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RESPIRATORY INFECTION

Genotypic variation in *Pneumocystis jirovecii* isolates in Britain

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Background: *Pneumocystis jirovecii* is the cause of *Pneumocystis* pneumonia (PCP) in immunosuppressed humans. Asymptomatic colonisation with *P jirovecii* may occur in patients with minor immunosuppression or chronic lung disease. The aim of this study was to describe the molecular epidemiology of *P jirovecii* in Britain over a period of 12.5 years.

Methods: Between January 1989 and July 2001 161 samples of *P jirovecii* were obtained from patients with PCP (n=119), patients colonised by *P jirovecii* (n=35), and from air spora (n=6). Genotyping of samples was performed at the mitochondrial large subunit rRNA (mt LSU rRNA).

Results: Genotype 1 (38%) was the most frequently identified genotype: genotypes 2 (26.6%), 3 (20.3%), and 4 (5%) were less common. Mixed infection (more than one genotype) was identified in 10% of samples. While genotype 1 was the most frequently detected type in both patients with PCP and those colonised by *P jirovecii* (38% and 42%, respectively), these groups differed in the relatively lower rate of detection of genotype 4 (2% v 17%) and the higher detection of mixed infection in those with PCP (13% v 3%). Detection of specific genotypes of *P jirovecii* was associated with the patient's place of residence (p=0.02). There was no association between specific genotypes and severity of PCP as measured by arterial oxygen tension (p=0.3).

Conclusions: The evidence of clustering of specific genotypes with patient's postcode of residence is consistent with the hypothesis of person to person transmission of *P jirovecii* via the airborne route. The lack of association between specific mt LSU rRNA genotypes and severity of PCP suggests that this locus is not implicated in the virulence of the organism.

The organism *Pneumocystis jirovecii* (previously called *Pneumocystis carinii* f sp *hominis*) is the cause of *Pneumocystis* pneumonia (PCP) in patients immunosuppressed by HIV infection, malignancy, transplantation, and therapeutic immunosuppression.^{1,2} Among HIV infected patients in the developed world, there has been a marked reduction in the incidence of PCP as a result of the use of specific prophylaxis and of highly active antiretroviral therapy (HAART).³ However, PCP remains a common opportunistic infection, particularly among those who are unaware of their HIV serostatus at presentation or who are intolerant of or non-adherent with HAART and/or prophylaxis.⁴ In the developing world, HIV infected patients without access to prophylaxis and HAART remain susceptible to PCP.^{5,6} There is increasing evidence that *P jirovecii* may also colonise the lungs of asymptomatic individuals with minor immunosuppression induced by HIV,^{7,8} malignancy,⁹ or long term receipt of glucocorticoids,¹⁰ immune competent persons with primary chronic lung disease,^{11–13} and pregnant women.¹⁴ It is hypothesised that these groups of individuals may act as a reservoir for human infection.^{8,15}

Study of the basic biology and epidemiology of *P jirovecii* has been hampered by the lack of a reliable method for culture. Differences among isolates of *P jirovecii* have been described using analysis of genetic polymorphisms at several loci.^{5,15–17} These differences have been used in studies of the epidemiology of *P jirovecii* infection in the United States, Europe, and in Africa.^{5,11,16,18} There are few data on the epidemiology of *P jirovecii* in Britain.¹⁹ The aim of this study was to describe the molecular epidemiology of *P jirovecii* in Britain over a 12 year period. Genotyping was carried out at the mitochondrial large subunit rRNA (mt LSU rRNA). Samples were obtained from patients immunosuppressed by

HIV or other causes who had PCP, from patients colonised by *P jirovecii*, and from samples of air spora.

METHODS

Samples

Between January 1989 and July 2001, 119 bronchoscopic alveolar lavage (BAL) fluid samples were obtained from 116 patients (109 men) with PCP (defined by typical clinical and radiological presentation, identification of *P jirovecii* cysts in BAL fluid by Grocott's methenamine silver staining, and by response to specific anti-pneumocystis therapy²⁰). Of the 116 patients (115 adults), 110 were HIV infected with CD4 counts ranging from 10 to 400 cells/μl (median 60); six were not HIV infected and were immunosuppressed by malignancy (n=5) or by immunosuppressive therapy for vasculitis (n=1). Three patients with PCP underwent a second bronchoscopic examination because of failure to respond to specific treatment; the interval between bronchoscopies was 17–23 days. In patients with PCP the following information was recorded from case note review: date of diagnosis of PCP, postcode of residence, admission oxygenation (PaO₂ breathing room air), and outcome.

Thirty six BAL fluid samples were from adult patients who did not have PCP and who were colonised with *P jirovecii*. Nineteen of these patients were HIV infected men with CD4 counts ranging from 10 to 370 cells/μl (median 20). All patients had alternative diagnoses, as defined previously;⁸ bacterial pneumonia in seven, pulmonary Kaposi sarcoma in five, cytomegalovirus pneumonitis in three, and *Mycobacterium avium-intracellulare* in three. Seventeen patients (14 men) were not HIV infected; their clinical details have been described

†Deceased.

elsewhere.¹⁰ Six samples of *P jirovecii* were obtained from air samples collected from spore traps situated in a rural environment, as previously reported.²¹ Thus, a total of 161 isolates of *P jirovecii* were studied. All patients undergoing bronchoscopy gave informed written consent and the study was performed within the guidelines of the Middlesex Hospital and Central Oxford Research Ethics Committees.

At the time of the diagnostic bronchoscopy an aliquot of BAL fluid was frozen at -20°C and subsequently transferred to the Weatherall Institute of Molecular Medicine, Oxford, for analysis. The BAL fluid samples were coded and analyses were carried out blind to the patients' details.

DNA extraction and amplification

P jirovecii DNA was extracted from BAL fluid samples as previously described.^{8 22-24} DNA amplification in patients with PCP was done using single round polymerase chain reaction (PCR) with primers pAZ102-H and pAZ102-E. In patients who were colonised with *P jirovecii* and for air samples nested PCR was carried out. In the first round conditions were as above, and in the second round the primers pAZ102-X and pAZ102-Y were used.^{8 22-24} Extreme caution was taken to prevent cross contamination of samples. For example, DNA extraction was performed in a separate room and PCR amplification and DNA sequencing were carried out in different areas of the laboratory. At each stage handling of samples was done in a laminar flow cabinet and a new set of disposable-tip micropipettes, tubes, and reagents was used for each experiment. In each DNA extraction and PCR amplification experiment negative controls (ultrapurified distilled water instead of *P jirovecii* DNA) were included. As a positive control, *P jirovecii* DNA from a patient with PCP was used in each PCR amplification experiment.

Sequencing of *P jirovecii*

Amplification products were purified and either cloned and sequenced, or sequenced directly, as previously described.^{5 8 22} DNA sequence data were analysed either with Chromas 1.62 software (Technelysium, Pty, Tewantin, Australia) and University of Wisconsin Computer Group software version 10.1 (Genetics Computer Group, Wisconsin, USA), or with Gap4 of the Staden package (MRC Rosalind Franklin Centre for Genomics Research, Cambridge, UK). Genotypes of *P jirovecii* were distinguished by identifying polymorphisms at position 85 and 248 of the mt LSU rRNA and were numbered using the method described by Beard *et al.*¹⁶

Statistical analysis

The χ^2 test and the analysis of variance F test were used for unadjusted tests of association between genotype of *P jirovecii* and categorical and continuous factors, respectively. Multinomial logistic regression with genotype as the outcome

was used to simultaneously examine the association of multiple factors. Mixed genotypes were considered as one category for analysis. In the three patients with PCP who underwent repeat sampling, only the first sample was used for genotypic analysis. Area of residence was assigned from postcodes into six categories: North, South, East and West London, Oxford region and "other". For associations with time a linear trend was tested, and "clustering by time" was tested by grouping time based on frequencies as 1989-91, 1992-3, 1994-6, and 1997-2001. A p value of <0.05 was considered significant. Statistical analyses were performed using STATA Version 8.

RESULTS

Genotype 1 (38%) was the most common type identified; genotypes 2 (26.6%), 3 (20.3%), and 4 (5%) were less common. In 10% of samples co-infection with two genotypes (mixed infection) was detected (table 1).

Among the limited number of air spora samples, genotype 2 (50%) was most frequently identified (fig 1), whereas mixed infection and genotype 4 were not identified. While genotype 1 was the most frequently detected type among both patients with PCP and in those colonised by *P jirovecii* (38% and 42% respectively, table 1), these two groups differed significantly ($p = 0.004$) in their genotype distribution due to the relatively lower detection of genotype 4 (2% v 17%) and higher detection of mixed infection among those with PCP (13% v 3%). The difference remained significant after adjusting for year of sample.

In patients with PCP, analysis of the association between detection of specific genotypes and year of diagnosis of PCP showed little association ($p = 0.31$, linear trend and $p = 0.12$, test for "time clustering"). Detection of specific genotypes of *P jirovecii* was associated with patient's place of residence ($p = 0.02$, fig 1). For example, among 28 patient samples from South London, 17 (61%) were genotype 1, but only one (4%) was genotype 3 and two (7%) were mixed infection. By contrast, of 36 samples from North London, eight (22%) were genotype 1, nine (25%) were genotype 3, and nine (25%) were mixed infection.

All patients with PCP survived; there was no association between specific genotypes of *P jirovecii* and severity of PCP as measured by Pao_2 ($p = 0.30$). In the three patients with PCP who underwent a repeat bronchoscopy, the same genotype of *P jirovecii* (type 2 in two patients and type 3 in one) was identified in the first and second BAL fluid samples. Genotype 1 was detected in the child with PCP.

DISCUSSION

This study from Britain is the first to describe genotyping of isolates of *P jirovecii* obtained contemporaneously from

Table 1 Genotypes of *P jirovecii* at the mt LSU rRNA locus in patients with PCP, patients colonised with *P jirovecii*, and in air spora

Genotype	Sample			Total	%
	PCP	Colonised	Air spora		
1	44	15	1	60	38
2	30	9	3	42	26.6
3	25	5	2	32	20.3
4	2	6	0	8	5.1
Mixed					
5 (1+3)	9	0	0	9	5.6
6 (1+2)	3	0	0	3	1.9
8 (1+4)	2	1	0	3	1.9
9 (2+3)	1	0	0	1	0.6
Total	116	36	6	158	100

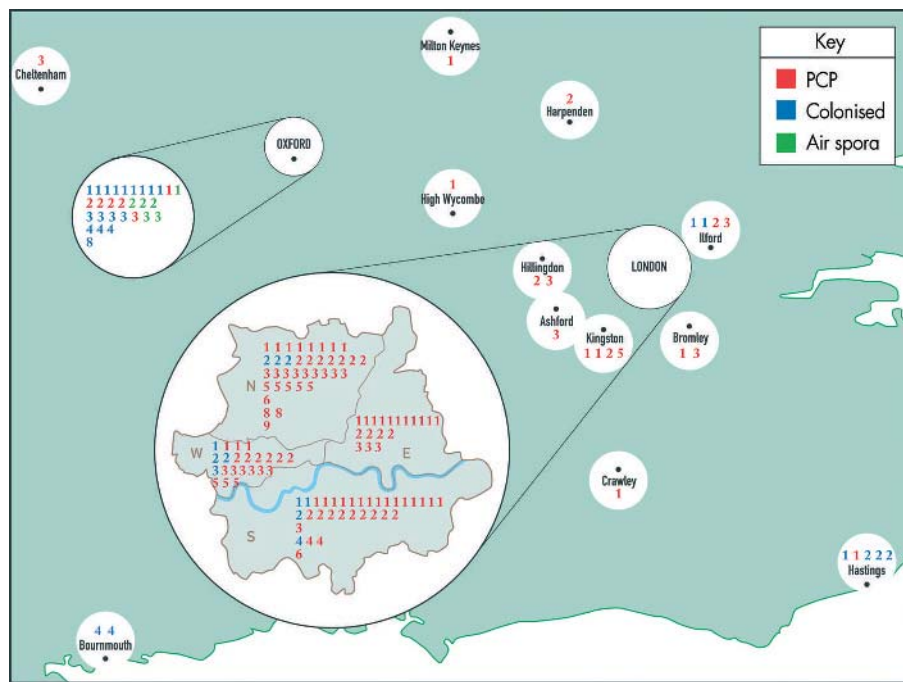


Figure 1 Map showing the geographical location of isolates of genotypes of *Pneumocystis jirovecii* in samples from patients with PCP (n = 116), patients colonised with *P jirovecii* (n = 36), and from air spora (n = 6).

patients with PCP, from patients colonised by *P jirovecii*, and from air spora. Analysis was performed at the mt LSU rRNA, which is an informative locus not under selective pressure from sulpha drug exposure that has been used previously in epidemiological studies.^{11–16} Of five different genotypes previously described at this locus, four were identified in this study. Use of single round PCR enabled detection of *P jirovecii* DNA in respiratory samples from patients with PCP, whereas nested PCR was needed to detect *P jirovecii* DNA in samples from those colonised by *P jirovecii* and in air spora, reflecting the lower levels of DNA in these samples.⁸

Genotype 1 was the commonest genotype identified in this study, accounting for 38% of isolates. Similar results have been reported in a multicentre study of 324 patients with PCP from the United States (genotype 1 frequency 38%)¹⁶ and a study of 79 isolates from patients with PCP and patients colonised by *P jirovecii* from Seville, Spain (genotype 1 frequency 49.3%).¹¹ By contrast, a preliminary study of 14 patients with PCP from Zimbabwe reported genotype 1 in only 28.6%; genotype 3 was the most frequently identified (57%).⁵ In the present study genotype 3 accounted for 20.3% of isolates, which contrasts with results from the USA (9.3% genotype 3)¹⁶ and from Seville (36.7% genotype 3).¹¹ Mixed infections (>1 genotype in a sample) were identified in 10% of isolates in this study; a similar rate of detection was reported in the study from USA (10.2%).¹⁶ This rate is almost three times greater than the rate of detection (3.7%) of mixed infection in the Spanish study.¹¹ The absence of change in the frequency of detection of different genotypes over the 12.5 years of the study suggests stability of this genetic locus and demonstrates its usefulness in longitudinal epidemiological studies of *P jirovecii* infection.

The distribution of genotypes in samples from patients with PCP and those colonised by *P jirovecii* differed significantly but also showed some similarities—for example, genotype 1 being most common. The distribution of genotypes in air spora was somewhat different, but the number of samples was limited. These genotypic data are consistent with the hypothesis that *Pneumocystis* is

transmitted via the airborne route from person to person. Further evidence from this study—which supports the hypothesis that PCP arises by de novo acquisition of infection—comes from the observation that, in patients with PCP, specific genotypes of *P jirovecii* were associated with residential postcode. Geographical clustering of cases of PCP by postcode (zip code) has previously been described in studies from Cincinnati²⁵ and San Francisco.^{26–27} Recent acquisition of infection may also be inferred from the finding that allelic variation patterns in isolates of *P jirovecii* from patients with PCP are correlated with patient's place of diagnosis and not their place of birth.¹⁶

This study found no correlation between specific genotypes of *P jirovecii* at the mt LSU rRNA locus and severity of PCP, as judged by the patient's admission Pao₂ (breathing room air). This observation corroborates the results of another study that showed a lack of association between severity of PCP (using admission Pao₂) and specific genotypes of *P jirovecii* at the internal transcribed spacer (ITS) regions of rRNA.²⁸ These data suggest that the genotype of *P jirovecii* at both the mt LSU rRNA and ITS loci does not influence PCP disease severity.

In the three patients who underwent repeat BAL during an episode of PCP because of failure to respond to specific treatment, there was no change in genotype at the mt LSU rRNA which suggests that *P jirovecii* does not mutate in the human host during an episode of PCP. Our observations confirm previous reports of four patients in the USA²⁹ and 10 in France³⁰ in whom no change in genotype was observed in patients with PCP who underwent repeat BAL after an interval of 7–30 days.

This study has shown the usefulness of genotyping at the mt LSU rRNA for describing the molecular epidemiology (particularly in longitudinal studies) of isolates of *P jirovecii* from patients with PCP, those colonised with the organism, and in samples of air spora. The genotype distribution in isolates from all three groups had similarities consistent with the concept of person to person transmission via the airborne route. An association between patient's postcode of residence

and specific genotypes further supports the concept of "recent" acquisition of infection. The lack of association between disease severity and genotype suggests that other loci are involved in determining the virulence of this organism and indicates that prospective studies are needed to address these issues.

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