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High Prevalence of *Pneumocystis jirovecii* Dihydropteroate Synthase Gene Mutations in Patients with a First Episode of *Pneumocystis* Pneumonia in Santiago, Chile, and Clinical Response to Trimethoprim-Sulfamethoxazole Therapy

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ABSTRACT Mutations in the dihydropteroate synthase (DHPS) gene of *Pneumocystis jirovecii* are associated with the failure of sulfa prophylaxis. They can develop by selection in patients receiving sulfa drugs or be acquired via person-to-person transmission. DHPS mutations raise concern about the decreasing efficacy of sulfa drugs, the main available therapeutic tool for *Pneumocystis* pneumonia (PCP). The prevalence of *Pneumocystis* DHPS mutations was examined in *Pneumocystis* isolates from 56 sulfa-prophylaxis-naive adults with a first episode of PCP from 2002 to 2010 in Santiago, Chile. Their clinical history was reviewed to analyze the effect of these mutations on response to trimethoprim-sulfamethoxazole (TMP-SMX) therapy and outcome. Mutant genotypes occurred in 22 (48%) of 46 HIV-infected patients and in 5 (50%) of 10 HIV-uninfected patients. Compared to patients with a wild-type genotype, those with mutant genotypes were more likely to experience sulfa treatment-limiting adverse reactions and to have a twice-longer duration of mechanical ventilation if mechanically ventilated. Specific genotypes did not associate with death, which occurred in none of the HIV-infected patients and in 50% of the non-HIV-infected patients. Chile has a high prevalence of DHPS mutations, which were presumably acquired through interhuman transmission because patients were not on sulfa prophylaxis. These results contrast with the low prevalence observed in other Latin American countries with similar usage of sulfa drugs, suggesting that additional sources of resistant genotypes may be possible. The twice-longer duration of mechanical ventilation in patients with mutant DHPS genotypes suggests a decreased efficacy of TMP-SMX and warrants collaborative studies to assess the relevance of DHPS mutations and further research to increase therapeutic options for PCP.

KEYWORDS DHPS, *Pneumocystis jirovecii*, sulfa resistance, sulfa drugs, sulfamethoxazole trimethoprim, dihydropteroate synthase

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Pneumonia caused by the nonculturable opportunistic fungus *Pneumocystis jirovecii* (PCP) is a major cause of morbidity and mortality among human immunodeficiency virus (HIV)-infected patients and other immunosuppressed patients (1). *Pneumocystis*, like other fungi, is unable to scavenge folic acid from the host and needs this gene for the synthesis of folate. Prophylaxis and treatment of this infection relies mostly on the use of the trimethoprim-sulfamethoxazole (TMP-SMX) combination to inhibit folate synthesis. TMP-SMX is widely available and has effectively reduced the incidence of PCP. Sulfa drug usage, however, has been associated with mutations in the active site of the dihydropteroate synthase (DHPS) enzyme in the *fas* gene of *P. jirovecii*, which also codes for dihydroneopterin aldolase and hydroxymethildihydropterin pyrophosphokinase. The other enzyme involved folic acid synthesis is dihydrofolate reductase (DHFR), which is coded in a separate gene. The trimethoprim component of TMP-SMX selectively inhibits the DHFR enzyme activity, and the sulfamethoxazole component inhibits the activity of DHPS in *Pneumocystis* and organisms, such as *Plasmodium falciparum* and *Streptococcus pneumoniae*, where DHPS and DHFR mutations have been documented as the mechanism by which sulfa resistance occurs (2, 3). Furthermore, most studies have shown an association between the chronic use of sulfa drugs administered as prophylaxis and the presence of DHPS mutations, suggesting that drug selection pressure is the mechanism by which an increase in DHPS mutants in *P. jirovecii* occurs (4–6). It is not clear whether short-term courses of sulfa drug in outpatient settings are sufficient for the selection pressure of DHPS mutants in *P. jirovecii*. Environments such as hospitals or outpatient clinics are probably more relevant for the selection of mutant *Pneumocystis* since patients on chronic sulfa drug therapy may accumulate mutant genotypes and be a reservoir for the transmission of resistant *P. jirovecii* strains. This notion is supported by the high prevalence of *P. jirovecii* DHPS mutants in patients with chronic bronchitis that had not received TMP-SMX in the previous 6 months and by the high transmissibility of *Pneumocystis* (7, 8). Germane to this work, mutations in the DHPS gene associate with decreased sulfa drug efficacy *in vitro* and with the failure of anti-*Pneumocystis* prophylaxis (9, 10). Thus, the detection of DHPS mutations in *P. jirovecii* infers that sulfa resistance might be developing and therefore affecting the efficacy of the first-line agent used for prophylaxis and the treatment of PCP.

The significance of DHPS mutations in clinical response and outcome of PCP treated with sulfa drugs is still controversial; data on the correlation between DHPS mutations and mortality from PCP are scarce, and the findings of retrospective studies have conflicted (11–13). It can be hypothesized, however, that the speed of response to sulfa treatment might be compromised in patients infected by DHPS mutant isolates, delaying the clearance of *Pneumocystis* and affecting interim outcomes of therapy such as, for example, the time connected to mechanical ventilation, oxygen requirements, and others.

DHPS mutant genotypes are selected by sulfa drug selection pressure and have been used as a marker to infer interhuman transmission of mutant *Pneumocystis* genotypes from sulfa drug-treated patients to individuals not treated with sulfonamides (14–16). The prevalence of these genotypes varies depending on geographical location, suggesting different patterns in the use of sulfa drugs. Recognition of the incidence of DHPS mutations in a particular region and whether mutations affect the response to anti-*P. jirovecii* therapy and prophylaxis is therefore warranted. There is a need for more studies characterizing PCP, including the incidence of DHPS mutations of *P. jirovecii* in Chile (17–19).

In the present study, we sought to report the incidence of DHPS mutations among adult patients without a history of prior use of anti-*P. jirovecii* prophylaxis and presenting with a first episode PCP in Santiago, Chile, describing their clinical presentation, response to therapy, and outcome.

RESULTS

Patients and sample characteristics. A total of 56 respiratory specimens corresponding to 56 adult patients with available medical history and who had not received

TMP-SMX or dapsone as *Pneumocystis* prophylaxis and presented with a first episode of PCP were found in our respiratory specimens collection. All were immunosuppressed: 46 (82%) were HIV-infected, and 10 (18%) had other causes of immunosuppression, including human T-cell leukemia virus type 1-associated T-cell lymphoma ($n = 1$), meningioma ($n = 1$), rheumatoid arthritis ($n = 1$), dermatomyositis ($n = 1$), psoriatic arthritis ($n = 1$), systemic lupus erythematosus ($n = 1$), Churg-Strauss syndrome ($n = 1$), myasthenia gravis ($n = 1$), chronic obstructive pulmonary disease ($n = 1$), and chronic renal failure ($n = 1$). The median age for HIV-infected patients was 38.5 years (range, 22 to 71), and for non-HIV-infected patients it was 56.5 years (range, 18 to 82). Forty-five (98%) of the 46 HIV-infected patients and 4 (40%) of the 10 non-HIV-infected patients were male. PCP was the AIDS-defining condition in 36 (78%) of the HIV-infected patients. Only one of the eight patients with HIV infection diagnosed prior to their PCP episode was receiving antiretroviral therapy. Median $CD4^+$ T-cell count among HIV-infected patients was <40 cells/μl (Table 1).

Five specimens were obtained during the period from 2002 to 2004, 16 during the period from 2005 to 2007, and 35 during the period from 2008 to 2010. These specimens consisted of bronchoalveolar lavage fluid ($n = 26$), tracheal aspirate ($n = 16$), spontaneously expectorated sputum ($n = 13$), and nasopharyngeal aspirate ($n = 1$).

Parameters to characterize the degree of severity of PCP were not standardized, and individual patients' receipt of supplemental oxygen and their arterial oxygen tension or saturation measurements (obtained while breathing room air) were not recorded systematically. However, all patients were admitted to the hospital, and the majority 54 (96%) required supplemental oxygen. No significant differences were detected in clinical parameters at admission (fever, cough, dyspnea, chest radiography, computed tomography, platelet count, serum albumin, lactate dehydrogenase, and C-reactive protein levels) among HIV-infected and uninfected patients.

Previous use of TMP-SMX. Hospital medical records were reviewed for use of trimethoprim-sulfamethoxazole (TMP-SMX). This sulfa drug combination is available in the Chilean therapeutic armamentarium, and occasionally prescribed for respiratory and urologic conditions. Except for two patients with asthma whose *P. jirovecii* isolates were DHPS genotype 1 (wild-type), only one other patient had chronic lung disease. None of the patients had urologic conditions referred in the medical history at hospital admission. No use of TMP-SMX as inpatients was documented. Outpatient and primary care medical records were not accessible.

Prevalence of DHPS mutations. Mutations in the DHPS gene were identified in 27 (48%) of 56 patients regardless of their underlying diagnosis (Table 2). Mutations occurred in one (20%) of the five isolates collected between 2002 and 2004, five (31%) of the 16 isolates collected between 2005 and 2007, and 21 (60%) of the 35 isolates collected between 2008 and 2010 ($P = 0.06$). Polymorphisms consisting of DHPS genotypes 2, 3, or 4 were present in 22 (48%) of the 46 HIV-infected patients and in 5 (50%) of the 10 non-HIV-infected patients with PCP. Coinfections with the wild-type genotype were more frequent in the HIV-infected group and were absent in non-HIV-infected patients with PCP (Table 2). No predominant pattern of mutation polymorphism was detected. We analyzed coinfections by grouping coinfections from 2002 to 2008, the first 6 years ($n = 13$), and those during the last 3 years from 2009 to 2012 ($n = 9$) of the study. Eight (61%) of thirteen coinfections during the first time period were compared to seven (77%) of nine coinfections in the second period ($P = 0.42$).

Clinical parameters and outcome. Anti-PCP treatment was initiated with TMP-SMX in 53 (95%) of the 56 patients. TMP-SMX combined with caspofungin or dapsone was used in two patients, and pyrimethamine-sulfadoxine was used in one patient. Among the HIV-infected patients, discontinuation of TMP-SMX, related to sulfa drug-related adverse events and not to treatment failure, was needed in 4 (18%) of 22 patients with mutant genotypes and in none of 24 patients with wild-type isolates ($P = 0.045$). Among HIV-infected patients requiring mechanical ventilation, the duration of mechanical ventilation among those harboring DHPS mutations was significantly longer, 11

TABLE 1 Demographic and clinical characteristics of 56 patients with a first episode of PCP[a]

Parameter	HIV-infected patients (n = 46)			Non-HIV-infected patients (n = 10)		
	WT DHPS genotype	Mutant DHPS genotype	P	WT DHPS genotype	Mutant DHPS genotype	P
Total patients	24/46 (52)	22/46 (48)		5/10 (50)	5/10 (50)	
Mean age in yrs (SD)	40 (12.2)	40.1 (9.3)	0.977	48.5 (24.7)	55.6 (23.3)	0.671
No. male	24/24 (100)	21/22 (95)	0.468	2/5 (40)	2/5 (40)	1
New HIV diagnosis	20/24 (83)	18/22 (82)	0.699	NA	NA	
Receipt of PCP prophylaxis	0/24 (0)	0/22 (0)	1	0/5	0/5	NA
Not receiving antiretroviral therapy	24/24 (100)	21/22 (96)	0.478	NA	NA	
Fever	15/24 (63)	12/22 (55)	0.765	3/5 (60)	2/3 (67)	1
Mean temp in °C (SD)	38.1 (0.85)	38.2 (0.98)	0.786			
Cough	18/24 (75)	19/22 (86)	0.464	3/5 (60)	3/4 (75)	1
Dyspnea	21/24 (88)	19/21 (91)	1	4/5 (80)	3/4 (75)	1
Chest radiograph	22/24	21/22		5/5	5/5	
Abnormal	21/22 (96)	21/21 (100)	1	5/5 (100)	5/5 (100)	
Bilateral interstitial infiltrates	18/22 (82)	16/21 (76)		1/5 (20)	1/5 (20)	NA
Another pattern	3/22 (14)[b]	5/21 (24)[c]		2/5 (40)[d]	4/5 (80)[e]	
Not determined				2/5 (40)		
Thoracic CT scan	15/24	15/22		3/5	3/5	
Abnormal	15/15 (100)	15/15 (100)	1	3/3 (100)	3/3 (100)	NA
Ground-glass infiltrates	12/15 (80)	14/15 (93)		1/3 (33)	1/3 (33)	
Another pattern	3/15 (20)[f]	1/15 (7)[g]		2/3 (67)[h]	2/3 (67)[i]	
Median CD4 cell count in cell/μl (IQR)	27 (11–57.5)	37 (18–64)	0.465	ND	ND	
No. of affected patients/total no.	20/24	19/22				
Median serum albumin in g/liter (IQR)	2.95 (2.37–3.40)	2.90 (2.40–3.30)	0.794	3.15 (2.35–3.43)	ND	
No. of affected patients/total no.	18/24	13/22		4/5		
Median serum LDH in IU/liter (IQR)	854.5 (568.8–1290)	863 (665–1089)	0.927	875 (558,8–3210)	1,099 (840–1405)	0.730
No. of affected patients/total no.	22/24	19/22		4/5	5/5	
Mean hematocrit in % (SD)	39.9 (6.39)	36.8 (4.72)	0.076	30.1 (8.84)	31.5 (9.54)	0.782
No. of affected patients/total no.	23/24	21/22		5/5	4/5	
Median C-reactive protein in mg/liter (IQR)	19.6 (4.8–99.9)	8.5 (1.1–86.2)	0.475	73 (3.40–217.0)	5,0 (1.90–50.25)	0.191
No. of affected patients/total no.	18/24	19/22		5/5	4/5	
Other lung pathology	2/24 (8)[j]	1/22 (5)[k]	1	3/5 (60)[l]	1/5 (20)[m]	0.524

[a]Values are expressed as the number of patients/total number of patients (%), except as stated otherwise in column 1. Abbreviations: DHPS, dyhydropteroate synthase; LDH, lactate dehydrogenase; IQR, interquartile range; n, total number of patients; NA, not applicable; ND, not determined.
[b]Bilateral interstitial infiltrates plus bilateral lobar consolidation (n = 1); bilateral interstitial infiltrates plus bilateral alveolar consolidation (n = 1); diffuse basal infiltrates (n = 1).
[c]Bilateral interstitial infiltrates plus air bronchogram and consolidation (n = 1); diffuse alveolar-interstitial pattern (n = 2); diffuse alveolar-interstitial pattern with apical cavitation (n = 1); right lobar consolidation (n = 1).
[d]Alveolar hemorrhage (n = 1); left pulmonary nodules (n = 1).
[e]Bilateral peribronchovascular thickening (n = 1); right basal consolidation (n = 1); bilateral alveolar consolidation plus air bronchogram (n = 1); right middle lobe consolidation (n = 1).
[f]Bilateral ground-glass infiltrates and bilateral consolidation (n = 1); noncharacteristic interstitial infiltrates (n = 1); diffuse infiltrates (n = 1).
[g]Bilateral ground-glass infiltrates and four cavitation-suggestive nodules (n = 1).
[h]Alveolar hemorrhage (n = 1); pneumomediastinum (n = 1).
[i]Bilateral ground-glass pattern and unilateral consolidation (n = 1); unilateral consolidation (n = 1).
[j]Bronchial asthma (n = 2).
[k]*Mycobacterium tuberculosis*.
[l]Dermatomyositis (n = 1); COPD plus cystic fibrosis (n = 1); bronchial asthma (n = 1).
[m]Myasthenia gravis.

days (interquartile range [IQR], 8 to 56), than in those with wild-type isolates, 6 days (IQR, 2 to 8; P = 0.017). There was a trend toward longer hospitalization in HIV-infected patients with mutations, i.e., 20 days (IQR, 10 to 42) compared to 11 days (IQR, 6 to 19) in HIV-infected patients with wild-type DHPS isolates (P = 0.073) (Table 3). All 46 (100%) HIV-infected patients survived, whereas only 5 (50%) of the 10 non-HIV-infected patients survived. Death was directly attributable to PCP in three of these patients.

DISCUSSION

Nearly half of the patients in this study, regardless of their HIV status, had *Pneumocystis* DHPS mutant genotypes despite having no prior receipt of sulfa drugs (sulfamethoxazole or dapsone) as prophylaxis for PCP, suggesting that human-to-human transmission was the most likely source of the acquisition of mutant isolates. Our results also showed that mechanically ventilated patients harboring *Pneumocystis* DHPS mutant

TABLE 2 *P. jirovecii* dihydropteroate synthase genotypes in respiratory specimens from 56 adult patients with a first episode of PCP

DHPS genotype[a]	No. positive/total no. (%)	
	HIV infected (n = 46)	Non-HIV infected (n = 10)
Wild-type		
G1	24/46 (52)	5/10 (50)
Mutant	22/46 (48)	5/10 (50)
Single mutant	2/22 (9)	2/5 (40)
G2 (position 165)	1/22 (5)	2/5 (40)
G3 (position 171)	1/22 (5)	0
Double mutant		
G4 (positions 165 + 171)	5/22 (23)	3/5 (60)
Coinfection with wild type	15/22 (68)	0
G5 (1 + 2)	9/22 (41)	0
G6 (1 + 3)	0	0
G7 (1 + 4)	6/22 (27)	0
Mixed single + double mutant		
G8 (2 + 4)	0	0

[a]Genotypes G1 to G8 were described previously (37).

genotypes had twice-longer durations of mechanical ventilation, suggesting that these mutations might have impacted the response to anti-*Pneumocystis* treatment with sulfamethoxazole-containing sulfa combinations. In addition, adverse drug reactions to sulfa treatment of PCP, necessitating treatment change, were observed more frequently among patients with mutant DHPS genotypes.

A high prevalence of DHPS mutations has also been reported in studies from other countries. For example, a recent study of AIDS-related PCP in Kampala, Uganda, documented that all 13 isolates of *P. jirovecii* harbored either single- or double-mutant DHPS genotypes, despite the fact that only two subjects had received TMP-SMX for PCP prophylaxis (20). This finding was ascribed to population-level selection pressure due to sulfa drug use for treatment of malaria caused by *Plasmodium falciparum* among the general population. *Pneumocystis* is not zoonotic; therefore, the absence of sulfa prophylaxis for PCP in our patients suggests that they likely acquired DHPS mutations through interhuman transmission (14, 16). The mechanism and role for selection pressure at a population level is, however, not clear because sulfa drugs are the third or fourth choice of antibiotic for the treatment of respiratory and urinary tract infections in both primary- and secondary-care settings in Chile. They account for approximately 5% of antibiotic prescriptions after synthetic penicillins, macrolides, cephalosporins, and quinolones (21). Furthermore, the use of the TMP-SMX combination has decreased since 2000 from approximately 7% to currently 2% of total antibiotic consumption in Chile (21). This consumption is similar to that reported in other Latin American countries (22), where the low usage of sulfa drugs parallels a low prevalence of DHPS mutations. Of note, an earlier United Kingdom study showed a 36% frequency of DHPS mutations in isolates of *P. jirovecii* in London, when there was population-level "selection pressure" from widespread use of sulfa drugs both as prophylaxis against PCP, and among the general population for the treatment of respiratory and urinary tract infections (23). Contemporaneously, a low prevalence (7.7%) of DHPS mutants was identified in Zimbabwe, where sulfa drugs are rarely used. When in the United Kingdom the selection pressure was removed, a predominance (80%) of wild-type genotypes was observed (23). Restriction measures for the use of antibiotics have been in place in Chile since 1998; however, there is no indication that the frequency of detection of DHPS mutants has decreased. In contrast, the possible increase in the proportion of DHPS mutations over time suggested in this study becomes paradoxical when the parallel decrease in use of sulfa drugs by national policies is considered. Therefore, the high frequency described in the present study seems too high to be explained solely by sulfa selection pressure within the human population.

TABLE 3 Dihydropteroate synthase genotypes and clinical outcomes among 56 patients with a first episode of PCP[a]

Treatment and/or outcome	HIV-infected patients ($n = 46$)			Non-HIV-infected patients ($n = 10$)		
	Wild-type DHPS genotype ($n = 24$)	Mutant DHPS genotype ($n = 22$)	*P*	Wild-type DHPS genotype ($n = 5$)	Mutant DHPS genotype ($n = 5$)	*P*
Initial treatment with trimethoprim-sulfamethoxazole	23/24 (96)[b]	20/22 (91)[c]	0.600	5/5 (100)	5/5 (100)	ND
Adverse effect	2/24 (8)[d]	4/22 (18)[e]	0.452	0/5 (0)	0/5 (0)	ND
Need for treatment change	0/24 (0)	4/22 (18)[f]	0.045	1/5 (20)[g]	0/5 (0)	ND
Sulfa allergies	1/24 (4)	4/22 (18)	0.178	0/5 (0)	0/5 (0)	ND
Adjunctive corticosteroids	21/24 (888)	15/22 (68)	0.159	4/5 (80)	4/5 (80)	ND
Duration of hospitalization (days)						
Median (IQR)	11 (6–19)	20 (10–42)	0.073	30 (14–40)	25 (8–37)	ND
No. with available data/total no.	19/24	16/22		5/5	5/5	
Required supplemental oxygen	24/24 (100)	20/22 (91)		5/5 (100)	5/5 (100)	
Median days (IQR)	11.5 (6.7–19.5)	14.5 (7.0–20)	0.203	34 (14–40)	17 (10–24)	ND
No. with available data/total no.	22/24	20/22		5/5	5/5	
Need for early ICU admission	8/22 (36)	7/20 (35)	0.763	1/5 (20)	3/4 (75)	ND
No. with available data/total no.	22/24	20/22		5/5	4/5	
Mechanical ventilation	9/24 (38)	7/22 (32)		4/5 (80)	5/5 (100)	
Median days (IQR)	6 (2.25–7.75)	11 (8–56)	0.017	25 (10–30)	14 (8–35)	ND
Pulmonary coinfection	7/24 (29)	5/22 (23)		3/5 (60)	2/5 (40)	
Bacterial	1[h]	3[i]		3[j]	2[k]	
Viral	4[l]	0		0	0	
Bacterial + viral	2[m]	2[n]		0	0	
Treatment outcome						
Survived	24/24 (100)	22/22 (100)		2/5 (40)	3/5 (60)	
Died	0 (0)	0 (0)		3/5 (60)	2/5 (40)	
Death due to PCP	0 (0)	0 (0)		2/3 (67)	1/2 (50)	

[a]Values are expressed as the number of patients/total number of patients (%), except as states otherwise in column 1.
[b]Pyrimethamine + sulfadoxine ($n = 1$).
[c]Caspofungin + trimethoprim-sulfamethoxazole ($n = 1$); dapsone + trimethoprim-sulfamethoxazole ($n = 1$).
[d]Rash ($n = 2$).
[e]Rash ($n = 2$); acute kidney injury ($n = 1$); rash, interstitial nephritis, hepatitis ($n = 1$).
[f]Change trimethoprim-sulfamethoxazole to: dapsone ($n = 2$); dapsone + clindamycin ($n = 2$).
[g]Treatment failure change to intravenous pentamidine.
[h]*Acinetobacter baumannii* ($n = 1$).
[i]*Streptococcus pneumoniae* ($n = 1$); *Klebsiella pneumoniae* ($n = 1$); *Stenotrophomonas maltophilia* ($n = 1$).
[j]*Pseudomonas aeruginosa* ($n = 1$); *Klebsiella pneumoniae* ($n = 1$); *Proteus mirallis* ($n = 1$).
[k]*Enterobacter cloacae* ($n = 2$).
[l]Cytomegalovirus (CMV).
[m]*Acinetobacter baumannii* + CMV ($n = 1$); *Enterobacter cloacae* + CMV ($n = 1$).
[n]β-Hemolytic streptococcus + CMV ($n = 1$); atypical mycobacteria + CMV ($n = 1$).

The restriction of antibiotic use in humans in Chile has not been accompanied by similar policies in veterinary settings, and currently there is a far greater use of antibiotics, including sulfa drugs, in the setting of pig, poultry, and fish farming than in humans in Chile (24). The veterinary use of sulfa drugs may select DHPS mutants on a much larger scale than that seen in human use, and the acquisition of bacterial genes generally responsible for metabolic or virulence traits via horizontal gene transfer has been documented in fungal species (25, 26). However, this type of resistance acquisition has never been described with *Pneumocystis*. Mutations in the DHPS gene of *P. jirovecii* have been shown to arise independently among multiple *Pneumocystis* strains (27). Therefore, the transmission of a single resistant clone of *P. jirovecii* appears to be very unlikely.

The finding that adverse drug reactions to sulfa treatment were more frequent among patients with mutant DHPS genotypes, who in turn required more frequent changes in anti-PCP treatment, does not have an immediately apparent explanation. Short courses of sulfa drugs, for example, as treatment for bronchitis or sinusitis, could not be identified from hospital records, and therefore the possibility of prior exposure in a primary care setting resulting in sensitization cannot be excluded. However, the two patients with asthma, as well as one additional patient with chronic obstructive lung disease, for whom the possibility of having received undocumented sulfa drug antibiotics in a primary-care setting was more likely, had wild-type DHPS genotype 1.

No difference in the proportion of patients with wild-type and mutant genotypes requiring mechanical ventilation was detected in the present study. This observation contrasts with a study by Crothers et al. (28), where 14.3% of patients with PCP and mutant *P. jirovecii* genotypes required mechanical ventilation compared to 2.5% of those with wild-type *P. jirovecii* genotypes ($P = 0.056$). However, mutant DHPS genotypes were associated with a longer duration of mechanical ventilation in the present series, and these data suggest that further research is needed (by way of a multicenter prospective study), since data from the present study infer a reduced ability of sulfa drug-based regimens to clear DHPS-mutant *P. jirovecii* from the lungs. Interestingly, the study by Crothers et al. also showed patients with mutant DHPS genotypes had a nonsignificant trend to longer overall hospital stay, but data on the duration of mechanical ventilation was not provided (28). The inability to culture *Pneumocystis in vitro* hinders antimicrobial sensitivity testing and makes proof of a mechanistic connection difficult. Moukhlis et al. (29) performed functional studies using a DHPS-deficient model of *Saccharomyces cerevisiae* experimentally complemented with *Pneumocystis* mutant and wild-type DHPS and documented a decreased susceptibility to sulfamethoxazole in *S. cerevisiae* isolates that were complemented with double-mutant genotypes. Their results, and related earlier work provide an *in vitro* correlation with our findings (9, 10, 29).

In the present study, we observed no association between mutant DHPS genotype and mortality. However, this was an observational study and so was not powered to show differences. Of interest, a prospective single cohort study from San Francisco General Hospital consisting of 301 patients with laboratory-confirmed PCP over a period of >10 years demonstrated that although the receipt of recent sulfa prophylaxis was associated with mutant genotypes of *P. jirovecii*, the detection of mutant DHPS genotypes was not associated with mortality. This observation conflicts with findings from other authors (13, 30).

The strengths of the present study are that it is the first from Chile to describe DHPS genotyping among *P. jirovecii* isolates from patients with PCP. Our results document a high frequency of DHPS mutants (48%) in anti-*P. jirovecii* sulfa-prophylaxis-naive patients with a first episode of PCP. This frequency is excessively high compared to the low prevalence of DHPS mutations in countries in the same continent, e.g., Brazil (0%) and Colombia (6.6%), that report similar patterns of sulfa drug usage in humans (22, 31, 32). Therefore, although our findings add further evidence to support the hypothesis of interhuman transmission as a mechanism of acquisition of mutant *P. jirovecii* types, additional mechanisms of acquisition may be possible. Significantly, our results also suggest that DHPS mutation can diminish the efficacy of sulfa drug treatment of PCP by documenting a significantly longer duration of mechanical ventilation in patients harboring mutant DHPS genotypes and highlight the need to increase the anti-*Pneumocystis* armamentarium. TMP-SMX is the only anti-*Pneumocystis* drug available in most of the world. Weaknesses of the present study include the lack of prospective, systematic acquisition of clinical data and the relatively small sample size.

In conclusion, we describe a high frequency of DHPS mutations among adult patients with first-episode of PCP who had not received sulfa drugs as PCP prophylaxis in Santiago, Chile. The likely explanation for this is interhuman transmission and selection pressure from sulfa drugs prescribed for other conditions. In addition, the potential role of the veterinary use of sulfa drugs in the selection of DHPS mutations for transmission to humans deserves further study. Patients with PCP and mutant DHPS genotypes were more likely to experience treatment-limiting adverse reactions to sulfa drug treatment and to require a longer duration of mechanical ventilation, thus suggesting a decrease in treatment efficacy. The final treatment outcome was not affected, since patients harboring mutant DHPS genotypes were not more likely to die. The prevalence of DHPS mutations in clinical isolates of *Pneumocystis* should be monitored, and their significance in delaying response to therapy needs to be confirmed in larger collaborative studies.

MATERIALS AND METHODS

Ethics review. The Ethics Committee for Studies in Humans of the University of Chile School of Medicine approved the study under protocol 00267. Clinical data were reported coded to the investigators and analyzed unlinked to the identity of the subjects. Informed consent for analyses of *Pneumocystis* isolates was not required.

Patients. Respiratory specimens from adult patients presenting to two hospital clinics in Santiago, Chile, between January 2002 and January 2010 who had not received TMP-SMX or dapsone (a sulfone) for anti-*Pneumocystis* prophylaxis and who had a first episode PCP were studied. Their clinical data were collected by means of retrospective hospital chart review, including patient demographics, past medical history, underlying cause of immunodeficiency (HIV infection, or other cause), receipt of anti-*Pneumocystis* prophylaxis with any agent, other drug treatments, clinical presentation, results of laboratory tests, and imaging (chest radiography and computed tomography), clinical course (including admission to the intensive care unit and the need for mechanical ventilation), receipt of adjunctive corticosteroids, response to anti-*Pneumocystis* treatment, the presence of copathogens, and outcome were recorded. Death attributable to PCP was defined as death caused by progressive respiratory failure.

Sample specimens. Fresh-frozen respiratory specimens were sent to the University of Chile School of Medicine for diagnosis of PCP. The samples were processed at arrival, and extracted DNA was kept at −20°C until DHPS genotype analyses. All analyses were performed "blind" to the patients' clinical details.

Diagnosis of PCP. *P. jirovecii* organisms were identified using either Grocott-Gomori methenamine silver staining or direct immunofluorescence (Merifluor pneumocystis; Meridian Biosciences, Inc.). In addition to staining, PCR with the standard primers pAZ102-E and pAZ102-H (33) designed to amplify the gene encoding the mitochondrial large subunit rRNA of *Pneumocystis*, and which amplifies all *Pneumocystis* species, was performed on all specimens prior to genotyping.

Processing of specimens. Samples were processed inside a biosafety cabinet using sterile precautions to avoid contamination at all times. They were homogenized with a sterile pipette, and a 200-μl aliquot was used for DNA extraction. DNA was extracted using a QIAamp DNA minikit (Qiagen, Valencia, CA) as described previously (34), and Platinum *Pfx* DNA polymerase (Invitrogen) was used for DNA amplification. Negative controls were included to monitor for cross-contamination during DNA extraction and purification steps. An internal control using the human β-globin gene was used in each sample to detect the inhibition of the PCR, i.e., false-negative results. Each sample was run undiluted and as a 1/5 dilution to assess for substrate inhibition. Amplification products were visualized by using ethidium bromide in 2% agarose gels.

Detection of mutations in the DHPS gene. The DHPS gene binding site was amplified using single-touchdown PCR with the primers DHPS-3 (5′-GCGCCTACACATATTATGGCCATTTTAAATC-3′) and DHPS-4 (5′-GGAACTTTCAACTTGGCAACCAC-3′), yielding an amplification product of 370 bp as previously described (11, 16, 35). Point mutations at positions 165 (G for T) and 171 (T for C) of the DHPS gene were detected using restriction fragment length polymorphism analysis with the restriction enzymes AccI (500 U, 10 U/μl; Promega) and HaeIII (2,500 U, 10 U/μl; Promega). Four DHPS allelic patterns were identified: wild type (genotype 1), single-mutant genotype 2 (point mutation at position 165), single-mutant genotype 3 (point mutation at position 171), and double-mutant genotype 4 (point mutations at positions 165 and 171), as previously described (36, 37). Mutations were classified according to the pattern of band polymorphisms visualized on 2% agarose gels stained with ethidium bromide.

Statistical analysis. GraphPad Prism5 software (San Diego, CA) was used for all statistical comparisons. Patient characteristics and clinical outcome were compared between mutant and wild-type groups in HIV-positive patients. Statistical comparisons were not performed in HIV-negative patients because there were too few of these patients. Qualitative characteristics were described using absolute frequency, and percentages and intergroup comparisons among mutant and wild-type groups were determined using the Fisher exact test. Abnormally distributed quantitative variables were described using medians and interquartile ranges (IQRs), and comparisons were made using the Mann-Whitney test. Normally distributed quantitative data were described using means and standard deviations (SD), and comparisons were made using an unpaired *t* test. A P value of <0.05 was considered significant. The proportions of DHPS mutants overtime were compared using χ^2 analysis. All comparisons were two tailed, and the confidence level was set at 95%.

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