

Pneumocystis jiroveci Genotypes in the Spanish Population

M. A. Montes-Cano,¹ C. de la Horra,¹ J. Martín-Juan,² J. M. Varela,¹ R. Torronteras,³ N. Respaldiza,¹ F. J. Medrano,¹ and E. J. Calderón¹

Departments of ¹Internal Medicine, ²Pneumology, and ³Microbiology Services, Virgen del Rocío University Hospital, Seville, Spain

This study describes the genotype distribution of *Pneumocystis jiroveci* in 79 respiratory samples obtained from 15 patients with acquired immunodeficiency syndrome (AIDS) with *P. jiroveci* pneumonia and 64 human immunodeficiency virus–negative subjects with different chronic pulmonary diseases. The genotyping was based in analysis of 2 independent genetic loci: the mitochondrial large subunit ribosomal RNA (mt LSU rRNA) fragment (assessed by direct sequencing) and the gene for dihydropteroate synthase (*DHPS*; assessed by restriction fragment–length polymorphism). The mt LSU rRNA analysis revealed the presence of 3 different polymorphisms for both populations. The major genotype, 85C/248C, was found to be significantly higher in patients with AIDS and *P. jiroveci* pneumonia than in patients with pulmonary disease. The rate of genotypes 85A/248C and 85T/248C was similar in both groups. The analysis of *DHPS* genotypes assesses the prevalence of its 4 possible genotypes, with 35.5% of genotypes related to sulfa resistance. The data suggest a common source of infection between both groups.

Pneumocystis jiroveci (previously known as *Pneumocystis carinii forma specialis hominis*) remains the most common opportunistic pathogen among HIV-infected persons [1, 2]. The incidence of *P. jiroveci* pneumonia (PcP) has decreased among patients with AIDS in developed countries with the use of specific chemoprophylaxis and, above all, with administration of HAART. Despite this, PcP is still an important cause of morbidity and mortality worldwide [3]. Today, the interest in *P. jiroveci* infection is not only confined to patients with AIDS; it also represents a common and serious opportunistic infection in other immunocompromised groups, such as organ transplant recipients [4], patients with autoimmune diseases who receive immunosuppressive therapy [5], and patients with neoplasias [6]. Moreover, recent studies show asymptomatic colonization with *P. jiroveci* in patients with chronic pul-

monary diseases [7]. However, it has not yet been established whether these people, who are sputum producers, could represent a source of infection for susceptible immunocompromised individuals.

In the absence of a reliable method to culture *P. jiroveci*, the basic biology and epidemiology of this fungus remain poorly understood. Recently, important genes have been identified for use in the analysis and characterization of *P. jiroveci*. The study of 2 different genetics loci—mitochondrial large subunit ribosomal RNA (mt LSU rRNA), which is involved in basic metabolic functions, and the gene for dihydropteroate synthase (*DHPS*), a target of sulfone and sulfonamide antimicrobial drugs—may provide information for understanding the epidemiology of *P. jiroveci*. Moreover, variations in the *DHPS* gene suggest that the widespread use of trimethoprim-sulfamethoxazole and dapsone may be exerting selective pressure on *P. jiroveci* genotypes circulating in humans [8, 9]. In this way, it is potentially useful as marker for changes in susceptibility levels, as well as valuable for characterization and typing of *P. jiroveci*.

Although different genotype studies in patients with AIDS and PcP have been reported in the United States [10] and north and central Europe [11, 12], there is

Received 10 December 2003; accepted 19 February 2004; electronically published 14 June 2004.

Reprints or correspondence: Dr. Enrique J. Calderón, Dept. of Internal Medicine, Virgen del Rocío University Hospital, 41013 Seville, Spain (ecs01se@nacom.es).

Clinical Infectious Diseases 2004;39:123–8

© 2004 by the Infectious Diseases Society of America. All rights reserved.
1058-4838/2004/3901-0020\$15.00

little information about the distribution of *P. jiroveci* genotypes in the south of Europe. The pattern of genetic variation in *P. jiroveci* isolated from nonimmunocompromised patients colonized by this pathogen is still poorly unknown and has only been studied in children [11].

The aim of the present study was to assess the genetic epidemiology of *P. jiroveci* by analysis of 2 independent loci in patients with AIDS and PcP, and in subjects with different chronic pulmonary diseases.

MATERIALS AND METHODS

Subjects. Included in this prospective study were the first 236 consecutive patients with any chronic pulmonary disease and 19 patients with AIDS who were suspected to have PcP between January 2001 and September 2003 in the Internal Medicine Department of the Virgen del Rocio University Hospital (Seville, Spain). A total of 133 patients had been admitted to the fibrobronchoscopy unit, and bronchoalveolar lavage (BAL) specimens were available for analysis. In the remaining 122 patients, only samples of sputum had been obtained. Every patient underwent a clinical and biological examination, which was performed with a standardized questionnaire, and BAL or sputum samples were obtained for analysis. When both samples were available, only BAL samples were analyzed. Additional patient data are shown in table 1.

During the study period, *P. jiroveci* was detected in 64 (27.1%) of 236 subjects with chronic pulmonary diseases (none of them with PcP) and 15 (78.9%) of 19 HIV-infected patients who were suspected of having PcP. Informed consent was obtained from all patients. The study had the approval of our hospital's ethics committee.

Detection of *P. jiroveci*. BAL and/or sputum specimens were obtained from the patients. After digestion with proteinase K at 56°C, DNA from *P. jiroveci* was extracted with a commercial kit from Qiagen. Two independent loci of the *P. jiroveci* genome were amplified: mt LSU rRNA and the *DHPS* gene. A 2-step protocol was used for amplification of mt LSU rRNA

fragment by nested PCR, as described elsewhere [13]. In brief, in the first amplification round, the external primers pAZ102-E (5'-GAT GGC TGT TTC CAA GCC CA-3') and pAZ102-H (5'-GTG TAC GTT GCA AAG TAC TC-3') were used. This yielded a 346-bp fragment. The second round of amplification used the primers pAZ102-X (5'-GTG AAA TAC AAA TCG GAC TAG G-3') and pAZ102-Y (5'-TCA CTT AAT ATT AAT TGG GGA GC-3') and yielded a 260-bp product. Both rounds of amplification underwent 40 cycles.

The single-copy gene of *DHPS* was amplified by the primers DHPS-3 (5'-GCG CCT ACA CAT ATT ATG GCC ATT TTA AAT C-3') and DHPS-4 (5'-GGA ACT TTC AAC TTG GCA ACC AC-3') [14] in all positive samples by nested PCR. The PCR mixture was prepared to a final volume of 25 μ L, which contained 3 μ L of DNA template, PCR buffer solution, 2.5 U Taq polymerase (Bioline), 0.2 mmol/L nucleotide triphosphate mixture, 20 pmol of each primer, and 2 mmol/L MgCl₂. A Touchdown-PCR protocol was used to amplify the samples, yielding a 370-bp fragment. After a hotstart step at 95°C for 10 min, a Touchdown procedure was assayed with a denaturation step at 94°C for 15 s. The primer annealing step followed: the temperature was decreased by 1°C per cycle from 72°C to 62°C for 30 s in the first 10 cycles. An extension step followed at 72°C for 15 s. In the next 40 cycles (cycles 11 to 50), the steps were 92°C for 15 s, 62°C for 30 s, and 72°C for 15 s. This was followed by a final extension step at 72°C for 5 min.

The amplification products were analyzed by electrophoresis on a 1.5% agarose gel containing ethidium bromide, and the bands were visualized with UV light. To prevent false-positive findings due to contamination, pipettes with filters were used at all stages. DNA extraction, preparation of the reaction mixture, PCR amplification, and detection were performed in different areas. To detect any cross-contamination, all PCR procedures were performed with a negative control of sterile water.

Genotype characterization of *P. jiroveci*. The PCR products from nested PCR were purified with Sephadryl S-400 columns (Amersham Pharmacia Biotech) and reamplified with

Table 1. Characteristics of patients included in the study.

HIV status ^a	Underlying diseases (no. of cases)	No. of sputum/BAL samples tested (n = 255)	No. of PCR-positive sputum/BAL samples (n = 79)	Total no. of PCR-positive patients/ no. tested (%)	No. of patients with previous sulfa exposure/ no. tested (%)
HIV positive (n = 19)	<i>Pneumocystis pneumonia</i> (15); bacterial pneumonia (4)	2/17	2/13	15/19 (78.9)	8/19 (42.1)
HIV negative (n = 236)	Lung cancer (17); cystic fibrosis (60); interstitial lung diseases (71); chronic obstructive pulmonary disease (88)	120/116	39/25	64/236 (27.1)	9/236 (3.8)

NOTE. BAL, bronchoalveolar lavage.

^a HIV status was analyzed by commercial EIA for HIV-1/HIV-2 (BioRad Multispot).

Table 2. *Pneumocystis jiroveci* genotypes found in Spanish patients.

Genotype	mt LSU rRNA		<i>DHPS</i> gene	
	Nucleotide position/identity	Percentage of genotypes	Nucleotide position/identity	Percentage of genotypes
1	85 C/248 C	49.3	165 (55)/A (Thr); 171 (57)/C (Pro)	64.5
2	85 A/248 C	10.1	165 (55)/G (Ala); 171 (57)/C (Pro)	12.9
3	85 T/248 C	36.7	165 (55)/A (Thr); 171 (57)/T (Ser)	9.6
4 ^a	85 C/248 T	...	165 (55)/G (Ala); 171 (57)/T (Ser)	3.2
Mixed	85C/248C and 85C/248T	3.7	...	9.6
	85C/248C and 85A/248C			
	85A/248C and 85T/248C			

^a mt LSU rRNA genotype 4 was only isolated in 1 patient with mixed infection.

ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). Then, for each reaction, 5 μ L of PCR product, 4 μ L of terminator ready reaction mix, and 3 pmol of primer were added. The extension products were purified by an ethanol precipitation procedure to remove the excess dye terminators. Each sample pellet was resuspended in 12.5 μ L of template suppression reagent and heated at 95°C for 3 min to denature. The electrophoresis was carried out on the ABI Prism 310 sequencer (PE Applied Biosystems) in accordance with the manufacturer's recommendations. The sequenced DNA fragments were analyzed by Sequence Navigator, version 1.0.1 (PE Applied Biosystems).

Restriction enzyme analysis. The 25 μ L of PCR–restriction fragment–length polymorphism (RFLP) was divided into 3 aliquots. One was used to confirm the presence of a 370-bp fragment from the *DHPS* gene. The second and third aliquots were used to identify the presence of wild-type versus mutations in codons 55 and 57 by RFLP with *AccI* and *HaeIII* (Roche Diagnostics), respectively. When the mutation is present, a 370-bp band appears. Likewise, after RFLP, 2 bands at 229 and 141 with *AccI*, and 221 bp and 149 bp with *HaeIII* in wild-type samples appear.

Statistical analysis. The χ^2 test or Fisher's exact test was used for assessing differences between proportions. Results were considered statistically significant at $P < .05$. Statistical analyses were performed by SPSS version 11.5 and the EpiInfo statistical program, version 6.5 (Centers for Disease Control and Prevention).

RESULTS

Amplification with specific primers. The mt LSU rRNA primers amplified a 260-bp fragment in 64 (27.1%) of 236 samples from pulmonary diseases cases and in 15 PcP cases in 19 HIV-positive patients. The *DHPS* primer sets amplified a 370-bp band in 20 (31.3%) of 64 positive samples obtained from patients with pulmonary disease and 11 (73.3%) of 15 samples obtained from patients with AIDS and PcP. All am-

plified fragments were examined for genetic polymorphisms by direct sequencing of mt LSU rRNA or RFLP of *DHPS* genes.

Genotype frequency. The results obtained are shown in table 2. From the 5 types described for the mt LSU rRNA locus [15], 4 genotypes were isolated in current study. Genotypes were distinguished on the basis of polymorphisms at nt 85 and 248. Genotype 1 was the most common (49.3%); genotype 2 (10.1%) was less common. Genotype 3 (36.7%) was the second most common genotype in our area. As shown in table 1, in 3.7% of samples analyzed, coinfection with multiple *P. jiroveci* strains could be detected. In 1 of these subjects, genotype 4 (85C/248T) was isolated (table 2).

DHPS gene analysis (table 2) revealed genotype 1 to be the most frequent among the cases analyzed; it accounted for 64.5% of the samples. Genotypes 2 and 3 occurred 12.9% and 9.6% of the time, respectively; and genotype 4 was uncommon, occurring only 3.2% of the time. In 3 (9.6%) of the 31 samples that amplified with *DHPS* primer sets, we found a mixture of genotypes, which demonstrates the possibility of coinfection in the subjects analyzed.

Taken together, the analysis from both genetic loci (mt LSU rRNA and *DHPS*) revealed that only 8 genotypes were found from the 12 possible combinations: 1/1 (29.3%), 1/2 (12.9%), 1/3 (6.4%), 1/4 (3.2%), 2/1 (3.2%), 3/1 (25.8%), 3/3 (3.2%), and mixed/mixed (16.1%). The most common multilocus genotypes consisted of combinations of the most common genotypes at each individual locus.

Genotype distribution in HIV-negative subjects with pulmonary diseases versus patients with AIDS and PcP. Figure 1 shows the rate of genotypes of the mt LSU rRNA region and the *DHPS* gene in subjects with pulmonary diseases versus patients with AIDS and PcP. For mt LSU rRNA analysis, in both groups of patients, genotype 1 (43.7% and 73.3%) was found to be the most frequent, but its presence was significantly higher in patients with AIDS and PcP ($P = .03$). Genotype 2 was the polymorphism with the lowest frequency (10.9% and 6.6%). Genotype 3 was most frequent in subjects with pul-

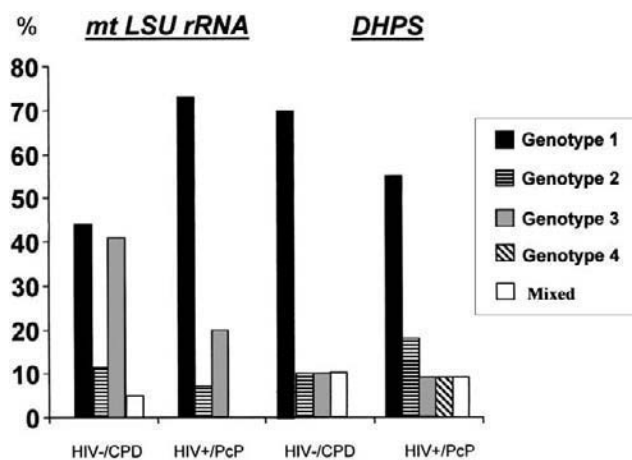


Figure 1. Genotype frequency in patients with pulmonary diseases (CPD) versus patients with AIDS and *Pneumocystis jiroveci* pneumonia (PcP) at mitochondrial large subunit ribosomal RNA (mt LSU rRNA) and DHPS loci. Genotype 1 represents polymorphism 85C/248C at mt LSU rRNA and 55 Trh/57 Pro at the DHPS gene. Genotype 2 represents polymorphism 85A/248C and 55 Ala/57 Pro. Genotype 3 represents polymorphism at 85T/248C and 55 Thr/57 Ser. Genotype 4 is only present in the DHPS gene (55 Ala/57 Ser). "Mixed" indicates the presence of >1 genotype.

monary disease (in comparison with patients with AIDS and PcP), but this finding was not statistically significant (40.6% vs. 20%; $P = .13$), probably because of the low number of cases. Finally, a mix of genotypes was only detected in the pulmonary disease group (4.6%).

Analysis of the DHPS locus in both groups revealed a wild-type frequency (55 Thr/57 Pro) of 70% in patients with chronic pulmonary diseases versus 54.5% in patients with AIDS and PcP ($P = .63$). The rate of genotypes 2 (mutation in codon 55) and 3 (mutation in codon 57) in 2 groups were similar: changes in codon 55 were found in 10% of patients with chronic pulmonary diseases and in 18% of patients with AIDS and PcP, and changes in codon 57 were found in 10% in patients with chronic pulmonary diseases and in 9% of patients with AIDS and PcP. Only 1 case of genotype 4 (mutations in codons 55/57) in the DHPS gene was found in AIDS-associated PcP. The frequency of mixed genotypes was similar in patients with pulmonary diseases and patients with AIDS and PcP.

DISCUSSION

To our knowledge, the study presented here is the first epidemiologic study performed in Spain in which the *P. jiroveci* genotypes have been analyzed in respiratory samples from subjects with chronic pulmonary diseases and patients with AIDS-associated PcP. The analysis reveals a high presence of genotypes 1 (85C/248C) and 3 (85T/248C), with a rate of 86% in mt LSU rRNA region, as well as a mutation rate of 35.5% in the DHPS gene.

As is shown in figure 1, the comparison of genotypes that focused on the mt LSU rRNA region indicated that genotype 1 occurred more often in patients with AIDS than in patients with chronic pulmonary disease, a finding that reached statistical significance. Genotype 2 (85A/248C) had a similar rate in both groups. Genotype 3 occurred most frequently in patients with chronic pulmonary diseases than in patients with AIDS. Finally, mixtures of *P. jiroveci* genotypes were found only in patients with chronic pulmonary diseases. However, a recent epidemiological study that involved patients with AIDS in the United States that analyzed mt LSU rRNA genotypes described a high frequency of genotype 1 (38%) and genotype 2 (36.7%) [10]. Comparison of this study and the data obtained in our population show a clear inversion between genotypes 2 and 3. Data also show an important significant difference in the mt LSU rRNA genotype between the groups included in the study: the patients with chronic pulmonary disease had a high rate of genotype 3, almost similar to that for genotype 1. In contrast, the patients with AIDS-associated PcP had a higher rate of genotype 1.

The analysis of mutations described in the DHPS gene does not reveal significant differences between both groups, but the wild type was most frequent in chronic pulmonary diseases (70%) with respect to patients with AIDS (54.5%). Genotype 4 (55 Ala/57 Ser) was only present in 1 patient with AIDS. Our study found a lower rate of DHPS mutations (30% in patients with chronic pulmonary diseases and 45.5% in patients with AIDS) than the 69% found in a study of patients with AIDS-associated PcP from the United States [10], but we found a similar rate to previous studies performed in Europe [16, 17].

In our study, the number of patients identified as having the DHPS gene seems to be lower. Only in 20 (31.2%) of 64 of subjects with chronic pulmonary diseases and in 11 (73.3%) of 15 of patients with AIDS and PcP were we able to detect DHPS genotypes. The lowest amplification rate obtained in patients with chronic pulmonary disease is perhaps related to a lower pathogen load in the samples, but is the first time that DHPS has been genotyped in this kind of patient. The rate of amplification for patients with AIDS is similar to that of previously published studies, in which the amplification rates were found to be ~67%–77% [10, 16, 18].

Our study focused on establishing the genotype distribution of *P. jiroveci* in our population. For this reason, we selected mt LSU rRNA because it has a high degree of genetic conservation, and because it is useful to detect intraspecific differences between populations [10]. The DHPS gene, which is related to sulfa drug resistance, was selected as a marker of mutation rate, which would permit us to assess information about the transmission source between patients with AIDS-associated PcP and patients with chronic pulmonary disease. Comparison of data obtained from patients with chronic pul-

monary diseases and patients with AIDS reveals a similar pattern of genotypes in the analysis of mt LSU rRNA region in samples isolated from the same city, providing support for the notion of a common infectious source. These results are in agreement with studies from European countries that suggest person-to-person transmission [11, 19, 20]. An experimental animal model has shown that *Pneumocystis* organisms are able to replicate in the lung alveolus of the immunocompetent host, thus keeping their infectious power intact, which suggests that patients with chronic pulmonary diseases who are colonized with this pathogen could be reservoirs and a source of infection in human populations [21].

The development of DHPS mutations has been associated with previous sulfa or sulfone prophylaxis [16]. In Spain, the use of trimethoprim-sulfamethoxazole is standard chemoprophylaxis against PcP in patients with AIDS; however, sulfa drugs are rarely used to treat infectious lung diseases. Instead, they are frequently treated with quinolones. The results obtained by RFLP show a greater presence of mutations in patients with AIDS than in patients with chronic pulmonary diseases. Thus, the study of both genes suggests a common source of infection and transmission of mutated *P. jiroveci* from patients with AIDS to patients with chronic pulmonary diseases.

The current results show the presence of only 8 genetic variations among combinations from the 4 possible genotypes detected at each individual locus. In addition, by multilocus typing, 1/1, 1/2, and 3/1 types were the ones most often found, with a rate of 67.7%. The analysis from data obtained by multilocus typing reveals a 16.1% coinfection rate, which is within the 10%–30% rate reported by other groups that used same approach [22, 23], but lower than the 69% rate found with single-strand conformation polymorphism analysis [24].

In summary, for the first time, *P. jiroveci* genotypes are described in patients from Spain on the basis of mt LSU rRNA and DHPS loci. We found a high presence of genotype 1 (85C/248C) and a clear inversion between genotypes 2 (85A/248C) and 3 (85T/248C), suggesting geographical variation; and we found a high rate of mutations in the DHPS gene. The same genotypic pattern in isolates from patients with AIDS and PcP and in subjects with chronic pulmonary diseases in the same geographical area suggests a possible common infectious source. This represents a step toward understanding the epidemiology of *P. jiroveci* infection, and further investigation to establish a relationship between genotype and virulence will be necessary.

Acknowledgments

We thank Virginia Nieto for assistance in preparing the article in manuscript.

Financial support. Fifth Framework programmer of the European

Commission (contract QLK2-2000-01369) and Spanish Ministry of Science and Technology (reference number SAF2003-06061).

Conflict of interest. All authors: No conflict.

References

- Stringer JR, Beard CB, Miller RF, Wakefield AE. A new name (*Pneumocystis jiroveci*) for *Pneumocystis* from humans. *Emerging Infect Dis* **2002**; 8:891–6.
- Calderon-Sandubete EJ, Varela-Aguilar JM, Medrano-Ortega FJ, et al. Historical perspective on *Pneumocystis carinii* infection. *Protist* **2002**; 153:303–10.
- Kaplan JE, Jones JL, Dykewicz CA. Protists as opportunistic pathogens: public health impact in the 1990s and beyond. *J Eukaryot Microbiol* **2000**; 47:15–20.
- Cardenal R, Medrano FJ, Varela JM, et al. *Pneumocystis carinii* pneumonia in heart transplant recipients. *Eur J Cardiothorac Surg* **2001**; 20:799–802.
- Ognibene FP, Shelhamer JH, Hoffman GS, et al. *Pneumocystis carinii* pneumonia: a major complication of immunosuppressive therapy in patients with Wegener's granulomatosis. *Am J Respir Crit Care Med* **1995**; 151:795–9.
- Varthalitis I, Aoun M, Daneau D, Meunier F. *Pneumocystis carinii* pneumonia in patients with cancer: an increasing incidence. *Cancer* **1993**; 71: 481–5.
- Maskell NA, Waine DJ, Lindley A, et al. Asymptomatic carriage of *Pneumocystis jiroveci* in subjects undergoing bronchoscopy: a prospective study. *Thorax* **2003**; 58:594–7.
- Huang L, Morris AM, Beard CB. *Pneumocystis carinii* dihydropteroate synthase gene mutations and treatment with sulfa or sulfone regimens: a proposal for standardized definitions for clinical evaluations. *J Eukaryot Microbiol* **2001**; (Suppl):180S–1S.
- Miller RF, Lindley AR, Ambrose HE, Malin AS, Wakefield AE. Genotypes of *Pneumocystis jiroveci* isolates obtained in Harare, Zimbabwe, and London, United Kingdom. *Antimicrob Agents Chemother* **2003**; 47:3979–81.
- Beard CB, Carter JL, Keely SP, et al. Genetic variation in *Pneumocystis carinii* isolates from different geographic regions: implications for transmission. *Emerg Infect Dis* **2000**; 6:265–72.
- Totet A, Respaldiza N, Pautard JC, Raccurt C, Nevez G. *Pneumocystis jiroveci* genotypes and primary infection. *Clin Infect Dis* **2003**; 36: 1340–2.
- Miller RF, Wakefield AE. *Pneumocystis carinii* genotypes and severity of pneumonia. *Lancet* **1999**; 353:2039–40.
- Wakefield AE, Pixley FJ, Banerji S, et al. Amplification of mitochondrial ribosomal RNA sequences from *Pneumocystis carinii* DNA of rat and human origin. *Mol Biochem Parasitol* **1990**; 43:69–76.
- Demanche C, Berthelemy M, Petit T, et al. Phylogeny of *Pneumocystis carinii* from 18 primate species confirms host specificity and suggests coevolution. *J Clin Microbiol* **2001**; 39:2126–33.
- Keely SP, Stringer JR, Baughman RP, Linke MJ, Walzer PD, Smulian AG. Genetic variation among *Pneumocystis carinii hominis* isolates in recurrent pneumocystosis. *J Infect Dis* **1995**; 172:595–8.
- Ma L, Kovacs JA, Cargnel A, Valerio A, Fantoni G, Atzori C. Mutations in the dihydropteroate synthase gene of human-derived *Pneumocystis carinii* isolates from Italy are infrequent but correlate with prior sulfa prophylaxis. *J Infect Dis* **2002**; 185:1530–2.
- Nahimana A, Rabodonirina M, Zanetti G, et al. Association between a specific *Pneumocystis jiroveci* dihydropteroate synthase mutation and failure of pyrimethamine/sulfadoxine prophylaxis in human immunodeficiency virus-positive and -negative patients. *J Infect Dis* **2003**; 188:1017–3.
- Helweg-Larsen J, Benfield TL, Eugen-Olsen J, Lundgren JD, Lundgren B. Effects of mutations in *Pneumocystis carinii* dihydropteroate synthase gene on outcome of AIDS-associated *P. carinii* pneumonia. *Lancet* **1999**; 354:1347–51.

19. Helweg-Larsen J, Tsolaki AG, Miller RF, Lundgren B, Wakefield AE. Clusters of *Pneumocystis carinii* pneumonia: analysis of person-to-person transmission by genotyping. QJM **1998**;91:813–20.
20. Dumoulin A, Mazars E, Seguy N, et al. Transmission of *Pneumocystis carinii* disease from immunocompetent contacts of infected hosts to susceptible hosts. Eur J Clin Microbiol Infect Dis **2000**;19:671–8.
21. Chabé M, Dei-Cas E, Creusy C, et al. Immunocompetent hosts as a reservoir of *Pneumocystis* organisms: histological and RT-PCR data demonstrate active replication. Eur J Clin Microbiol Infect Dis **2004**;23:89–97.
22. Lee CH, Helweg-Larsen J, Tang X, et al. Update on *Pneumocystis carinii* f sp *hominis* typing based on nucleotide sequence variations in internal transcribed spacer regions of rRNA genes. J Clin Microbiol **1998**;36:734–41.
23. Keely SP, Baughman RP, Smulian AG, Dohn MN, Stringer JR. Source of *Pneumocystis carinii* in recurrent episodes of pneumonia in AIDS patients. AIDS **1996**;10:881–8.
24. Hauser PM, Blanc DS, Bille J, Francioli P. Typing methods to approach *Pneumocystis carinii* genetic heterogeneity. FEMS Immunol Med Microbiol **1998**;22:27–35.