Increase in Prevalence of *Pneumocystis carinii* Mutations in Patients with AIDS and *P. carinii* Pneumonia, in the United States and China

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This study of *Pneumocystis carinii* dihydropteroate synthase (DHPS) mutations in patients with AIDS who have *P. carinii* pneumonia compares the change in the prevalence of such mutations in the United States, where sulfa-drug prophylaxis is widespread, to that in China, where it is infrequent. The DHPS gene from 145 US patients presenting during 1983–2001 and from 15 Chinese patients presenting during 1998–2001 was amplified by polymerase chain reaction and was sequenced. In the United States, 40% of patients had DHPS mutations; 38% received sulfa-drug prophylaxis. Mutation prevalence increased to 70% during 2000–2001, from 25% during 1994–1995 (P < .01). In China, 7% of patients had DHPS mutations; none received sulfa-drug prophylaxis. The prevalence of *P. carinii* DHPS mutations has markedly increased in the United States but remains low in China.

Studies performed during 1997–2002 show that the prevalence of *Pneumocystis carinii* dihydropteroate synthase (DHPS) mutations at amino acid positions 55 and 57 in US patients with AIDS and *Pneumocystis carinii* pneumonia (PCP) was 45%–65% [1–7]. Several findings indirectly suggest that the high overall prevalence of DHPS mutations may be a result of selective evolutionary pressure due to widespread use of sulfa-drug prophylaxis against PCP. For example, DHPS polymorphisms are nonsynonymous (i.e., all result in changes in the encoded amino acids) [1, 4]. In addition, mutations in the *P. carinii* DHPS gene occur up to 4.5 times more commonly in patients with AIDS and PCP who have received sulfa-drug prophylaxis than in those who have not received it [1–7]. Furthermore, a longer duration of sulfa-drug prophylaxis increases the chance that a *P. carinii* DHPS mutation will develop [4]. Finally, there are geographical variations in the prevalence of these mutations, perhaps reflecting regional variations in clinical practices’ preferences in the choice of sulfa drugs for prophylaxis against PCP [4, 5, 7].

These studies have addressed whether the prevalence of DHPS mutations has changed over time [1,4], but it remains unresolved whether they have increased from the onset of the AIDS epidemic to the early 2000s. The possibility that DHPS mutations may be the result of widespread sulfa-drug use would be strengthened by the demonstration that mutations have increased since the 1980s, before trimethoprim-sulfamethoxazole (TMP-SMZ) became the first-choice agent for prophylaxis against PCP in patients with AIDS [8]. Also, demonstration of a low frequency of mutations in countries where sulfa-drug prophylaxis is currently infrequent, such as China, would provide additional support to the theory that DHPS mutations occur under the evolutionary pressure of sulfa-drug use. For these reasons, we performed this study to compare, for the period from the 1980s to the early 2000s, the change in prevalence of *P. carinii* DHPS mutations in US patients with AIDS and PCP to that in such patients in China.

**Patients, materials, and methods.** Respiratory specimens and clinical data from 145 US patients with AIDS and PCP that were collected from 5 centers during 1983–2001, were analyzed according to year of diagnosis of PCP; 117 of these patients represented pooled data from 2 previously published trials [1, 4]. Specimens from patients from the University of Michigan Medical Center (Ann Arbor), Brigham and Women’s Hospital (Boston, MA), Indiana University Medical Center (Indianapolis), Wayne State University Medical Center (Detroit, MI), and Denver Health Medical Center (Denver, CO) were obtained for diagnostic purposes. In addition, samples from 15 Chinese patients with AIDS patients that were collected during 1998–2001, from 2 hospitals (Peking Union Medical College Hospital [Beijing] and the You An Hospital [Beijing]) were also obtained for diagnostic purposes. Chinese patients treated for PCP were excluded if no clinical data had been recorded or if a diagnostic specimen either had not been obtained or had been lost. The overall prevalence of DHPS mutations may be a result of selective evolutionary pressure due to widespread use of sulfa-drug prophylaxis against PCP. For example, DHPS polymorphisms are nonsynonymous (i.e., all result in changes in the encoded amino acids) [1, 4]. In addition, mutations in the *P. carinii* DHPS gene occur up to 4.5 times more commonly in patients with AIDS and PCP who have received sulfa-drug prophylaxis than in those who have not received it [1–7]. Furthermore, a longer duration of sulfa-drug prophylaxis increases the chance that a *P. carinii* DHPS mutation will develop [4]. Finally, there are geographical variations in the prevalence of these mutations, perhaps reflecting regional variations in clinical practices’ preferences in the choice of sulfa drugs for prophylaxis against PCP [4, 5, 7].

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been discarded. Preserved alcohol-fixed slides, paraffin-embedded tissue for cytopathology analysis, or frozen samples obtained from bronchoalveolar-lavage or sputum-induction specimens were delivered to The University of Michigan for analysis by polymerase chain reaction (PCR). The institutional review board at each institution approved the study.

Information regarding use of a sulfonamide chemoprophylactic agent—that is, dapsone or TMP-SMX—was abstracted from the patients’ charts by individuals who did not have knowledge of the results of the PCR analysis for *P. carinii* DHPS. Significant sulfonamide prophylaxis was defined as the use of sulfonamide drugs for at least 1 month during the 4 months preceding the date of diagnosis of PCP.

DNA was extracted from all samples and then was amplified by PCR in a PTC-100 programmable thermal controller (MJ Research), as described elsewhere [1]. If bands were present, then the DNA was sequenced directly. Bands were absent in 80 specimens, which subsequently were subjected to nested PCR, as described elsewhere [1]. Reaction products were purified on 1.2% low-melt agarose (GIBCO BRL) extracted by use of a Gel Extraction Kit (Qiagen) and were sequenced on an Applied Biosystems Model 373a automated DNA sequencer using ANEST primers. DNA and amino acid sequences were aligned by MacVector version 5.0 (International Biotechnologies). Results were analyzed according to year of diagnosis of PCP and year of sulfonamide prophylaxis.

Statistical calculations were performed by Epi Info (version 6.02; Centers for Disease Control and Prevention). Associations between sulfonamide use and DHPS polymorphisms were determined by 2-tailed Fisher’s exact test. *P* < .05 was considered to be significant.

**Results.** DNA was successfully extracted from specimens from 145 (73%) of 198 patients; these patients were included in the study. The 145 US patients with AIDS and PCP, whose DHPS gene was PCR-amplified from stored specimens obtained during 1983–2001, had a mean age ± SD of 39 ± 11 years (range, 26–61 years); 76% were men; and the mean CD4 cell count ± SD was 76 ± 59 cells/mm³ (range, 0–214 cells/mm³). The mean HIV load ± SD, available for 107 (74%) of the 145 patients (and determined after May 1995), was 4.1 ± 2.6 log₃ copies/mL. A total of 56 (38%) of the 145 patients had received prior sulfonamide prophylaxis against PCP; 9 (6%) of the 145 patients had experienced an episode of PCP, and none had experienced an episode of toxoplasmosis. Samples from 98 (68%) of the 145 patients were successfully amplified by a single round of PCR, yielding sequences that were 720–742 nt long. Samples from the remaining 47 (32%) of the 145 patients were amplified by nested PCR; for these, the sequences measured were 300–323 nt long.

The wild-type *P. carinii* DHPS sequence (Thr55/Pro57) was seen in 87 (60%) of the 145 US patients with AIDS, and mutations (Ala55/Ser57) were present in 58 (40%) of these 145 patients (table 1). Mutations were not present in any of the 26 patients who had experienced an episode of PCP prior to 1994 (table 2). Mutation prevalence increased to 70% (14 mutations/20 specimens) during 2000–2001, from 25% (6 mutations/24 specimens) during 1994–1995 (*P* < .01). Twelve of the 20 specimens obtained during 2000–2001 were from The University of Michigan Medical Center; the remaining 8 were from Brigham and Women’s Hospital. Mutations were first observed in patients with prior sulfonamide prophylaxis, and their frequency increased from 46% during 1994–1995 to 88% during 2000–2001. Subsequently, mutations appeared in those without sulfonamide prophylaxis, increasing from 38% during 1996–1997 to 54% during 2000–2001.

The 15 Chinese patients with AIDS and PCP, whose DHPS gene was PCR-amplified from stored specimens obtained during 1998–2001, had a mean age ± SD of 37 ± 16 years (range, 24–54 years); 54% were men; and the mean CD4 cell count ± SD was 46 ± 41 cells/mm³ (range, 8–126 cells/mm³). Data on the HIV load was not available for any of these 15 patients, and none had either received prior sulfonamide prophylaxis against PCP or experienced an episode of PCP or toxoplasmosis. The wild-type *P. carinii* DHPS sequence was seen in 14 (93%) of these 15 patients, and 1 (7%) of the 15 patients had a single mutation at position 55, resulting in an amino acid

| Table 1. Sequence patterns of *Pneumocystis carinii* dihydropteroate synthase, in 145 patients from the United States and 15 patients from China. |
|-----------------|----------------|----------------|
| Patients, no. (country) | Position 55 | Position 57 |
| 87 (United States); 14 (China) | Thr | Pro |
| 58 (United States); 0 (China) | Ala | Ser |
| 0 (United States); 1 (China) | Thr | Ala |

| Table 2. Mutations in *Pneumocystis carinii* dihydropteroate synthase, in US patients with AIDS and *P. carinii* pneumonia, by years of diagnosis and sulfonamide prophylaxis. |
|-----------------|----------------|----------------|
| Time period | Sulfa-drug prophylaxis | No sulfa-drug prophylaxis | Total |
| 1983–1993 | 0/3 (0) | 0/23 (0) | 0/26 (0) |
| 1996–1997 | 12/15 (80) | 8/21 (38) | 20/36 (55) |
| 1998–1999 | 12/16 (75) | 6/23 (26) | 18/39 (46) |
| 2000–2001 | 8/9 (88) | 6/11 (54) | 14/20 (70)a |
| Total | 38/56 (68) | 20/89 (22) | 58/145 (40) |

a Mutation-prevalence increase between 2000–2001 and 1994–1995 is statistically significant (*P* < .01).
change from threonine to alanine (table 1). No patient had a double DHPS mutation at amino acid positions 55 and 57.

Discussion. The present study shows that, in the United States, P. carinii DHPS mutations have appeared recently and that their the prevalence has increased. The increase in these mutations corresponds with the widespread use of sulfa drugs for prophylaxis against PCP in patients with AIDS, which occurred during the late 1980s after these drugs were deemed the agent of choice for this purpose [8]. It is unlikely that the geographic location where US specimens were obtained could account for the recent increase in mutations, because specimens analyzed during 2000–2001 were not from locales with the highest reported prevalence of DHPS [4]. In contrast, DHPS mutations were infrequent in a small number of patients from a region in China, a country where sulfa-drug prophylaxis for PCP is uncommon. The small number of Chinese specimens reflects the practice in that country—to empirically treat patients without obtaining diagnostic respiratory specimens. Together, the findings from the United States and China lend support to the hypothesis that the DHPS gene has been under selective evolutionary pressure such as that which would occur from prophylaxis with sulfa drugs.

An increasing prevalence of genotypic or phenotypic drug resistance has occurred in several microorganisms, in association with increasing drug use over time. For example, during the past decade there has been an increase in primary protease mutations noted in antiretroviral-naïve HIV-infected patients, reflecting the widespread use of these agents since their licensure in 1996 [9]. Furthermore, in malaria, mutations that accumulate over time tend to impart greater degrees of drug resistance [10]. Moreover, the prevalence of pneumococcal resistance to penicillin and multiple other antibiotics is an example of how increasing drug resistance in one organism has occurred in parallel with widespread antibiotic use over time [11].

It is possible that DHPS-mutant P. carinii strains, once present, could spread to patients who are not receiving prophylaxis, because PCP may represent a newly acquired infection rather than a reactivation of one acquired previously [12]. This hypothesis could account for the increasing prevalence of P. carinii DHPS-gene mutations in those in the present study who were not receiving prophylaxis. It is possible, however, that some patients whom we classified as not having sulfa-drug prophylaxis did indeed have exposure to this type of drug—but that both the retrospective nature of the study and the case definition used did not identify them as such. Nonetheless, although previous studies have not shown that DHPS mutations are associated with specific strain types, which might be expected if person-to-person spread of mutant strains occurs [12], the demonstration that mutant strains have begun to appear recently in those not receiving prophylaxis is consistent with person-to-person spread of mutant strains. A similar explanation has been proposed to account for the recent and increasing appearance of primary protease mutations, which has been witnessed in antiretroviral-naïve HIV-infected patients after widespread use of these agents [9].

Several factors suggest that Thr55→Ala and Pro57→Ser substitutions in the P. carinii DHPS enzyme could lead to a reduction in sulfa-drug sensitivity. First, binding of pterin substrate to Thr55 is interrupted because alanine lacks the hydroxyl group required for this interaction [13]. Second, Ser57 may disrupt pterin binding by altering the position of Arg56 [13]. Third, both DHPS mutations are similar to those in other organisms—for example, Mycobacterium leprae, Toxoplasma gondii, Plasmodium falciparum, and Escherichia coli—that demonstrate sulfa-drug resistance [13]. For these organisms, a relationship between genetic mutations and phenotypic sulfa-drug resistance has been directly shown by measurements of the effects that sulfa drugs have on in vitro growth or DHPS enzymatic activity. However, this has not directly been shown in P. carinii, because the organism cannot be cultured from isolates from patients to determine drug sensitivities.

Notwithstanding these considerations, the clinical significance of DHPS mutations in P. carinii remains speculative. The occurrence of PCP in patients receiving sulfa-drug prophylaxis suggests that DHPS mutations may confer a low-level resistance that can overcome the inhibitory effects of doses used for prophylaxis. Whether DHPS mutations have a clinically relevant affect on response to higher, therapeutic doses of sulfa drugs seems less likely, because 3 [1, 4, 15] of 4 studies [1, 2, 4, 15] have shown that the outcome of treatment with sulfa drugs in patients with wild-type P. carinii is not better than that in patients with the mutant form. In addition, another study has shown that sulfa-drug treatment fails in only 28% of patients with DHPS mutations [1]. Together, these findings suggest that the mutations at amino acid positions 55 and 57 in the P. carinii DHPS have, at most, a small effect on overall response to therapy.

Nevertheless, it remains possible that, if a strain with additional DHPS mutations arises, a much higher level of sulfa-drug resistance may occur, which could result in a significant loss of response to treatment with sulfa-drug agents—the most effective drugs for treatment of PCP. In other microorganisms, in fact, there already is precedence for a correlation with increasing number of drug-resistant mutations conferring a higher level of drug resistance to that agent. For example, a greater number of pneumococcal penicillin–binding mutations in penicillin-binding proteins 2B and PBP 2X are associated with a greater degree of decreased binding affinity and a greater degree of penicillin resistance [14]. In addition, a greater number of HIV mutations in certain protease-inhibitor genes lead to a greater degree of phenotypic resistance to certain agents (e.g., lopinavir), which is also the case with mutations of nucleoside agents (e.g., azidothymidine) [9].
In summary, the findings of the present study support the hypothesis that DHPS strains may occur under selective pressure resulting from widespread sulfa-drug use. These findings may have particular relevance for developing countries, where PCP has recently been recognized as a significant AIDS-associated opportunistic infection [16]. There will likely be an increase in sulfa-drug use in developing countries, as a result of the provisional World Health Organization recommendations for sulfa-drug use to reduce mortality [16]. It will be important for prospective studies to determine whether, like the United States, developing countries also experience, over time, an association between an increase in sulfa-drug use and an increase in \( \text{P. carinii} \) mutations. These studies should also monitor whether DHPS polymorphisms at amino acid positions other than 55 and 57 will occur—and, if so, whether these mutations will result in a higher level of sulfa-drug resistance that could possibly result in a reduced response to sulfa-drug therapy.

References