatment.

Antimalarial drug sensitivity assay. *Plasmodium vivax*-infected blood was centrifuged at 2,000 rpm at 4°C for 5 minutes. After the plasma and buffy coat were removed by using a Plasmodipur® filter, (Euro Diagnostica, Apeldoorn, The Netherlands) the packed erythrocytes were washed three times

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in RPMI 1640 medium and resuspended to give a 3% cell suspension in complete medium as described previously.[22] A 50-µL cell suspension was added to triplicate wells of a predosed antimalarial microtiter plate. We assessed seven drugs: quinine, artesunate, chloroquine, mefloquine, piperaquine, pyrimethamine, and primaquine (Table 1). Artesunate was prepared in triplicate wells in 96-well plates as described previously,[25] and 50 µL of erythrocyte suspension was added into each well. Each drug concentration was tested in duplicate. After adding the erythrocytes, the lid was placed over the plate and the plate was then shaken gently to dissolve the drug. The samples were incubated at 37°C in an atmosphere of 5% CO_2 for 40–44 hours, depending on the stage of the parasite before culturing. At the end of the incubation, thick and thin blood films were made from each well. Wells without drugs were included as controls. The experiments were performed in triplicate.

Evaluation of antimalarial drug susceptibility. Thick and thin blood films were fixed with methanol, stained with Field's stain, and examined under a microscope. Smears were made on a 1-cm^2 grid. The number of schizonts containing more than 8 nuclei were counted per 1 cm^2 (50% hematocrit of 1 µL of erythrocyte suspension). Activity was expressed as the percentage of inhibition compared with control (no drug).

Data analysis. Results of parasite schizogony after 40–44 hours culture at each drug concentration were fitted to a sigmoid curve by using Win-nonlin™ version 3.1 computer software (Pharsight Corp., St. Louis, MO) to determine the concentration causing 50% inhibition (IC_{50}) of schizont development. Correlations were assessed by the method of Spearman.

RESULTS

A total of 24 synchronous isolates were obtained during 2005–2007. The mean (SD) percentage of ring-stage parasites on peripheral blood films per 100 infected erythrocytes was 84 (3)%. Parasitemia before testing varied between 18 and 1,850 asexual parasites in 200 leukocytes. The geometric mean (95% confidence interval [CI]) was 435 (194–676). All isolates showed development to mature schizonts in the control wells and could be evaluated for drug susceptibility. Tests were run in parallel for the seven antimalarial drugs. After 44–48 hours of incubation, the geometric mean (95% CI) number of schizonts in the control wells of the seven parallel series was 86 (7–165) per 1 cm^2 (50% hematocrit of 1 µL of erythrocyte suspension). The overall coefficient of variation for the counting of the individual isolates control wells was 7.9%. The variation in counting between two observers was analyzed by kappa analysis. The agreement was 55%.

Drug concentrations that showed 50% inhibition of schizont maturation were derived from sigmoid plots and are summarized in Table 2. The data were compared with those from previous studies[25] in Thailand by using the same methods. For most of isolates (21 of 24), complete inhibition of schizont maturation occurred in wells containing 10 µg/mL of quinine, chloroquine, mefloquine, and pyrimethamine. For all isolates, complete inhibition of schizont maturation occurred in wells containing 22 ng/mL of artesunate and 800 ng/mL of piperaquine. For 50% of isolates (10 of 19), complete inhibition schizont maturation occurred in wells containing 500 ng/mL of primaquine. The other 50% were completely inhibited at a concentration of 1 µg/mL of primaquine. There were positive correlations between IC_{50} values for quinine and mefloquine (r = 0.6, P = 0.004), piperaquine and chloroquine (r = 0.6, P = 0.008), and piperaquine and primaquine (r = 0.5, P = 0.01).

TABLE 1
Drug concentration ranges tested

Drug	Drug concentration (base)
Quinine	1 ng/mL–100 µg/mL
Piperaquine	6 ng/mL–20 µg/mL
Chloroquine	1 ng/mL–100 µg/mL
Mefloquine	1 ng/mL–100 µg/mL
Primaquine	15 ng/mL–500 ng/mL
Pyrimethamine	1 ng/mL–100 µg/mL
Artesunate	0.6 ng/mL–200 ng/mL

DISCUSSION

The total number of reported cases of *P. vivax* malaria in the Republic of Korea peaked in 2001 when there were 298,058 cases, and has subsequently decreased to 47,354 in 2003 and 34,485 in 2004.[5] Most cases were in military personnel who received chloroquine and primaquine prophylaxis. The duration of prophylaxis was extended from 16 weeks to 22 weeks since 2001.[26] Chloroquine and primaquine have been used for the radical cure of patients with *P. vivax* malaria that originated in the Republic of Korea.[5,27,28]

There have been no previous reports of *in vitro* susceptibility of *P. vivax* in the Republic of Korea. In this study, we evaluated the susceptibility of *P. vivax* from the Republic of Korea to antimalarial drugs by assessment of schizont maturation of *P. vivax*. This method is reliable and reproducible, but differs in some respects from other *in vitro* methods. It is possible that the IC_{50} values reported here may not be directly comparable with those obtained by using other methods.

When compared with *P. vivax* isolates from Thailand using this method, *P. vivax* from the Republic of Korea was more sensitive to quinine and mefloquine, but equally sensitive to chloroquine and artesunate. Reduced *P. vivax* mefloquine and quinine susceptibility in Thailand may have resulted from the use of mefloquine as first-line treatment for patients with *P. falciparum* malaria over the past 24 years because there is a high rate of mixed infections, and therefore consistent exposure of *P. vivax* relapses and newly acquired *P. vivax* infections to relatively low levels of mefloquine. Mefloquine has not been used in the Korean peninsula. In this study, the susceptibility to pyrimethamine was evaluated in the presence of folic acid (a competitive antagonist to antifolate activity) because *P. vivax* needs folic acid for complete schizogony.[25] Therefore,

TABLE 2
Susceptibility of *Plasmodium vivax* in the Republic of Korea to antimalarial drugs using the schizont maturation inhibition assay compared with results from Thailand*

Drug	Geometric mean IC_{50} (ng/mL) (95% CI)	
	Isolates from Thailand[15] (n = 50)	Isolates from the Republic of Korea (n = 24)
Quinine	308	59 (54–75)
Chloroquine	50	39 (22–282)
Mefloquine	127	39 (35–67)
Pyrimethamine	8	137 (89–280)
Artesunate	0.5	0.58 (0.5–0.8)
Primaquine	ND	121 (98–232)
Piperaquine	ND	27 (17–-58)

*Data are the mean of geometric 50% inhibition concentration (IC_{50}) and 95% confidence interval (CI). ND = no data.

the IC_{50} of pyrimethamine in this study might not reflect directly the *in vivo* sensitivity, although the value obtained could be useful for sequential monitoring of antifolate resistance. These data also provide baseline sensitivity of *P. vivax* to primaquine and piperaquine. The trends of *P. vivax* susceptibility to antimalarial drugs need to be monitored continuously to detect the emergence of drug resistance

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