MAJOR ARTICLE

Interhuman Transmission as a Potential Key Parameter for Geographical Variation in the Prevalence of *Pneumocystis jirovecii* Dihydropteroate Synthase Mutations

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Background. *Pneumocystis jirovecii* dihydropteroate synthase (DHPS) mutations are associated with failure of prophylaxis with sulfa drugs. This retrospective study sought to better understand the geographical variation in the prevalence of these mutations.

Methods. DHPS polymorphisms in 394 clinical specimens from immunosuppressed patients who received a diagnosis of *P. jirovecii* pneumonia and who were hospitalized in 3 European cities were examined using polymerase chain reaction (PCR) single-strand conformation polymorphism. Demographic and clinical characteristics were obtained from patients' medical charts.

Results. Of the 394 patients, 79 (20%) were infected with a *P. jirovecii* strain harboring one or both of the previously reported DHPS mutations. The prevalence of DHPS mutations was significantly higher in Lyon than in Switzerland (33.0% vs 7.5%; $P < .001$). The proportion of patients with no evidence of sulfa exposure who harbored a mutant *P. jirovecii* DHPS genotype was significantly higher in Lyon than in Switzerland (29.7% vs 3.0%; $P < .001$). During the study period in Lyon, in contrast to the Swiss hospitals, measures to prevent dissemination of *P. jirovecii* from patients with *P. jirovecii* pneumonia were generally not implemented, and most patients received suboptimal prophylaxis, the failure of which was strictly associated with mutated *P. jirovecii.* Thus, nosocomial interhuman transmission of mutated strains directly or indirectly from other individuals in whom selection of mutants occurred may explain the high proportion of mutations without sulfa exposure in Lyon.

Conclusions. Interhuman transmission of *P. jirovecii,* rather than selection pressure by sulfa prophylaxis, may play a predominant role in the geographical variation in the prevalence in the *P. jirovecii* DHPS mutations.

The circulation of *Pneumocystis jirovecii* strains among putative human reservoirs, including patients with *P. jirovecii* pneumonia (PCP) and colonized or healthy adults and infants, remains poorly understood. Comprehension of epidemiological patterns of transmission and acquisition of *P. jirovecii* is crucial to improve prevention of the disease. This is also true in developing countries where PCP is probably a major cause of illness and death [1, 2]. However, at the present time, diagnosis of PCP in these countries is mainly clinical, and reliable studies using bronchoalveolar lavage specimens are scarce. Thus, most of the data about *P. jirovecii* epidemiology are still expected to come from developed countries, despite the decrease in the number of PCP cases, which is related to better access to highly active antiretroviral therapy.

Most drugs used for prevention and treatment of PCP target enzymes that are involved in the biosynthesis of folic acid. Sulfa drugs (sulfamethoxazole, dapsone, and sulfadoxine) inhibit dihydropteroate synthase

Received 5 February 2010; accepted 20 April 2010; electronically published 6 July 2010.

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Clinical Infectious Diseases 2010; 51(4):e28–e33

1058-4838/2010/5104-00E1$15.00
DOI: 10.1086/655145

Table 1. Demographic and Clinical Characteristics of 305 Human Immunodeficiency Virus (HIV)–Positive and HIV-Negative Patients Who Received a Diagnosis of *Pneumocystis jirovecii* Pneumonia in Lyon, Lausanne, and Zurich Hospitals

Characteristics	Patients who received sulfa prophylaxis[a]	Patients who received no or other prophylaxis[b]	Overall
HIV negative	9	64	73 (100)
Age, median years (range)	49 (20–76)	36 (4–82)	55 (4–82)
Sex			
Male	8	39	47 (64.4)
Female	1	25	26 (35.6)
HIV positive	25	207	232 (100)
Age, median years (range)	35 (23–56)	36 (4–69)	36 (4–69)
Sex			
Male	20	170	190 (82)
Female	5	37	42 (18)
HIV risk factor			
Homosexual	14	89	103 (44.4)
Heterosexual	7	58	65 (28)
Injection drug user	2	28	30 (12.9)
Other	2	32	34 (14.7)
CD4 cell count, median cells/μL (range)	9 (0–99)	31 (0–390)	29 (0–390)
Cerebral toxoplasmosis	0	0	0

NOTE. Data are no. (%) of patients, unless indicated otherwise.

[a] Includes 14 patients who received sulfadoxine-pyrimethamine, 10 patients who received sulfamethoxazole-trimethoprim, and 10 patients who received dapsone.

[b] Patients who received prophylaxis with pentamidine ($n = 31$) or atovaquone ($n = 2$) are included in this category.

(DHPS), whereas trimethoprim and pyrimethamine target dihydrofolate reductase. Cotrimoxazole, the combination of sulfamethoxazole and trimethoprim, is the drug of choice, whereas Fansidar, the combination of sulfadoxine and pyrimethamine, is rarely used. In the past, several studies reported the association between failure of sulfa prophylaxis or exposure to sulfa drugs and substitutions of 2 amino acids within the putative sulfa binding site of DHPS at positions 55 (threonine to alanine; mutation M1) and 57 (proline to serine; M2) [3–5]. They are observed either as single mutations (M1 or M2) or a double mutation (M3). The *P. jirovecii* DHPS sequence with no substitutions was defined as the wild type.

The DHPS mutations not only raise questions about the acquisition of resistance to sulfa drugs by *P. jirovecii* but also can be useful as an epidemiological marker to investigate *P. jirovecii* circulation among its reservoirs. The characterization of the parameters influencing the geographic variation in allelic frequency at the DHPS locus may prove to be informative. We therefore analyzed this locus in a large collection of clinical specimens from 3 Western European cities and focused on the epidemiological implications of the results. (Preliminary results of this study were presented as a short note in a conference proceeding [6].)

PATIENTS, MATERIALS, AND METHODS

Patients and specimens. A total of 394 human immunodeficiency virus (HIV)–negative and HIV-infected patients who received a diagnosis of PCP and who were hospitalized at 1 of the 7 hospitals in 3 distinct European cities are included in this study. Five hospitals were in Lyon (France), and 2 were in Switzerland (Centre Hospitalier Universitaire Vaudois in Lausanne and University Hospital in Zurich). Diagnosis of PCP was performed by methenamine silver staining in Lyon and Lausanne and by immunostaining in Zurich (Dako). Consent was obtained from all patients. Study protocols and patient consent forms were approved by each site's institutional review board. Lyon is ~400 km from Zurich, and Lausanne is located at approximately mid-distance between Lyon and Zurich. Lyon, Zurich, and Lausanne include 1.75, 1.1, and 0.3 million inhabitants, respectively. The 394 bronchoalveolar lavage specimens were collected from April 1993 to November 1997 for Lyon's hospitals, from October 1990 to February 2000 for the Lausanne hospital, and in June 1991 as well as from August 1994 to October 1998 for the Zurich hospital. Part of the 194 specimens from Lyon were analyzed previously to study the association of DHPS mutations with different prophylactic sulfa

Table 2. *Pneumocystis jirovecii* Dihydropteroate Synthase (DHPS) Genotypes of 394 Human Immunodeficiency Virus (HIV)–Negative and HIV-Positive Patients Who Received a Diagnosis of *P. jirovecii* Pneumonia in 3 European Cities

DHPS genotype	Lyon	Lausanne	Zurich
WT	130 (67.0)	131 (93.6)	54 (90.0)
Mutant			
M1	0 (0)	2 (1.4)	0 (0)
M2	32 (16.5)	0 (0)	0 (0)
M3	9 (4.6)	2 (1.4)	2 (3.3)
Mixture[a]			
WT + M1	0 (0)	1 (0.7)	0 (0)
WT + M2	6 (3.1)	3 (2.2)	3 (5)
WT + M3	13 (6.7)	1 (0.7)	1 (1.7)
M2 + M3	4 (2.1)	0 (0)	0 (0)
Total			
WT	130 (67.0)	131 (93.6)	54 (90.0)
Mutant (M1, M2, M3)	64 (33.0)	9 (6.4)	6 (10.0)

NOTE. Data are no. (%) of patients. WT, wild type.

[a] Patients with 2 DHPS alleles, which suggested coinfection with 2 *P. jirovecii* types. The specimens were classified in the corresponding mutant category (see text).

drugs (158 specimens [7]) and to investigate the possibility of interhuman transmission within a cluster of patients with PCP (39 patients [8]). Specimens were stored at −20°C before analysis.

Patients' characteristics. Specific information on demographic and clinical characteristics and PCP chemoprophylaxis were obtained from patients' medical charts. Patients were considered to have received sulfa prophylaxis if they received sulfamethoxazole-trimethoprim, pyrimethamine-sulfadoxine, or dapsone during a period of 3 months preceding the date of diagnosis of PCP. The duration of prophylaxis ranged from 1 week to the entire 3 months. These definitions were chosen because the data in the described clusters of PCP suggest that the incubation period of de novo infection is 3 weeks to 3 months [8]. All chart reviews were done without knowledge of the DHPS genotyping results.

DHPS extraction and genotyping. DNA was extracted from specimens using QIAamp DNA Blood Mini Kit (Qiagen). A region of 318 base pairs spanning the *P. jirovecii* DHPS binding site was amplified and genotyped using the PCR single-strand conformation polymorphism (SSCP) technique as described elsewhere [7]. In this technique, each DHPS allele is identified by a specific SSCP pattern made of 2 DNA bands, each corresponding to 1 of the 2 single strands of the allele.

Statistical analysis. Possible associations were analyzed by χ^2 test. A P value <.05 was considered statistically significant. Statistical analyses were done with STATA/IC statistical software (version 10.1; Stata).

RESULTS

Patients. Of the 394 patients included in the study, 194 (49.2%) were from Lyon, 140 (35.5%) were from Lausanne, and the remaining 60 (15.2%) were from Zurich. Complete demographic and clinical data were available for 305 patients (158, 109, and 38 patients from Lyon, Lausanne, and Zurich, respectively) (Table 1). According to the medical charts, 34 patients were receiving sulfa prophylaxis (14 received pyrimethamine-sulfadoxine, 10 received sulfamethoxazole-trimethoprim, and 10 received dapsone). The 14 patients who received pyrimethamine-sulfadoxine were from Lyon; in 3 of the 5 hospitals of this city, pyrimethamine-sulfadoxine was the first-choice regimen for anti–*P. jirovecii* prophylaxis. Nevertheless, this prophylaxis was suboptimal because it was at a dosage lower than recommended (1 or 3 tablets of 500 mg sulfadoxine plus 25 mg pyrimethamine every 2 weeks vs 2 tablets per week [9]). In the 2 other hospitals in Lyon, aerosolized pentamidine was most frequently used as prophylaxis. By mid-1996, all susceptible patients in Lyon were given appropriate prophylaxis with sulfamethoxazole-trimethoprim. Adequate prophylaxis with dapsone or sulfamethoxazole-trimethoprim was used in the 2 Swiss hospitals. A review of the entire available medical history of the patients suggested that no patients had received sulfa drugs for any purpose other than for PCP, and none had received sulfadiazine as treatment for toxoplasmosis prior to the episode of PCP. Except for 2 patients receiving pyrimethamine-sulfadoxine who stopped prophylaxis 7 days before the diagnosis of PCP was made, all patients were receiving prophylaxis at the time of PCP occurrence. There were no significant differences in clinical or biologic parameters among the patients from the different cities and hospitals or between those who received prophylaxis and those who did not receive prophylaxis.

Genotypes and prevalence of DHPS. Eight different *P. jirovecii* DHPS genotypes were detected using PCR SSCP for the 394 specimens (Table 2). The predominant genotype observed among patients from each city was the wild-type allele (67.0% in Lyon, 93.6% in Lausanne, and 90.0% in Zurich). The remaining specimens contained either only 1 mutant genotype or a mixture of DHPS genotypes, including most often a mutant and a wild-type allele. The latter category comprised 32 specimens (8.1%) and suggested coinfection with at least 2 *P. jirovecii* strains. These specimens were classified in the corresponding mutant category in further analyses. Four specimens displaying a mixture of M2 and M3 genotypes were classified in the M3 category. The predominant mutant in Lyon was M2 (38 [59.4%] of 64 mutants). The proportion of mutant DHPS strains was 33.0% in Lyon but was only 6.4% in Lausanne and 10.0% in Zurich. The prevalence of mutant DHPS was significantly higher in Lyon than in Switzerland (64 [33.0%] of 194 vs 15 [7.5%] of 200; $P < .001$). In contrast, the difference in

Table 3. *Pneumocystis jirovecii* Dihydropteroate Synthase (DHPS) Genotypes in 305 Patients with *P. jirovecii* Pneumonia Who Received or Did Not Receive Sulfa Prophylaxis in 3 European Cities

City, DHPS genotype	Received sulfa prophylaxis (%)	Received no or other prophylaxis (%)
Lyon (5 hospitals)		
WT	4 (20.0)	97 (70.3)
Mutant	16 (80.0)	41 (29.7)
Lausanne		
WT	3 (30.0)	98 (99.0)
Mutant	7 (70.0)	1 (1.0)
Zurich		
WT	3 (75.0)	31 (91.2)
Mutant	1 (25.0)	3 (8.8)
All[a]		
WT	10 (29.4)	226 (83.4)
Mutant	24 (69.6)	45 (16.6)

NOTE. Data are no. (%) of patients. WT, wild type.
[a] $P < .001$.

prevalence of mutants between the Zurich and Lausanne hospitals ($P = .56$) was not statistically significant. There were no significant differences in age, sex, HIV status, HIV risk factor, and CD4 cell count among patients infected with mutant *P. jirovecii* and those infected with wild-type strains.

Association between DHPS mutations and sulfa prophylaxis. Among the 305 patients with complete medical charts available, the specimens obtained from patients who received sulfa prophylaxis were more likely to harbor *P. jirovecii* mutant DHPS strains than were those from patients who did not receive any sulfa prophylaxis (24 [70.6%] of 34 patients vs 45 [16.6%] of 271; $P < .001$) (Table 3). The proportion of patients receiving sulfa prophylaxis was not significantly higher in the Lyon hospitals than in the Swiss hospitals (20 [12.7%] of 158 vs 14 [9.5%] of 147; $P = .48$). DHPS mutations were present in 16.6% of the patients who were not exposed to sulfa prophylaxis. This proportion was significantly higher in the Lyon hospitals than in the Swiss hospitals (41 [29.7%] of 138 vs 4 [3.0%] of 133; $P < .001$).

DISCUSSION

In this retrospective study of the prevalence of *P. jirovecii* mutant DHPS genotypes among patients in 3 cities, we found a significantly higher prevalence of DHPS mutants in Lyon than in Zurich and Lausanne. In contrast, there were no significant differences in the prevalence between Zurich and Lausanne, as well as among the 5 hospitals in Lyon [7]. Furthermore, we observed that patients receiving sulfa prophylaxis were more likely to harbor a mutant *P. jirovecii* strain than were those not receiving prophylaxis, that DHPS mutations were also present in patients who had apparently not been exposed to sulfa drugs, and that the proportion of the latter category of patients was significantly higher in the Lyon hospitals than in the Swiss hospitals. The limitations of the study included the lack of contemporary data, as well as the lack of clinical information for 23% of the patients, but there are no reasons to think that these affected the conclusions drawn.

Geographic variation in allelic frequency at the DHPS locus among cities in a restricted area, or even within a single city, has been reported in several countries [10–13]. The proportion of patients receiving PCP prophylaxis with sulfa drugs was thought to be the key parameter to explain these variations. Indeed, the pressure exerted by the sulfa drug can select the mutant strains within a patient receiving sulfa for prophylaxis [14, 15]. Selection of the mutations by sulfa drugs was also strongly supported by the fact that, in the 5 hospitals in Lyon, the M2 mutation was specifically selected by suboptimal prophylaxis with the combination of sulfadoxine and pyrimethamine, which was used only in this city [7]. However, sulfa prophylaxis might not be the only explanation, because *P. jirovecii* mutant strains were also found in the absence of prophylactic or therapeutic exposure to sulfa drugs [5, 11]. Although it is difficult to rule out that these patients have received any sulfa drugs, such cases are more likely to result from acquisition of a mutated strain via interhuman transmission from other individuals in whom selection of mutants occurred. Supporting this hypothesis, the place of the PCP diagnosis was an independent risk factor for the acquisition of a mutant *P. jirovecii* DHPS genotype, after adjustment for other variables, including sulfa exposure, in the multivariate analyses [10, 11]. Interhuman transmission might have been direct, but indirect transmission through carriers such as health care workers, physicians, or asymptomatic immunosuppressed patients cannot be ruled out.

The higher prevalence of mutant DHPS in Lyon than in Switzerland was not because of a higher proportion of patients receiving prophylaxis. Indeed, the proportion of patients with no evidence of sulfa exposure that harbored a mutant *P. jirovecii* DHPS genotype was significantly higher in the Lyon hospitals, compared with the Swiss hospitals. Two facts suggest a role of person-to-person transmission in the acquisition of mutated *P. jirovecii* during the study period in Lyon. First, a policy of isolating patients according to their underlying disease or because of the occurrence of a PCP episode was not implemented in 4 of the 5 hospitals. In particular, HIV-infected patients with PCP were not separated from organ transplant recipients in 1 of the 5 hospitals. In this hospital, we previously reported molecular evidence of interhuman transmission among a cluster of 10 renal transplant recipients with PCP [8]. Second, suboptimal prophylaxis using Fansidar was the first choice in 3 of the 5 hospitals in Lyon. Failure of such prophylaxis in 14 pa-

tients was strictly associated with mutation M2 [7]. Dissemination of these strains may have provoked secondary cases, possibly corresponding to the 24 patients with mutation M2 in Lyon without evidence of exposure to sulfa drugs. In contrast, dissemination of *P. jirovecii* was prevented by isolation measures and adequate prophylaxis used in the 2 Swiss hospitals.

The DHPS wild-type genotype was present in the majority of samples from the 3 cities and the mean prevalence of mutant genotypes was 20%. This prevalence is within the range of values reported in Europe—from a low of 3% in Portugal [16] to a high of 47% in England [17]. A similar prevalence was reported in Australia (13%) [18] and Japan (29%) [19]. The limited data from developing countries seem to indicate that the prevalence may be lower there than in the developed countries: 0% in Brazil [20] and China [21], 7% in Zimbabwe [22], and 12% in Thailand [23]. By contrast, the rates reported in the United States were generally higher, ranging from 26% [3] to 81% [24]. This difference could be explained at least partially by the fact that a smaller proportion of patients were receiving prophylaxis in Europe than in the United States. Indeed, this proportion was only 12% in our study but was 38% and 64% in the studies by Huang et al [11] and Kazanjian et al [5], respectively. Nevertheless, the present study suggests that acquisition of mutant strains by interhuman transmission may also have played an important role in some studies. The mutant strains acquired in the absence of sulfa exposure reported [5, 11] may be examples of such interhuman transmission events, nosocomial or not.

In conclusion, we describe a geographical variation in the prevalence of the *P. jirovecii* DHPS mutations, which may be caused by interhuman transmission in one city, possibly linked to the absence of measures to prevent nosocomial dissemination of the fungus and to a suboptimal prophylaxis regimen. Our results suggest that interhuman transmission may play a predominant role in *P. jirovecii* epidemiology in some hospitals. This should be kept in mind in the perspective of a possible emergence of *P. jirovecii* strains resistant to sulfa drugs. In the absence of isolation measures, interhuman transmission may accelerate the selection of strains clinically resistant to sulfa drugs by favoring the accumulation of mutations under the pressure of sulfa prophylaxis. This may become more pronounced in developing countries because of the widespread use of sulfa drugs to treat many bacterial diseases.

Acknowledgments

We thank the physicians responsible for the involved wards for access to patients' charts. We are indebted to Arlette Cruchon for DNA extraction.

Financial support. Swiss National Program on AIDS Research (3345-65407); Swiss National Science Foundation (310030-124998); Centre de Coordination de la Lutte contre les Infections Nosocomiales Sud-Est et Hospices Civils de Lyon; Swiss Federal Office for Education and Science for participation in EUROCARINII project, Framework V Program, European Commission (QLK2-CT-2000-01369); North-South fellowship from the University of Lausanne (to A.N.).

Potential conflicts of interest. All authors: no conflicts.

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