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Mutational Analysis of *Pneumocystis jirovecii* Dihydropteroate Synthase and Dihydrofolate Reductase Genes in HIV-Infected Patients in China

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We investigated *Pneumocystis jirovecii* dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR) genes for mutations in 25 Chinese HIV-infected patients with *P. jirovecii* pneumonia. We identified DHPS mutations in 3 (12%) patients and DHFR mutations in 1 (4%) patient. The prevalence of DHPS and DHFR mutations in China remains low, as it does in other developing countries.

Pneumocystis jirovecii pneumonia (PCP) is a common and serious opportunistic infection in immunocompromised patients, especially in patients with AIDS. The first-line drug for the prophylaxis and treatment of PCP is the combination of trimethoprim and sulfamethoxazole (TMP-SMX), which targets dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS), respectively. Numerous studies from different countries have shown that mutations in the *P. jirovecii* DHPS gene are highly prevalent and are associated with prior exposure to and failure of TMP-SMX and dapsone, primarily when administered as prophylaxis, which strongly suggests that *P. jirovecii* is developing resistance to sulfa drugs (1–4). In contrast, there have been fewer reports of mutations in the *P. jirovecii* DHFR gene, and there has been no strong evidence to suggest that *P. jirovecii* is developing mutations related to resistance to TMP or other DHFR-inhibiting drugs. Despite a continuing increase in the morbidity and mortality rates associated with HIV/AIDS in China (5), there have been very few studies of *P. jirovecii* DHPS mutations, and all reports have included small numbers of patients (1, 6). There have been no reports characterizing *P. jirovecii* DHFR mutations. The aim of the present study was to examine the prevalence of *P. jirovecii* DHPS and DHFR mutations in HIV-infected patients with PCP from a hospital in Guangzhou, China.

From November 2009 to May 2013, we enrolled a total of 45 patients from the Guangzhou No. 8 Hospital in Guangzhou, China, who (i) were HIV positive, (ii) had at least two of three symptoms (cough, fever, and dyspnea), and (iii) had interstitial and/or alveolar diffuse infiltrates as shown with chest radiographs. Bronchoalveolar lavage fluid (BALF) samples were obtained from all the patients. Informed consent was obtained from all the study participants, and ethical clearance to conduct the study was obtained from the Guangzhou No. 8 Hospital Ethical Committee. Of the 45 patients, 13 had positive findings by Gomori methenamine silver staining of BALF or lung biopsy specimen samples (confirmed PCP), and 32 had negative findings by staining, although PCP was clinically suspected in 14 of the cases. Demographic and other clinical information from these patients was obtained from their medical records (as summarized in Table 1). None of the patients received anti-*Pneumocystis* prophylaxis or treatment before they were diagnosed with PCP.

DNA was extracted from all samples using the QIAamp DNA minikit (Qiagen) according to the manufacturer's instructions. The entire *P. jirovecii* DHPS and DHFR genes for each sample were amplified by PCR using AmpliTaq Gold DNA polymerase (Applied Biosystems) under the conditions described elsewhere (2). To obtain PCR products of sufficient quality and quantity for sequence analysis, we performed a primary PCR for each sample and secondary nested PCR for those samples for which the initial PCR products were not visible or were very faint on agarose gels. Initially, all positive PCR products were directly sequenced using internal primers. If there was any indication of mixed sequences present in the sequence chromatograms, the PCR products were cloned into TOPO vector (Invitrogen), and multiple plasmid clones were sequenced. To minimize potential PCR errors, at least 2 clones were used to confirm a nucleotide change. All nucleotide changes were confirmed by at least two independent PCR and sequencing experiments. Any nucleotide change that led to a change in the predicted amino acid sequence is referred to as a mutation or a nonsynonymous mutation. DNA sequencing was carried out commercially by Macrogen USA, Inc. Nucleotide sequences were analyzed using Sequencher software version 4.10.1 (Gene Codes Corporation) and MacVector version 12.6 (MacVector, Inc.).

Statistical analyses were conducted using SPSS version 17 (SPSS, Inc.). We compared the demographic and clinical data between patients with and without positive amplification of *P. jirovecii* DNA using the Student *t* test or the Kruskal-Wallis test for continuous variables and the chi-square test for categorical variables. A P value of <0.05 was considered to be significant.

We tested a total of 45 BALF specimens; the DHPS and DHFR genes were successfully amplified by PCR in 25, while the remaining 20 showed no amplification of either gene. Of the 25 PCR-

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TABLE 1 Comparison of HIV-infected patients with and those without positive results in PCR amplification of *P. jirovecii* DHPS and DHFR genes

Clinical information	PCR-positive patients ($n = 25$)	PCR-negative patients ($n = 20$)	*P* value
Age (mean ± SD) (yr)	39.3 ± 9.6	40.8 ± 9.0	0.56
Male sex (no. [%])	22 (88)	17 (85)	0.45
CD4 cell count (median [range]) (cells/μl)	20 (5–61)	72 (3–474)	0.03
CD4 cell count of <100 cells/ml (no. [%])	25 (100)	12 (60)	0.002
New HIV diagnosis (no. [%])	25 (100)	10 (50)	0.0001
PCP prophylaxis (no. [%])	0 (0)	6 (30)	0.012
Received >3 mo of antiretrovirals (no. [%])	0	9 (45)	0.001
Confirmed PCP (no. [%])[a]	13 (52)	0 (0)	0.0001
Outcome, survival (no. [%])	25 (100)	20 (100)	1

[a] Confirmed by Gomori staining. All 20 patients negative by PCR were also negative by Gomori staining.

TABLE 2 *P. jirovecii* DHPS and DHFR mutations in 25 HIV-infected patients with *P. jirovecii* pneumonia in Guangzhou, China

No. of patients	DHPS gene nucleotide(s) at position[a]:		DHFR gene nucleotide(s) at position[a]:	
	163	169	188	312
8[b]	A	C	A	T
4	A	C	A	C
9	A	C	A	T and C[c]
1	A	C	A and G[c]	C
2	A and G[c]	C and T[c]	A	T
1	A	C and T[c]	A	T and C[c]

[a] Relative to the full-length DHPS and DHFR genes of *P. jirovecii* (GenBank accession no. AF139132 and AF090368, respectively).
[b] Wild type for DHPS and DHFR genes.
[c] Confirmed by sequencing of plasmid clones. Double mutations were present in at least 3 clones for each patient.

positive cases, 13 cases had confirmed PCP, 10 cases had suspected PCP, and 2 cases were not diagnosed with PCP. The $CD4^+$ T cell counts were lower in PCR-positive patients than in PCR-negative patients, and all of the PCR-positive cases were also newly diagnosed with HIV infection, compared to 50% of the PCR-negative cases. No PCR-positive cases were receiving anti-*Pneumocystis* prophylaxis or antiretroviral therapy, versus 30% and 45%, respectively, of the PCR-negative patients (Table 1). There were no significant differences in any demographic or clinical characteristics between the patients with and those without *P. jirovecii* DHPS mutations (data not shown).

Of the 25 samples in which the *P. jirovecii* DHPS gene was amplified, 22 showed the wild-type DHPS sequence (with nucleotides A and C at positions 163 and 169, respectively), 2 showed a mixture of the wild-type and mutant nucleotides in these two positions, and the remaining 1 showed the wild-type nucleotide at position 163 and a mixture of the wild-type and mutant nucleotides at position 169 (Table 2). The mutations resulted in amino acid changes (nonsynonymous mutations) from threonine to alanine and proline to serine at codons 55 and 57, respectively, as described previously (2). There were no nucleotide changes in the other positions.

For the same 25 samples, sequencing of the *P. jirovecii* DHFR gene identified 10 samples with the wild-type DHFR sequence (nucleotides A and T at positions 188 and 312, respectively), 4 with a synonymous nucleotide change from T to C at position 312, 10 with a mixture of T and C at position 312, and 1 with a synonymous nucleotide change from T to C at position 312 and a mixture of A and G at position 188 (Table 2). The nucleotide change from A to G at position 188 results in an amino acid change from Glu to Gly at codon 63 (nonsynonymous mutation). Nucleotide changes at both positions have been reported previously (2, 7). There were no nucleotide changes in the other positions analyzed.

While 13 of the 25 samples showed nucleotide changes in either the DHPS or DHFR gene, only one showed nucleotide changes in both genes (with a synonymous nucleotide change in DHFR and a nonsynonymous mutation in DHPS). None of the patients in this study had known prior exposure to any drug with anti-*Pneumocystis* activity, including dapsone or TMP-SMX. Thus, the mutations detected cannot be attributed to the use of antifolate drugs in these patients. To our knowledge, this study is the largest to have examined *P. jirovecii* DHPS mutations and the first to report *P. jirovecii* DHFR mutations in patients from China. The prevalence of *P. jirovecii* DHPS mutations in this study (3/25, 12%) is not statistically significantly higher than those of two previous studies from China, which reported prevalences of 6.6% (1/15, $P = 1$) and 0% (0/10, $P = 0.542$) in samples collected during 1998 to 2001 (1) and 2007 to 2008 (6), respectively. The small sample sizes and relatively low frequencies among these studies preclude any conclusions about trends in prevalence over time. The overall prevalence of *P. jirovecii* DHPS mutations in China is similar to those reported from other developing countries (3, 8–12) but lower than those reported from developed countries (1–4) and may reflect the less frequent utilization of TMP-SMX or dapsone for PCP prophylaxis in developing countries.

Prior studies identified great variability in the frequencies of mutations in the *P. jirovecii* DHFR gene (7–9, 13, 14). In the present study, we detected nucleotide changes in the DHFR gene in 15 (60%) of 25 samples, although only 1 (4%) had a nonsynonymous mutation. This is similar to the prevalences reported in other Asian countries, including Japan (13) and Thailand (9), where approximately 60% of samples harbored nucleotide changes in the DHFR gene, most of which were synonymous. However, this prevalence is higher than those reported in the United States (3%, all synonymous [2]), South Africa (15%, all nonsynonymous [8]), Uganda (8%, all synonymous [10]), and Portugal (27% overall, of which 4% were nonsynonymous [14]). None of these studies linked nucleotide changes in the DHFR gene to antifolate prophylaxis for PCP, and given that the vast majority of the nucleotide changes are synonymous, they likely represent polymorphisms rather than antibiotic-related mutations. There has been only one report of a positive association between *P. jirovecii* DHFR mutations and the use of DHFR inhibitors, specifically pyrimethamine (7). The low overall prevalence of nonsynonymous DHFR mutations worldwide may reflect a low drug pressure on DHFR by TMP.

In conclusion, the present study demonstrates that the prevalence of *P. jirovecii* DHPS and DHFR mutations in recent HIV-infected patients in China (2009 to 2013) remains low, as it does in other developing countries. There will likely be an increase in PCP cases in China due to the continuing growth in the number of

patients with HIV/AIDS (5) and likely an increase in TMP-SMX use in China due to the provisional World Health Organization recommendations for TMP-SMX use to reduce the mortality rate of PCP (15); therefore, it will be important to continue monitoring the prevalence of *P. jirovecii* DHPS and DHPS mutations to identify any changes that may impact the therapeutic efficacies of sulfa drugs.

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