MAJOR ARTICLE

# Limited Asymptomatic Carriage of *Pneumocystis jiroveci* in Human Immunodeficiency Virus–Infected Patients

**Ann E. Wakefield,[1,a] Austin R. Lindley,[1] Helen E. Ambrose,[1] Cecile-Marie Denis,[1,3] and Robert F. Miller[2]**

[1]Molecular Infectious Diseases Group, Department of Paediatrics, Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, and [2]Department of Sexually Transmitted Diseases, Windeyer Institute of Medical Sciences, Royal Free and University College Medical School, University College London, London, United Kingdom; [3]Faculté Libre des Sciences, Lille, France

**Forty-seven bronchoalveolar lavage fluid samples from 16 human immunodeficiency virus (HIV)–infected patients were used to test the latency model of *Pneumocystis* infection in the human host. Identification of DNA sequence polymorphisms at 4 independent loci were used to genotype *Pneumocystis jiroveci* from the 35 samples that contained detectable *P. jiroveci* DNA. Eighteen of those 35 samples came from patients who did not have *Pneumocystis* pneumonia (PCP) and had confirmed alternative diagnoses. Seven patients had asymptomatic carriage of *P. jiroveci* over periods of ≤9.5 months after an episode of PCP, and in all 7 cases, a change in genotype from that in the original episode of PCP was observed. The absence of *P. jiroveci* DNA in one-fourth of the 47 samples and the observed changes in genotype during asymptomatic carriage do not support the latency model of infection. Asymptomatic carriage in HIV-infected patients may play a role in transmission of *P. jiroveci* and may even supply a reservoir for future infections.**

The route of transmission of human *Pneumocystis* infection is still not understood. It was long thought that *Pneumocystis jiroveci* organisms were acquired during childhood and persisted throughout life in the lungs in a dormant phase [1, 2]. Subsequent immunosuppression of the host, resulting from a variety of causes, permitted the fungus to propagate and to cause disease. According to this theory of transmission, there was little reason to protect immunosuppressed adults from possible exposure to *Pneumocystis;* acquisition of the infection was thought to take place early in life and not at the time of immunosuppression. The latency hypothesis, however, has recently been challenged. Several studies have suggested that, in the immunocompetent human host, *Pneumocystis* organisms are frequently acquired and cleared by the immune system, rather than being acquired during childhood and persisting for a lifetime. This reinfection hypothesis has important public health implications, because it suggests that susceptible individuals could be protected from *Pneumocystis* infection.

Data refuting the latency hypothesis come from animal studies. It has been shown in a rat model that *Pneumocystis* organisms were eliminated from the lungs after *Pneumocystis* pneumonia (PCP) and that persistence of latent organisms was limited [3]. Similar results have been obtained using the severe combined immunodeficiency (SCID) mouse model [4]. In addition, some studies of human infection have indicated the

Received 9 October 2002; accepted 20 November 2002; electronically published 6 March 2003.

This study was carried out within the guidelines of the Middlesex Hospital Clinical Investigations Panel. All patients gave informed written consent for bronchoscopy.

Financial support: Royal Society (to A.E.W.); Wellcome Trust (to A.E.W.); Medical Research Council (to H.E.A. and C.-M.D.); Fifth Framework Programme of the European Commission (grant QLK2-CT-2000-01369 to A.R.L.); Camden Primary Care Trust (R.F.M.).

[a] Deceased.

Reprints or correspondence: Dr. Robert F. Miller, Dept. of Sexually Transmitted Diseases, Windeyer Institute of Medical Sciences, Royal Free and University College Medical School, University College London, Mortimer Market Centre, WC1E 6AU London, United Kingdom (rmiller@gum.ucl.ac.uk).

**The Journal of Infectious Diseases 2003; 187:901–8**



0022-1899/2003/18706-0004$15.00

lack of *Pneumocystis* in the lungs of immunocompetent adults [5]. However, although it is important to test the latency hypothesis, the hypothesis is difficult to disprove, because it is impossible to demonstrate the complete absence of a particular organism in the human lung. Support for the reinfection model comes from studies involving patients with recurrent episodes of PCP in which a genetically different type of *P. jiroveci* was associated with each episode [6–9]. Further evidence comes from a study in which allelic variation patterns in isolates of *P. jiroveci* correlated with patients' place of diagnosis, rather than their place of birth, which strongly supports recent transmission, rather than latency [10].

As another approach, to question the latency hypothesis in the human host, we examined multiple bronchoalveolar lavage (BAL) fluid samples from 16 human immunodeficiency virus (HIV)–infected patients that were collected over a period of months to years. If latent *P. jiroveci* organisms were present throughout life in these patients, then the immunosuppression associated with HIV infection would provide a permissive environment, and the endogenous *P. jiroveci* organisms would proliferate.

## SUBJECTS, MATERIALS, AND METHODS

***Samples.*** Forty-seven BAL fluid samples from 16 HIV-infected patients (2–6 samples/patient) were collected between 1990 and 2001. The interval between samples obtained from a single patient ranged from 2 weeks to 33 months. All patients were known to be HIV-1 antibody positive and had been admitted to the specialist HIV/AIDS inpatient care facility at University College London hospitals for investigation of respiratory symptoms. For each patient, the CD4 lymphocyte count and use of prophylaxis against *P. jiroveci* pneumonia were recorded. All bronchoscopies were performed by R.F.M. PCP was defined by typical clinical and radiographic presentation [11], with demonstration of *P. jiroveci* cysts in BAL fluid by Grocott methenamine silver staining and response to anti-*Pneumocystis* therapy. Bacterial pneumonia was defined by the acute onset of respiratory symptoms, focal or diffuse radiographic abnormalities, identification of a specific bacterial pathogen in BAL fluid and/or blood, and response to specific antibiotics in conventional doses [12]. Disseminated *Mycobacterium avium* complex infection was defined by staining and culture of the organism from BAL fluid and blood and/or bone marrow. Cytomegalovirus (CMV) pneumonitis was defined by identification in BAL fluid of typical intranuclear and intracytoplasmic inclusions and by a positive result of testing for CMV (detection of early antigen by fluorescent foci) [13]. Pulmonary Kaposi sarcoma was defined by visualization during bronchoscopy of multiple violaceous lesions [14] and identification of multiple skin lesions. Patients received anti-*Pneumocystis* prophylaxis according to published guidelines [15]. All samples were analyzed by individuals who were blinded to clinical details.

***Detection of* P. jiroveci *using DNA amplification.*** DNA extraction of the samples was carried out using the QIAamp DNA mini kit (Qiagen), according to the manufacturer's protocol, with 2 exceptions: the proteinase K step was extended to 1 h, and DNA was eluted from the column in 50 $\mu$L, rather than 200 $\mu$L, of AE buffer. Detection of *P. jiroveci* DNA was carried out using a nested polymerase chain reaction (PCR) at the genes encoding the mitochondrial large subunit (mt LSU) rRNA and the mitochondrial small subunit (mt SSU) rRNA. For PCR at the mt LSU rRNA locus, the primer pair pAZ102-H and pAZ102-E was used in the first round of amplification, followed by pAZ102-X and pAZ102-Y in the second round of amplification, as described elsewhere [16–18]. For PCR at the mt SSU rRNA locus, the primer pair pAZ112-10F and pAZ112-10R was used, followed by pAZ112-13 and pAZ112-14, as described elsewhere [16, 19]. To monitor for false-negative PCR caused by inhibition of DNA amplification, a control PCR assay, using the conserved human $\beta$-globin gene sequence, was used. A positive result of human $\beta$-globin PCR, using primer pair BGLO1 (5′-ACACAACTGTGTTCACTAGCA-3′) and BGLO2 (5′-CAACTTCATCCACGTTCACC-3′) with an annealing temperature of 55°C and 40 cycles, demonstrated that DNA amplification was possible for a given sample [20].

*Taq* DNA polymerase (Promega) was used throughout the study. To avoid contamination, negative controls were included in each experiment in both DNA extraction and amplification, DNA extraction and PCR were performed in a laminar flow cabinet, and disposable tips, tubes, and reagent aliquots were used.

**P. jiroveci *multilocus genotyping.*** Multilocus genotyping was carried out at 4 loci, those encoding mt LSU rRNA, mt SSU rRNA, dihydropteroate synthase (DHPS), and superoxide dismutase (SOD). PCR at the mt LSU rRNA and mt SSU rRNA loci was carried out as described above. For the DHPS locus, the primer pair DHPS1 and DHPS2 was used in the first round of PCR, followed by DHPS3 and DHPS4 (corresponding to $A_{HUM}$ and BN) in the second round [21, 22]. For the SOD locus, the primer pair SOD7 and SOD8 was used in the first round, followed by SOD9 and SOD10 in the second round [23]. The PCR protocol for both the DHPS and the SOD locus was the same as that for the mt LSU rRNA locus.

Amplification products were purified using the SpinPrep PCR Clean-up Kit (Novagen) and sequenced directly using the ABI Prism Big Dye Terminator Cycling Sequencing Ready Reaction Kit, version 2.0 (Applied Biosystems). The program was 96°C for 30 s, 50°C for 15 s, and 60°C for 4 min for 25 cycles. The reactions were precipitated using Pellet Paint NF Co-Precipitant (Novagen) and run on an ABI 377 automated se-

quencer (Applied Biosystems). DNA sequence data were analyzed using Gap4 of the Staden package (Genetics Computer Group).

At the mt LSU rRNA locus, genotypes were distinguished on the basis of polymorphisms at nucleotide positions 85 and 248, as described elsewhere [6, 16]. At the mt SSU rRNA locus, genotypes were distinguished by polymorphisms at nucleotide positions 216 and 299 (numbering according to Hunter and Wakefield [19]). At the DHPS locus, genotypes were distinguished on the basis of polymorphisms at nucleotide positions 165 and 171 (corresponding to amino acid substitutions at positions 55 and 57), as described elsewhere [21]. At the SOD locus, genotypes were distinguished by polymorphisms at nucleotide positions 110 and 215 [23].

## RESULTS

***Detection of* P. jiroveci *DNA.*** *P. jiroveci* DNA was detected using PCR at 2 different loci, the locus encoding mt LSU rRNA and the locus encoding mt SSU rRNA. *P. jiroveci* DNA was detected using a single-round PCR at the mt LSU rRNA and mt SSU rRNA loci in samples from 14 patients with clinical and laboratory-confirmed PCP (table 1). This was consistent with the findings of another study, in which a positive result of a single-round PCR at the mt LSU rRNA locus in a BAL fluid sample indicated clinical disease [17].

Another patient with confirmed PCP had *P. jiroveci* DNA that was detectable only by nested PCR at the mt LSU and SSU rRNA loci, which indicates lower levels of DNA [21] (table 1). Two patients had an atypical presentation of PCP (table 1); both had respiratory symptoms and negative findings of silver staining of BAL fluid. In one, a thoracic computed tomographic scan showed multiple nodules and patchy focal ground glass shadowing; the patient responded clinically and radiographically to intravenous pentamidine. In the other patient, a chest radiograph showed multiple pneumatocoels with associated patchy ground glass shadowing. A clinical and radiographic response occurred with a combination of clindamycin and primaquine. In both patients, *P. jiroveci* DNA was detected in BAL fluid by nested PCR.

Thirty samples were from patients who had clinical and laboratory diagnoses other than PCP, including bacterial pneumonia, disseminated *Mycobacterium avium* complex infection, CMV pneumonitis, and pulmonary Kaposi sarcoma. *P. jiroveci* DNA was found in 16 samples when nested PCR, a more sensitive test capable of detecting much lower levels of *Pneumocystis* DNA, was used (table 1). The presence of *P. jiroveci* DNA in these samples, in the absence of clinical symptoms of PCP, suggests that these patients had asymptomatic carriage of *Pneumocystis*.

No *P. jiroveci* DNA was detected in a total of 12 samples from 7 patients by nested PCR at the mt LSU rRNA and mt SSU rRNA loci (table 1). All of the samples from 2 patients (2 samples from patient 4, obtained 4 months apart, and 3 samples from patient 10, obtained over a period of 33 months) were negative for *P. jiroveci* DNA, indicating that the finding was consistent (table 2). Two of these patients (patients 4 and 5) died within a month after a sample was obtained in which no *P. jiroveci* DNA was found; necropsy failed to find *P. jiroveci* in the lungs of these patients by histochemical staining (table 2). The lack of detectable *P. jiroveci* in some of the samples from these 7 patients was not due to weaker immunosuppression; the CD4 lymphocyte counts of these patients ranged from 10 to 380 cells/$\mu$L (median, 50 cells/$\mu$L) and were similar to

**Table 1. Detection of *Pneumocystis jiroveci* DNA in 47 bronchoalveolar lavage fluid samples obtained during respiratory episodes in 16 human immunodeficiency virus–infected patients.**

| Diagnosis | Samples in which *P. jiroveci* DNA was detected | | | Samples in which *P. jiroveci* DNA was not detected ($n$ = 12) | Total |
|---|---|---|---|---|---|
| | High levels[a] ($n$ = 16) | Low levels[b] ($n$ = 10) | Very low levels[c] ($n$ = 9) | | |
| *P. jiroveci* pneumonia | D88, D124, D155, D282, D319, D320, D325, D332, D339, D609, D631, D633, D764, D818[d] | D742, D670,[e] D744[e] | | | 17 |
| Bacterial pneumonia | D120[f] | D19, D54, D203, D344, D669 | D378, D399,[d] D540 | D87, D95, D606, D661[d] | 13 |
| CMV pneumonitis | | D125 | D131, D753 | D469, D510 | 5 |
| dMAC infection | | | D177, D281 | D184, D774 | 4 |
| PKS | D477[g] | D418 | D871, D902 | D701, D820, D851, D965 | 8 |

**NOTE.** CMV, cytomegalovirus; dMAC, disseminated *Mycobacterium avium* complex; mt LSU, mitochondrial large subunit; mt SSU, mitochondrial small subunit; PCR, polymerase chain reaction; PKS, pulmonary Kaposi sarcoma.

[a] *P. jiroveci* DNA detected at the mt LSU and mt SSU rRNA loci by single-round PCR.

[b] *P. jiroveci* DNA detected at the mt LSU and mt SSU rRNA loci by nested PCR; single-round PCR was negative at these loci.

[c] *P. jiroveci* DNA detected only at the mt LSU rRNA locus by nested PCR; single-round PCR at the mt LSU rRNA locus and both single-round and nested PCR at the mt SSU rRNA locus were negative.

[d] Patient also had pulmonary Kaposi sarcoma.

[e] Patient had atypical *P. jiroveci* pneumonia.

[f] Patient had *P. jiroveci* pneumonia 4 weeks before sample was obtained.

[g] Patient died of severe *P. jiroveci* pneumonia 4 weeks after sample was obtained.

**Table 2. Laboratory data for patients for whom at least 1 bronchoalveolar lavage fluid sample had no detectable *Pneumocystis jiroveci* DNA.**

| | First sample | | | | Second sample | | | | Third sample | | | | Fourth sample | | | | Fifth sample | | | | Sixth sample | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Patient | Code | Diagnosis | *P. jiroveci* DNA | Interval,[a] months | Code | Diagnosis | *P. jiroveci* DNA | Interval,[a] months | Code | Diagnosis | *P. jiroveci* DNA | Interval,[a] months | Code | Diagnosis | *P. jiroveci* DNA | Interval,[a] months | Code | Diagnosis | *P. jiroveci* DNA | Interval,[a] months | Code | Diagnosis | *P. jiroveci* DNA |
| 3 | D19 | Bac | + | 9.5 | D124 | PCP | + | 6 | D177 | dMAC | + | 0.5 | D184 | dMAC | − | 9 | D281 | dMAC | + | 7 | D344 | dMAC/Bac | + |
| 4 | D469 | CMV | − | 4 | D510[b] | CMV | − | | | | | | | | | | | | | | | | |
| 5 | D742 | PCP | + | 0.75 | D753 | CMV | + | 3.5 | D774[b] | dMAC | − | | | | | | | | | | | | |
| 9 | D54 | Bac | + | 5 | D87 | Bac | − | 0.75 | D95 | Bac | − | 9.5 | D203 | Bac | + | | | | | | | | |
| 10 | D820 | PKS | − | 5 | D851 | PKS | − | 28 | D965 | PKS | − | | | | | | | | | | | | |
| 12 | D540 | Bac | + | 2 | D606 | Bac | − | 5 | D661 | PKS/Bac | − | | | | | | | | | | | | |
| 15 | D701 | PKS | − | 3.5 | D818 | PKS/PCP | + | | | | | | | | | | | | | | | | |

**NOTE.** Bac, bacterial pneumonia; CMV, cytomegalovirus pneumonitis; dMAC, disseminated *Mycobacterium avium* complex infection; PCP, *Pneumocystis* pneumonia; PKS, pulmonary Kaposi sarcoma; +, detected; −, not detected.

[a] Interval between the times at which 2 samples were obtained.

[b] Patient died 4 weeks after sample was obtained; no *P. jiroveci* was detected at necropsy.

those of the other patients in this study (range, 10–370 cells/$\mu$L; median, 30 cells/$\mu$L). Six of the 7 patients were receiving anti-*Pneumocystis* prophylaxis; patient 15 was not (table 2).

***Change in* P. jiroveci *genotype in multiple samples from a single patient.*** Multilocus genotyping was carried out on 14 samples from 7 patients in whom asymptomatic carriage of *P. jiroveci* was detected after an episode of PCP; the interval between samples ranged from 0.75 to 9.5 months (table 3). In all 7 patients, the *P. jiroveci* genotype found in samples obtained during asymptomatic carriage was different from that found in samples from the episode of PCP. All patients were receiving prophylaxis.

Asymptomatic carriage of *P. jiroveci* was found in 2 patients (patients 1 and 3) who had bacterial pneumonia 4 and 9.5 months before an episode of PCP. Both patients were receiving prophylaxis. Multilocus genotyping showed that, in both of these patients, the type of *P. jiroveci* observed during the subclinical infection was the same as that causing the clinical disease.

***Duration of asymptomatic carriage of* P. jiroveci.** In 5 patients, the duration of asymptomatic carriage of *P. jiroveci* ranged from 0.5 to 16.5 months (table 4). In 2 of these patients (patients 6 and 9), a change of genotype was detected in samples obtained up to 14.5 months apart; in the other 3 patients, the *P. jiroveci* genotype was the same in all samples. Four of the 5 patients were receiving prophylaxis; patient 11 was not.

## DISCUSSION

This study sought to question the hypothesis that *P. jiroveci* infection in the human host is caused by latent carriage of fungus acquired during childhood by examining multiple BAL fluid samples obtained from immunosuppressed HIV-infected patients with and without PCP. We found that not all samples contained detectable *P. jiroveci* DNA. This finding indicated that, although patients were immunologically challenged by HIV infection, no proliferation of *Pneumocystis* had occurred, which suggests that these individuals were not carrying latent *P. jiroveci* infection in their lungs. Samples were obtained over a period of 2.5 years from 1 patient with a CD4 lymphocyte count of <200 cells/$\mu$L, and there was no evidence of *P. jiroveci* at any point. Since the 1980s, increasingly sensitive techniques have been developed for detecting *P. jiroveci* DNA in human respiratory samples [2, 5, 6, 8, 17, 18, 22, 24–26]. This increase in sensitivity has enabled detection of *P. jiroveci* in individuals in whom it was not expected, for example, in adults with mild immunosuppression induced by HIV infection [26, 27] and immunocompetent individuals with primary pulmonary disorders [28–31]. By contrast, although the use of nested PCR at the mt LSU rRNA locus to distinguish genotypes is very sensitive and can identify 1 or 2 *P. jiroveci* organisms in a respiratory sample [32], it is possible that the BAL fluid samples in this study in which no *P. jiroveci* DNA was found by nested PCR at the mt LSU rRNA locus did, in fact, contain *P. jiroveci* at very low levels, levels below the threshold for detection by this technique.

In the present study, identification of *P. jiroveci* DNA at the mt LSU rRNA locus after a single round of PCR on a BAL fluid sample was only made in samples from patients with clinical and laboratory-confirmed PCP, samples from patients who had recently recovered from PCP, and samples obtained immediately before presentation with PCP. This finding confirms and extends observations published elsewhere [17, 24].

**Table 3. Laboratory data for patients with alternative diagnoses and asymptomatic *Pneumocystis jiroveci* carriage after an episode of *Pneumocystis* pneumonia (PCP).**

| | First sample | | | | | | | | | | | Second sample | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Nucleotide at indicated position | | | | | | | | | | | Nucleotide at indicated position | | | | | | | | |
| | | | mt LSU | | mt SSU | | SOD | | DHPS | | | | | mt LSU | | mt SSU | | SOD | | DHPS | | |
| Patient | Code | Diagnosis | 85 | 248 | 216 | 299 | 110 | 215 | 165 | 171 | Interval,[a] months | Code | Diagnosis | 85 | 248 | 216 | 299 | 110 | 215 | 165 | 171 | Genotype change |
| 1 | D88 | PCP | A | C | G | G | C | T | G | C | 2 | D120 | Bac | C | T | T | A | T | C | G | C | Yes |
| 2 | D339 | PCP | C | C | G | G | T | C | A | C | 4 | D378 | Bac | A | C | ND | ND | ND | ND | ND | ND | Yes |
| 3 | D124 | PCP | T/C[b] | C | T | G | C | T | A | C | 6 | D177 | dMAC | A | C | ND | ND | ND | ND | ND | ND | Yes |
| 5 | D742 | PCP | T | C | T | G | ND | ND | ND | ND | 0.75 | D753 | CMV | A | C | ND | ND | ND | ND | ND | ND | Yes |
| 6 | D332 | PCP | A | C | G | A | T | C | G | C | 9.5 | D418 | PKS | T | C | T | G | ND | ND | ND | ND | Yes |
| 7 | D319 | PCP | C | C | G | G | C | T | A | C | 8.5 | D399 | PKS/Bac | A | C | ND | ND | ND | ND | ND | ND | Yes |
| 14 | D631 | PCP | T | C | T | G | T | C | G | C | 5 | D669 | Bac | A | C | G | G | ND | ND | ND | ND | Yes |

**NOTE.** Bac, bacterial pneumonia; CMV, cytomegalovirus pneumonitis; DHPS, dihydropteroate synthase; dMAC, disseminated *Mycobacterium avium* complex infection; mt LSU, mitochondrial large subunit rRNA; mt SSU, mitochondrial small subunit rRNA; ND, *P. jiroveci* was not detected at this locus in this sample; PKS, pulmonary Kaposi sarcoma; SOD, superoxide dismutase.

[a] Interval between the times at which 2 samples were obtained.

[b] Mixed infection.

**Table 4. Laboratory data for patients with alternative diagnoses and asymptomatic *Pneumocystis jiroveci* carriage for whom *P. jiroveci* DNA was detected in ⩾2 bronchoalveolar lavage fluid samples**

| | First sample | | | | | | | Second sample | | | | | | | Third sample | | | | | | | Fourth sample | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Nucleotide at indicated position | | | | | | | Nucleotide at indicated position | | | | | | | Nucleotide at indicated position | | | | | | | Nucleotide at indicated position | | | | | |
| | | | mt LSU | | mt SSU | | | | | mt LSU | | mt SSU | | | | | mt LSU | | mt SSU | | | | | mt LSU | | mt SSU | | | |
| Patient | Code | Diagnosis | 85 | 248 | 215 | 299 | Interval,[a] months | Code | Diagnosis | 85 | 248 | 215 | 299 | Interval,[a] months | Code | Diagnosis | 85 | 248 | 215 | 299 | Interval,[a] months | Code | Diagnosis | 85 | 248 | 215 | 299 | Genotype change |
| 3 | D177 | dMAC | A | C | ND | ND | 0.5 | D184 | dMAC | ND | ND | ND | ND | 9 | D281 | dMAC | A | C | ND | ND | 7 | D344 | dMAC/Bac | A | C | G | G | No |
| 6 | D418 | PKS | T | C | T | G | 6 | D477 | PKS[b] | C | C | G | G | | | | | | | | | | | | | | | Yes |
| 8 | D125 | CMV | C | C | G | G | 0.5 | D131 | CMV | C | C | ND | ND | | | | | | | | | | | | | | | No |
| 9 | D54 | Bac | A | C | G | G | 4 | D87 | Bac | ND | ND | ND | ND | 0.75 | D95 | Bac | ND | ND | ND | ND | 9.5 | D203 | Bac | T | C | T | A | Yes |
| 11 | D871 | PKS | C | T | ND | ND | 9 | D902 | PKS | C | T | ND | ND | | | | | | | | | | | | | | | No |

**NOTE.** Bac, bacterial pneumonia; CMV, cytomegalovirus pneumonitis; dMAC, disseminated *Mycobacterium avium* complex infection; mt LSU, mitochondrial large subunit rRNA; mt SSU, mitochondrial small subunit rRNA; ND, *P. jiroveci* DNA was not detected at this locus in this sample; PKS, pulmonary Kaposi sarcoma.

[a] Interval between the times at which 2 samples were obtained.

[b] Patient died of severe *P. jiroveci* pneumonia 4 weeks after sample was obtained.

Patients with radiologically atypical PCP, despite negative findings of histochemical staining, also had detectable *P. jiroveci* DNA in BAL fluid, but this could only be identified by nested PCR, a more sensitive technique, at the mt LSU and SSU rRNA loci. This finding has been described elsewhere for patients with atypical (granulomatous) PCP [33].

Asymptomatic carriage of *P. jiroveci* was shown by detection of *P. jiroveci* DNA in a BAL fluid sample only by nested PCR at the mt LSU rRNA locus. Not all of these patients had detectable *P. jiroveci* DNA at the mt SSU rRNA locus, which indicates that PCR was more sensitive at the mt LSU rRNA locus than at the mt SSU rRNA locus. In 7 patients, *P. jiroveci* DNA was detected in BAL fluid obtained 0.75–9.5 months after treatment of an episode of PCP. In all 7 patients, the genotype found at follow-up was different from that identified during the acute episode of PCP. It is postulated that high-dose treatment of PCP resulted in clearance of the infection and that, because patients were vulnerable as a result of their level of immunosuppression, reinfection from an external reservoir took place despite prophylaxis. These data support the reinfection hypothesis.

On the other hand, use of *Pneumocystis* prophylaxis did not always result in clearance of infection. In some patients, asymptomatic carriage was demonstrated in consecutive samples obtained over the course of several months, which suggests that, although prophylaxis had prevented development of PCP in these individuals, it merely contained the infection. In 2 of these patients, asymptomatic carriage subsequently led to development of PCP.

In this study, multilocus typing was carried out at 4 independent loci. Five different types of *P. jiroveci* have been identified on the basis of differences at the mt LSU rRNA locus [6], 2 have been identified on the basis of differences at the mt SSU rRNA locus [19], and 4 types have been identified on the basis of differences at the DHPS locus [21], which is under selective pressure from sulfa drug exposure [34–36]. Two genotypes have been identified at the SOD locus [23]. Using these 4 loci to genotype *P. jiroveci* enabled discrimination between 9 different genotypes (5.6%) of a potential total of 160 genotypes (4 types of *P. jiroveci* were identified at the mt SSU rRNA locus in this study). By contrast, genotyping at the internal transcribed spacer (ITS) regions shows 27 types at ITS region 1 and 30 types at ITS region 2, yielding a potential of 810 different types, of which 87 (10.7%) have been described [26, 37].

The chief advantage of multilocus typing is that there is a greater sensitivity for detecting differences when several loci are examined concurrently than when a single locus is examined [38, 39]. In addition, by looking at polymorphisms at multiple loci, further information is available for strain characterization, and "strain" typing is achieved, rather than simply "gene" typing. Hauser et al. [40] used multilocus typing at 4 independent loci—ITS region 1, the intron of the nuclear 26S rRNA gene, the mt LSU rRNA locus, and the intron 6 region of the $\beta$-tubulin gene—to describe differences in isolates of *P. jiroveci*. Amplification by PCR was followed by identification using single-strand confirmation polymorphism analysis. A total of 35 genotypes (16.2%) of a potential total of 216 types were identified.

Multilocus typing was not possible for all samples from patients with asymptomatic carriage of *P. jiroveci* in the present study. In some of these, we were able to genotype only at multicopy genes (i.e., at the mt LSU and SSU rRNA loci), and genotyping at the single-copy DHPS and SOD genes was not possible, an observation consistent with the presence of low levels of *P. jiroveci* DNA in these samples [18]. The change in genotype of *P. jiroveci* identified by multilocus typing in these sequential samples suggests that these patients were exposed to different types of *P. jiroveci* over time [18] and provides further evidence to support the model of reinfection, rather than latency.

When we identified changes at the DHPS locus, they were always accompanied by changes at $\geqslant$1 other independent locus. If changes at the DHPS locus were due to selective pressure resulting from exposure to sulfa drugs [32–34], then it would be predicted that no differences would be observed at other independent loci. This suggests that mutations at the DHPS locus were not evolving within individual patients and further supports the idea that exogenous reinfection by different genotypes of *P. jiroveci* is occurring [41].

A large number of samples from patients in this study without clinically apparent disease had detectable *P. jiroveci* DNA at low levels in BAL fluid (35 of 47 samples; 74.5%). This suggests that asymptomatic individuals who had demonstrable carriage of *P. jiroveci* DNA over periods of up to several months might act a reservoir of *P. jiroveci* infection. Furthermore, this patient group might represent an important and as yet unrecognized element in the transmission of disease [27, 29].

It is important, from a public health perspective, to determine whether the reinfection or the latency model of human *P. jiroveci* infection occurs; if reinfection during adult life can occur, then susceptible patients should be protected from potential sources of infection. The data from this study demonstrate that multilocus typing may be used to define differences in *P. jiroveci* isolates and that not all immunosuppressed HIV-infected patients have detectable *P. jiroveci* in their lungs. The absence of *P. jiroveci* in some patients and the observed change in genotype in patients with asymptomatic carriage strongly support the reinfection model of human infection. Asymptomatic carriage in HIV-infected patients may play a role in transmission of *P. jiroveci*. This patient group may be a reservoir for future infections in other susceptible patients.

## References

1. Stringer JR, Cushion MT, Wakefield AE. New nomenclature for the genus *Pneumocystis*. J Eukaryot Microbiol **2001**; 49(Suppl):184S–9S.

2. Stringer JR, Beard CB, Miller RF, Wakefield AE. A new name *(Pneumocystis jiroveci)* for *Pneumocystis* from humans. Emerg Infect Dis **2002**; 8:891–6.
3. Vargas SL, Hughes WT, Wakefield AE, Oz HS. Limited persistence in and subsequent elimination of *Pneumocystis carinii* from the lungs after *P. carinii* pneumonia. J Infect Dis **1995**; 172:506–10.
4. Chen W, Gigliotti F, Harmsen AG. Latency is not an inevitable outcome of infection with *Pneumocystis carinii*. Infect Immun **1993**; 61: 5406–9.
5. Peters SE, Wakefield AE, Sinclair K, Millard PR, Hopkin JM. A search for *Pneumocystis carinii* in post mortem lungs by DNA amplification. J Pathol **1992**; 166:195–8.
6. 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.
7. Keely SP, Boughman RP, Smulian AG, Dohn MN, Stringer JR. Source of *Pneumocystis carinii* in recurrent episodes of pneumonia in AIDS patients. AIDS **1996**; 10:881–8.
8. Keely SP, Stringer JR. Sequences of *Pneumocystis carinii* f sp *hominis* strains associated with recurrent pneumonia vary at multiple loci. J Clin Microbiol **1997**; 35:2745–7.
9. Tsolaki AG, Miller RF, Underwood AP, Banerji S, Wakefield AE. Genetic diversity at the internal transcribed spacer regions of the rRNA operon among isolates of *Pneumocystis carinii* from AIDS patients with recurrent pneumonia. J Infect Dis **1996**; 174:141–56.
10. 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.
11. Miller RF, Millar AB, Weller IVD, Semple SJG. Empirical treatment without bronchoscopy for *Pneumocystis carinii* pneumonia in the acquired immunodeficiency syndrome. Thorax **1989**; 44:559–64.
12. Miller RF, Loveday C, Holton J, Sharvell Y, Patel G, Brink NS. Community-based respiratory viral infections in HIV positive patients with lower respiratory tract disease: a prospective bronchoscopic study. Genitourin Med **1996**; 72:9–11.
13. Millar AB, Patou G, Miller RF, et al. Cytomegalovirus in the lungs of patients with AIDS. Am Rev Respir Dis **1990**; 141:1474–7.
14. Miller RF, Tomlinson MC, Cottrill CP, Donald JJ, Spittle MF, Semple SJG. Bronchopulmonary Kaposi's sarcoma in patients with AIDS. Thorax **1992**; 47:721–5.
15. US Public Health Service (USPHS) and Infectious Diseases Society of America (IDSA). 1999 USPHS/IDSA guidelines for the prevention of opportunistic infections in persons infected with human immunodeficiency virus. MMWR Morb Mortal Wkly Rep **1999**; 48(RR-10):1–59, 61–6.
16. Tsolaki AG, Beckers P, Wakefield AE. Pre-AIDS era isolates of *Pneumocystis carinii* f sp *hominis:* high genotypic similarity with contemporary isolates. J Clin Microbiol **1998**; 36:90–3.
17. Wakefield AE, Pixley FJ, Banerji S, et al. Detection of *Pneumocystis carinii* with DNA amplification. Lancet **1990**; 336:451–3.
18. Miller RF, Ambrose HE, Wakefield AE. *Pneumocystis carinii* f sp *hominis* DNA in immunocompetent health care workers in contact with patients with *P. carinii* pneumonia. J Clin Microbiol **2001**; 39: 3877–82.
19. Hunter JAC, Wakefield AE. Genetic divergence at the mitochondrial small subunit ribosomal RNA gene among isolates of *Pneumocystis carinii* from five mammalian host species. J Eukaryot Microbiol **1996**; 43(Suppl): 24S–5S.
20. Saiki RK, Scharf S, Faloona F, et al. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anaemia. Science **1985**; 230:1350–4.
21. Lane BR, Ast JC, Hossler PA, et al. Dihydropteroate synthase polymorphisms in *Pneumocystis carinii*. J Infect Dis **1997**; 175:482–5.
22. Miller RF, Ambrose HE, Novelli V, Wakefield AE. Probable mother-to-infant transmission of *Pneumocystis carinii* f sp *hominis* infection. J Clin Microbiol **2002**; 40:1555–7.
23. Denis CM, Mazars E, Guyot K, et al. Genetic divergence at the SODA locus of six different *formae specialis* of *Pneumocystis carinii*. Med Mycol **2000**; 38:289–300.
24. Tsolaki AG, Miller RF, Wakefield AE. Oropharyngeal samples for genotyping and monitoring of response to treatment in AIDS patients with *Pneumocystis carinii* pneumonia. J Med Microbiol **1999**; 48:897–905.
25. Wakefield AE. DNA sequences identical to *Pneumocystis carinii* f sp *carinii* and *Pneumocystis carinii* f sp *hominis* in samples of air spora. J Clin Microbiol **1996**; 34:1754–9.
26. 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.
27. Leigh TR, Kangro HO, Gazzard BG, Jeffries DJ, Collins JV. DNA amplification by the polymerase chain reaction to detect sub-clinical *Pneumocystis carinii* colonization in HIV-positive and HIV-negative homosexuals with and without respiratory symptoms. Respir Med **1993**; 87:525–9.
28. Nevez G, Raccurt C, Jounieaux V, Dei-Cas E, Mazars E. Pneumocystosis versus pulmonary *Pneumocystis carinii* colonization in HIV-negative and HIV-positive patients. AIDS **1999**; 13:535–6.
29. Sing A, Roggenkamp A, Autenrieth IB, Heesemann J. *Pneumocystis carinii* carriage in immunocompetent patients with primary pulmonary disorders as detected by single or nested PCR. J Clin Microbiol **1999**; 37: 3409–10.
30. Armbruster C, Hassl A, Kriwanek S. *Pneumocystis carinii* colonization in the absence of immune suppression. Scand J Infect Dis **1997**; 29:591–3.
31. Calderon EJ, Regordan C, Medrano FJ, Ollero M, Varela JM. *Pneumocystis carinii* infection in patients with chronic bronchial disease. Lancet **1996**; 347:977.
32. Peters SE, Wakefield AE, Banerji S, Hopkin JM. Quantification of the detection of *Pneumocystis carinii* by DNA amplification. Mol Cell Probes **1992**; 6:115–7.
33. Wakefield AE, Miller RF, Guiver LA, Hopkin JM. Granulomatous *Pneumocystis carinii* pneumonia: DNA amplification studies on bronchoscopic alveolar lavage samples. J Clin Pathol **1994**; 47:664–6.
34. Huang L, Beard CB, Creasman J, et al. Sulfa or sulfone prophylaxis and geographic region predict mutations in the *Pneumocystis carinii* dihydropteroate synthase gene. J Infect Dis **2000**; 182:1192–8.
35. Kazanjian P, Armstrong W, Hossler PA, et al. *Pneumocystis carinii* mutations are associated with duration of sulfa or sulfone prophylaxis exposure in AIDS patients. J Infect Dis **2000**; 182:551–7.
36. Ma L, Kovacs JA. Genetic analysis of multiple loci suggests that mutations in the *Pneumocystis carinii* f sp *hominis* dihydropteroate synthase gene arose independently in multiple strains. Antimicrob Agents Chemother **2001**; 45:3213–5.
37. Nimri LF, Moura INS, Huang L, et al. Genetic diversity of *Pneumocystis carinii* f sp *hominis* based on variations in nucleotide sequences of internal transcribed spacers of rRNA genes. J Clin Microbiol **2002**; 40: 1146–51.
38. Maiden MCJ, Bygraves JA, Feil E, et al. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci **1998**; 95:3140–5.
39. Feavers IM, Gray SJ, Urwin R, et al. Multilocus sequence typing and antigen gene sequencing in the investigation of a meningococcal disease outbreak. J Clin Microbiol **1999**; 37:3883–7.
40. Hauser PM, Blanc DS, Sudre P, et al. Genetic diversity of *Pneumocystis carinii* in HIV-positive and -negative patients as revealed by PCR-SSCP typing. AIDS **2001**; 15:461–6.
41. Huang L, Friedly J, Morris AM, et al. *Pneumocystis carinii* dihydropteroate synthase genotypes in HIV-infected persons residing in San Francisco: possible implications for disease transmission. J Eukaryot Microbiol **2001**; 48(Suppl):137S–8S.