MAJOR ARTICLE

Association between a Specific *Pneumocystis jiroveci* Dihydropteroate Synthase Mutation and Failure of Pyrimethamine/Sulfadoxine Prophylaxis in Human Immunodeficiency Virus–Positive and –Negative Patients

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To investigate the possible association between different prophylactic sulfa drugs and the genotype of the *Pneumocystis jiroveci* dihydropteroate synthase (DHPS) gene, we examined DHPS polymorphisms in clinical specimens from 158 immunosuppressed patients (38 HIV-negative and 120 HIV-positive), using polymerase chain reaction–single-strand conformation polymorphism. Fifty-seven (36.1%) of 158 patients were infected with a mutant DHPS genotype. All patients who developed *P. jiroveci* pneumonia (PcP) while receiving pyrimethamine/sulfadoxine (PM/SD) prophylaxis ($n = 14$) had a strain harboring DHPS with an amino acid change at position 57 (Pro→Ser). This mutation was only present in 20 (14%) of 144 patients not receiving prophylaxis ($P < .001$). Hospitalization in a specific hospital was an independent risk factor for having *P. jiroveci* harboring the same DHPS mutation, which indirectly supports that interhuman transmission may affect the dissemination of the mutant strains.

Pneumocystis carinii special form *hominis*, which has recently been renamed *Pneumocystis jiroveci* [1, 2], is one of the most common opportunistic pathogens causing severe life-threatening pneumonia in immunocompromised patients. Cotrimoxazole, the combination of trimethoprim (TMP) and sulfamethoxazole (SMZ), is the drug of choice for the treatment and prophylaxis of *P. jiroveci* pneumonia (PcP) [3, 4]. TMP is an inhibitor of dihydrofolate reductase (DHFR), whereas SMZ inhibits dihydropteroate synthase (DHPS). Both enzymes are involved in the biosynthesis of folic acid. Experiments in animal models [5, 6] have suggested that the anti-*Pneumocystis* activity of TMP/SMZ is due only to SMZ. Dapsone is a derivative of sulfa drugs (sulfone) that is also frequently used for prophylaxis and targets DHPS. The widespread use of sulfa drugs in the prevention and treatment of PcP in recent years has been found to be associated with an increase in the prevalence of specific mutations in the gene coding for DHPS [7–9]. These mutations are similar to those known to confer sulfa drug resistance in other pathogens [10, 11]. The *P. jiroveci* DHPS mutations have been associated with a failure of TMP/SMZ in prophylaxis [7– 9, 12–17] and possibly in treatment [8, 12].

Some studies have reported the efficacy of pyrimethamine/sulfadoxine (PM/SD) in the prevention of PcP

Received 20 February 2003; accepted 17 April 2003; electronically published 26 September 2003.

Financial support: Swiss National Program on AIDS Research (grant 3345-65407); 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 the EUROCARINII project, Framework V Program , European Commission (contract QLK2-CT-2000-01369); North-South fellowship from the University of Lausanne (support to A.N.)

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The Journal of Infectious Diseases 2003; 188:1017–23

0022-1899/2003/18807-0012$15.00

and toxoplasmosis in human immunodeficiency virus (HIV)–infected individuals [18–20]. Like TMP/SMZ, PM/SD is a combination of a DHFR inhibitor (PM) and DHPS inhibitor (SD). PM/SD is not recommended as prophylaxis against PcP for HIV-infected patients in the US Public Health Service/Infectious Disease Society of America guidelines [3] or for HIV-negative patients [4], because its efficacy has not been well documented. No study has yet investigated the possibility of *P. jiroveci* DHPS mutations conferring resistance to PM/SD. To investigate the contribution of the use of different sulfa drugs in the epidemiology of DHPS mutations, we conducted an epidemiological study of *P. jiroveci* DHPS mutations in our collection of clinical specimens from patients who had received different prophylactic agents, including PM/SD.

PATIENTS, MATERIALS, AND METHODS

Characteristics of patients. Specific information on demographic and clinical characteristics, chemoprophylaxis, treatment regimens, and PcP outcome were obtained from patients' medical charts. Patients were considered as having received sulfa prophylaxis if they had received TMP/SMZ, PM/SD, or dapsone during the 3 months preceding the date of diagnosis with PcP. The duration of sulfa prophylaxis ranged from 7 days to the entire 3-month period. In 3 hospitals (A, D, and E), PM/SD was the first-choice regimen for prophylaxis at a dose of 1 or 3 tablets every 2 weeks (first and fifteenth day of the month), whereas, in the 2 other hospitals, aerosolized pentamidine was most frequently used as prophylaxis. The failure of sulfa prophylaxis was defined as the development of PcP in patients who received sulfa prophylaxis. Death attributed to PcP was defined as death occurring in the hospital and for which the treating physician had recorded PcP as the primary cause of death. All chart abstractions were done without knowledge of the DHPS genotyping results. Consent was obtained from all patients. Study protocols and patient consent forms were approved by each site's institutional review board,

Specimens. Specimens were 167 bronchoalveolar lavage (BAL) samples obtained from 158 HIV-positive and -negative immunosuppressed patients with confirmed PcP who were hospitalized in 5 hospitals in Lyon, France: hospital A, 62 patients; hospital B, 43 patients; hospital C, 30 patients; hospital D, l6 patients; and hospital E, 7 patients. BAL specimens were collected between April 1993 and December 1996 and were stored at −20°C before analysis. The 167 BAL specimens from 158 cases corresponded to all available specimens and represented 66% of the PcP cases that occurred during that period in the 5 hospitals (7 patients had a subsequent episode of PcP that was excluded from the present analysis). To set up the polymerase chain reaction (PCR)–single-strand conformation polymorphism (SSCP) method to differentiate the different DHPS alleles, specimens collected from a hospital in Lausanne, Switzerland, were also analyzed.

P. jiroveci *DHPS amplification and genotyping.* DNA was extracted from BAL specimens by use of the QIAamp DNA Blood Mini Kit (QIAGEN). A region of 318 bp spanning the putative drug binding site of the DHPS, in which *P. jiroveci* mutations were observed, was amplified using PCR primers Ahum [21] and Bhs′ (5′-ACCTTCCCCCACTTATATC-3′). PCR was carried out with reagents of the HotStar Taq DNA Polymerase Kit (QIAGEN). PCR conditions included a hot start for 10 min at 95°C, followed by 35 cycles consisting of 30 s at 94°C, 30 s at 52°C, and 1 min at 72°C. The reaction ended with 5 min of extension at 72°C.

P. jiroveci DHPS genotyping was done using the PCR-SSCP technique described elsewhere [22], with migration at 4°C for 4 h and 15 min in Pharmacia-Biosciences delect buffer. DNA sequencing was done as described elsewhere [23].

Statistical analysis. To compare the patients infected by *P. jiroveci* strains harboring DHPS mutations with those infected by a wild-type (wt) DHPS genotype, we used the 2-sided Wilcoxon rank sum test for continuous variables and the χ^2 and Fisher's exact tests for proportions. The significance level was .05 in all tests. Significant univariate predictors of infection with a mutant DHPS strain were then candidates for inclusion in a logistic-regression model that was built through a forward selection process. The model was then tested for confounding by each of the excluded covariates. The Wald test was used to report the significance level of the predictors in the final model. Predictors of death attributed to PcP were investigated using the same strategy. Statistical analyses were done with STATA statistical software (version 6.0; Stata).

RESULTS

Patients. One hundred sixty-seven BAL specimens from 158 patients diagnosed with PcP in 5 hospitals in Lyon, France, were included in the study. The majority ($n = 120$; 76%) of the patients were HIV infected, and 24% (38/158) were HIV-negative but had various other causes of immunodeficiency (lung, kidney, and heart transplantation; chemotherapy for malignant lymphoma; sarcoidosis; leucocytoclastic vasculitis; chronic myeloid leukemia; chronic lymphocytic leukemia; and lung cancer). The characteristics of the patients are reported in table 1, according to HIV serological status and prophylactic regimen. There were no significant differences in demographic or clinical characteristics between patients who harbored *P. jiroveci* with DHPS mutations and those who did not. The patients' medical charts stated that no patient has received sulfa drugs for any other purpose than PcP, and none received sulfadiazine as a treatment for toxoplasmosis before the diagnosis of PcP.

Table 1. Characteristics of 38 human immunodeficiency virus (HIV)–negative and 120 HIV-positive patients with *Pneumocystis jiroveci* pneumonia (PcP) from 5 hospitals in Lyon.

Characteristic	Sulfa prophylaxis[a]	Other or no prophylaxis[b]	Overall
HIV-negative (n = 38 [24%])	8	30	38 (100)
Age, mean ± SD (range), years	44 ± 14 (20–61)	50 ± 20 (4–73)	49 ± 19 (4–73)
Sex			
Male	7	17	24 (63.2)
Female	1	13	14 (36.8)
HIV-positive (n = 120 [76%])	12 (10)	108 (90)	120 (100)
Age, mean ± SD (range), years	39 ± 10 (28–56)	38 ± 10 (4–69)	38 ± 10 (4–69)
Sex			
Male	11	92	103 (85.8)
Female	1	16	17 (14.2)
HIV risk factor			
Homosexual	7	44	51 (42.5)
Heterosexual	2	35	37 (30.8)
Intravenous drug user	1	6	7 (5.9)
Other	2	23	25 (20.8)
CD4 cell count, median cells/μL (range)[c]	7.5 (0–58)	31 (0–390)	28 (0–390)
Cerebral toxoplasmosis	0	0	0

NOTE. Data are no. (%), except where noted.

[a] Patients who received pyrimethamine/sulfadoxine (n = 14), trimethoprim and sulfamethoxazole (n = 5), or dapsone (n = 1).

[b] Patients who received pentamidine (n = 23) or atovaquone (n = 2) prophylaxis or no prophylaxis at all (n = 113).

[c] CD4 cell counts at the time of diagnosis of PcP were documented in the medical chart for 113 of 120 HIV-positive patients; 3 patients had a CD4 cell count >200 cells/μL (231, 242, and 390).

Detection of DHPS alleles using PCR-SSCP. A portion of the *P. jiroveci* DHPS locus (318 bp) was amplified by a single-round PCR from 167 BAL specimens. Four different SSCP patterns made of 2 bands corresponding to the 2 strands of the PCR product were observed in our collection of specimens (figure 1). Three PCR products producing each of the 4 patterns were sequenced. Each pattern was found to correspond to a single DHPS allele. One allele had a threonine at position 55 and a proline at position 57 and was defined as WT, because it was the allele found in patients before the introduction of sulfa drug prophylaxis in 1989 [7–9], as well as in most *P. carinii* special forms infecting various mammals [24]. Two alleles harbored a single mutation, at nt 165 or 171, leading to an amino acid change within the putative sulfa-binding site at positions 55 (Thr→Ala, M1 for mutation 1) or 57 (Pro→Ser, M2). The fourth allele presented both substitutions (M3). These 4 different DHPS alleles are identical to those that have been described in other studies [7–9]. We did not observe other DHPS polymorphisms. The M1 allele was actually displayed only by 3 specimens collected in Lausanne's hospital. The stability of the DHPS locus over time was assessed by analyzing 2–3 consecutive BAL specimens obtained within intervals of 8–21 days during the same PcP episode in 8 patients. No variation in the DHPS genotype was observed in these patients.

Six different *P. jiroveci* DHPS genotypes were observed among the 158 patients with PcP (table 2). The majority (n = 101; 63.9%) of the patients were infected with *P. jiroveci* harboring the WT DHPS. The remaining patients were infected with either a mutant DHPS strain only (n = 38; 24.0%) or with a mixture of 2 DHPS genotypes (n = 19; 12.1%) including, most often, a mutant and a wt DHPS allele. To simplify the subsequent analyses, specimens that contained a mixture of WT and mutant DHPS genotypes were classified in the corresponding mutant category. The mixture of M2 and M3 DHPS genotypes (2 patients) was classified in the M3 DHPS genotype category. The proportion of the specimens with mutant DHPS varied from 25% to 57%, according to the hospital (figure 2). There were no significant differences among the proportions of the specimens with mutant DHPS observed in the 5 hospitals (P = .21, χ^2 test).

Predictors of infection with* P. jiroveci *harboring DHPS mutations. The patients who received sulfa prophylaxis (16 [80.0%] of 20) were more likely to harbor *P. jiroveci* mutant DHPS strains than those who did not (41 [29.7%] of 138; P = .00002, Fisher's exact test; figure 2). A review of the entire medical history of the patients revealed that 32 of 41 patients with DHPS mutations had never received sulfa drug prophylaxis or treatment at any given time. Nine patients had had a short exposure (maximum, 2 weeks) to sulfa drugs >3 months before the PcP episode but were switched to pentamidine be-

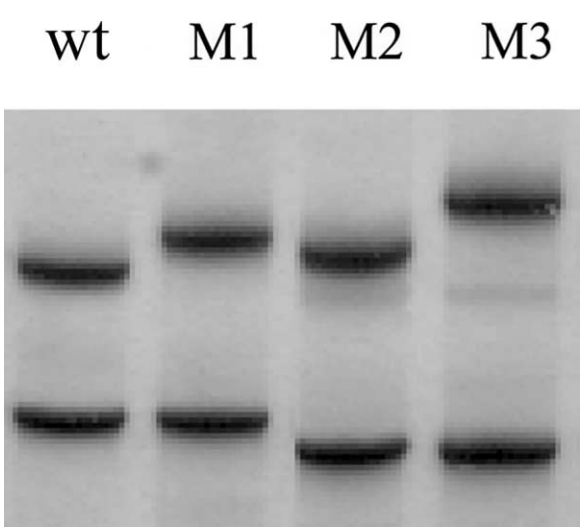

Figure 1. Single-strand conformation polymorphism (SSCP) detection of the 4 different *Pneumocystis jiroveci* dihydropteroate synthase (DHPS) alleles. Each band corresponds to 1 of the 2 single strands of the polymerase chain reaction (PCR) product. M1, mutation 1 (Thr55Ala); M2, mutation 2 (Pro57Ser); M3, mutation 3 (Thr55Ala, Pro57Ser); wt, wild type.

cause of adverse effects. There was no significant difference in the rate of DHPS mutations between patients with and those without pentamidine prophylaxis

We then investigated predictors of infection by M2 DHPS genotype, the most frequent mutation we found. Results of the univariate analysis are shown in table 3. HIV infection, hospitalization in hospital A, and anti-PcP prophylaxis (particularly with sulfa drugs) were predictors of infection caused by *P. jiroveci* harboring the M2 DHPS genotype. In multivariate analysis, sulfa prophylaxis and hospitalization in hospital A were independent risk factors for having *P. jiroveci* harboring this mutation (table 4). Because the 14 patients receiving PM/SD prophylaxis were all infected with the *P. jiroveci* M2 DHPS mutant, we could not precisely quantify the association between PM/SD and this mutation through multivariate analysis. Hospitalization in hospital A remained the only independent predictor of the M2 DHPS genotype when the analysis was restricted to patients not receiving PM/SD prophylaxis (odds ratio, 3.90 [95% confidence interval, 1.44–10.53]; $P = .007$).

DHPS mutations in patients without prophylaxis. Thirty-one (27.4%) of 113 patients who, according to their medical records, did not receive any prophylaxis (i.e., no sulfa, pentamidine, or atovaquone prophylaxis) had a mutant DHPS strain. Moreover, 9 (20.5%) of 44 patients who presented with PcP as the first symptom of HIV infection (and who had no prophylaxis) also harbored mutant DHPS strains.

Risk factors associated with death attributed to PcP. In univariate analysis, the rates of death attributed to PcP was similar in patients who had a *P. jiroveci* mutant DHPS and those who did not. Death attributed to PcP was only associated with HIV-negative status (12/38 [32%] vs. 18/120 [15%] in HIV-positive patients; $P = .032$) and older age (mean age, 46 years in nonsurvivors vs. 39 years in survivors; $P = .043$). However, these 2 predictors were no longer significant in multivariate analysis.

DISCUSSION

In the present study involving 158 patients diagnosed with PcP, the PCR-SSCP method was rapid and efficient for the identification of the *P. jiroveci* DHPS alleles that were associated with the failure of sulfa prophylaxis. We and others have previously described that the PCR-SSCP method is suitable to detect single–base-pair polymorphisms [22] and for genotyping [23, 25, 26]. The DHPS locus was found to be stable at least over 3 weeks, which is an essential feature for epidemiological studies.

DHPS mutations were found in 80.0% of the immunosuppressed patients with failure of sulfa prophylaxis, whereas only 29.7% of the patients without such prophylaxis harbored a mutant DHPS strain. This association is significant and consistent with the results of other investigators, who found DHPS mutations in 19.0%–84.6% of the patients receiving sulfa prophylaxis at PcP occurrence, compared with 4.0%–47.5% of patients not receiving this prophylaxis [8, 9, 12–17]. All of these observations strongly suggest that the mutations are selected by drug pressure.

The present study shows a significant association between a specific DHPS mutation (M2) and 1 prophylactic agent (PM/SD). This association further supports the selection of DHPS mutations by sulfa drugs. It also suggests that the type of DHPS mutation selected may depend on the type of prophylactic agent

Table 2. *Pneumocystis jiroveci* dihydropteroate synthase (DHPS) genotypes in 158 patients with *P. jiroveci* pneumonia from 5 hospitals in Lyon, as revealed by polymerase chain reaction–single-strand conformation polymorphism.

DHPS genotype	No. (%) of patients
wt	101 (63.9)
Mutant	
M2	29 (18.3)
M3	9 (5.7)
Mixed[a]	
wt + M2	5 (3.2)
wt + M3	12 (7.6)
M2 + M3	2 (1.3)

NOTE. M2, mutation 2 (Pro57Ser); M3, mutation 3 (Thr55Ala, Pro57Ser). wt, wild type.

[a] Patients harbored 2 DHPS alleles, which suggests coinfection with 2 *P. jiroveci* types.

Table 3. Univariate analysis of demographic and clinical risk factors for infection with *Pneumocystis jiroveci* dihydropteroate synthase genotype M2, in 158 patients with *P. jiroveci* pneumonia from 5 hospitals in Lyon.

Risk factor	Genotype M2		*P*
	Negative (n = 124)	Positive (n = 34)	
Male sex	99 (80)	28 (82)	.813
Hospitalization in hospital A	40 (32)	22 (65)	.001
Prophylaxis (all regimens[a])	28 (23)	17 (50)	.003
Sulfa prophylaxis (PM/SD, TMP/SMZ, dapsone)	5 (4)	15 (44)	<.001
PM/SD prophylaxis	0 (0)	14 (41)	<.001
HIV infection	101 (81)	19 (56)	.003
Age, mean (range), years	41 (4–73)	39 (4–61)	.876
CD4 cell count, mean cells/μL (range)	60 (0–390)	36 (4–242)	.233

NOTE. Data are no. (%) of patients, except where noted. HIV, human immunodeficiency virus; PM/SD, pyrimethamine-sulfadoxine; TMP/SMZ, trimethoprim-sulfamethoxazole.

[a] All regimens included PM/SD, TMP/SMZ, dapsone, pentamidine, and atovaquone prophylaxis.

used. In agreement with this observation, the M3 DHPS genotype was the allele most often observed in studies where TMP/SMZ or high-dose dapsone (100 mg daily) were first-choice regimens for prophylaxis (44%–98% of M3 allele among mutated alleles) [12–16]. In only 1 study was the frequency of the allele much lower (only 13% of M3 in patients receiving TMP/SMZ) [9]. On the other hand, the M1 DHPS genotype was the most frequent allele (57%) in Italian patients receiving low-dose dapsone prophylaxis (50 mg daily) [17]. These observations suggest that 2 DHPS mutations may be required for TMP/SMZ or high-dose dapsone prophylaxis failure, whereas 1 DHPS mutation may be sufficient to cause the failure of PM/SD or low-dose dapsone prophylaxis. This may be due to a lower affinity of SD for DHPS than SMZ or dapsone. However, it is also possible that other factors, such as geographic location, play a role in the prevalence of the different types of DHPS mutations. On the basis of the homology of the 3-dimensional structure of DHPS with *Escherichia coli* [27], the 2-aa changes observed in *P. jiroveci* DHPS are located in the active site of the enzyme. The replacement of Thr by Ala at position 55 and/or of Pro by Ser at position 57 may alter the position of the critical Arg56 involved in binding to sulfa drugs and, thereby, may decrease the affinity to these drugs.

All patients who developed PcP under PM/SD prophylaxis were infected with *P. jiroveci* harboring the M2 DHPS genotype. This finding could be partially explained by the fact that some patients in Lyon were receiving suboptimal prophylaxis at the time of PcP. Indeed, they were receiving 1 or 3 tablets of PM/SD every 2 weeks, whereas only a dose of 1 to 2 tablets weekly has been reported to be effective [18–20]. An alternative explanation could be that PM/SD prophylaxis may be ineffective in preventing PcP in patients who acquire de novo a *P. jiroveci* strain harboring the M2 mutation and that such strains were less prevalent in the other studies. In agreement with this hypothesis, the M2 DHPS genotype was rarely observed in studies that have reported DHPS genotyping data [7–8, 12–17]. The efficacy of PM/SD prophylaxis to prevent PcP in immunosuppressed patients requires further investigation.

Hospitalization in hospital A was another independent risk factor for having *P. jiroveci* harboring the M2 DHPS genotype. Thirty-four percent of the patients in this hospital were HIV-negative transplant recipients or patients with hematologic diseases who were hospitalized several weeks before developing PcP, which suggests that they may have acquired *P. jiroveci* within the hospital either from a common source or by direct or indirect contact with a patient with active PcP (authors' unpublished data). There was no evidence that patients had contacts or shared a potential common source outside the hospital. Several circumstances in hospital A may actually have contributed to interhuman transmission. There was no policy of isolation between potentially infectious and susceptible patients, the anti-PcP prophylaxis was suboptimal in most patients, and, when permitted by their general condition, patients were allowed to move freely about the units and to share a TV room.

The interhuman transmission of mutant DHPS strains, either within a hospital or in the community, is further supported by

Table 4. Multivariate analysis of demographic and clinical risk factors for infection with *Pneumocystis jiroveci* dihydropteroate synthase genotype M2 among the 158 patients with *P. jiroveci* pneumonia from 5 hospitals in Lyon.

Risk factor	Adjusted OR (95% CI)	*P*
Sulfa prophylaxis	26.04 (7.38–91.92)	<.001
Hospitalization in hospital A	5.53 (2.05–14.97)	.001

NOTE. CI, confidence interval; OR, odds ratio.

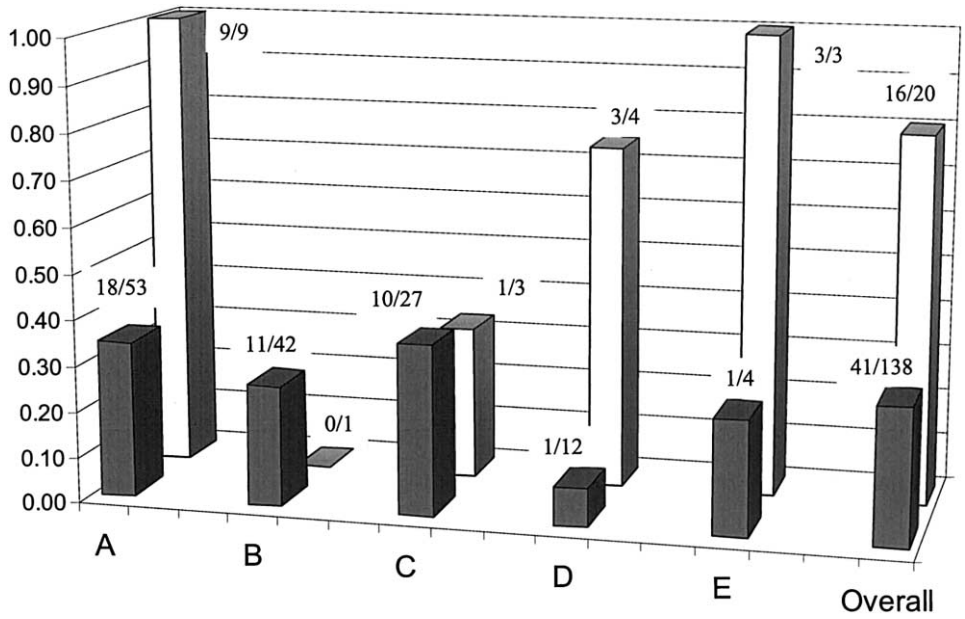

Figure 2. Proportions of mutant *Pneumocystis jiroveci* dihydropteroate synthase, according to hospital and sulfa prophylaxis, in the 158 patients with *P. jiroveci* pneumonia. *White bars,* patients whose medical chart documented sulfa prophylaxis. *Black bars,* patients whose charts did not document sulfa prophylaxis prescription. Overall, data from 5 hospitals were combined.

the presence of *P. jiroveci* mutant DHPS strains in patients whose medical charts did not document any anti-PcP prophylaxis or who presented with PcP as the first manifestation of their HIV infection. The presence of *P. jiroveci* DHPS mutations among patients newly diagnosed with HIV also has been described elsewhere [9, 14]. Although it is difficult to rule out that these patients have received sulfa drugs for a reason unrelated to HIV infection, such cases are more likely to represent direct or indirect cross-infections from patients exposed to prophylaxis. The interhuman transmission of *P. jiroveci* was also suggested by the observation of clusters of PcP cases [28–30] and the finding of *P. jiroveci* DNA in the noses of immunocompetent health care workers who were in contact with a patient who had PcP [31].

We did not observe any difference in the rate of death attributed to PcP between patients who were infected with *P. jiroveci* mutant DHPS strains and those infected with *P. jiroveci* harboring WT DHPS alleles. This is consistent with results obtained by others [8] but contrasts with those of Navin et al. [15], who reported a high rate of death attributable to PcP among patients infected with *P. jiroveci* harboring WT DHPS alleles. The reason for this discrepancy is presently unknown. Virulence factors not related to the DHPS locus might be involved. A specific *P. jiroveci* genotype has been associated with a more severe clinical presentation [32]. HIV-negative status and older age were associated with a higher rate of death attributed to PcP in univariate analysis. The fact that these 2 predictors did not reach statistical significance in multivariate analyses may be due to mutual confounding (the HIV-negative patients being older than HIV-positive patients).

In summary, the present results show an association between the failure of PM/SD prophylaxis and a specific DHPS mutation (M2), which further suggests that sulfa drug pressure is responsible for the selection of DHPS mutations and that *P. jiroveci* sulfa drug resistance is emerging. They also suggest that interhuman transmission is involved in the dissemination of the DHPS mutations and that being infected with *P. jiroveci* mutant DHPS strains (independently of the type of DHPS mutation) does not affect the outcome of disease.

Acknowledgments

We thank the physicians responsible for the involved wards in Lyon for access to patients' charts, particularly J. L. Touraine, D. Peyramond, and C. Trepo. We also thank S. Picot for storage of the specimens. We are indebted to Arlette Cruchon for DNA extraction. The present work was submitted by A. Nahimana as part of his fulfillment for a PhD degree at the University of Lausanne.

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