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# *Pneumocystis jirovecii* colonisation in patients with interstitial lung disease

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## ABSTRACT

A prospective study was conducted to determine the prevalence of colonisation by *Pneumocystis jirovecii* in 80 consecutive patients who required bronchoscopy and bronchoalveolar lavage (BAL) following suspicion of interstitial lung disease (ILD). The mtLSU rRNA gene of *P. jirovecii* was identified by nested PCR in BAL samples. Patients with ILDs were divided into three groups: group A comprised those with idiopathic interstitial pneumonias; group B comprised those with sarcoidosis; and group C comprised those with other ILDs. The overall prevalence of *P. jirovecii* carriage was 33.8%, with colonisation rates of 37.8%, 18.8% and 37% in groups A, B and C, respectively (p not significant). There were more smokers among the carriers, but there were no other significant differences between carriers and non-carriers. The high prevalence of *P. jirovecii* carriers found among immunocompetent patients with ILDs in Spain suggests a possible role of *P. jirovecii* in the natural history of these diseases.

**Keywords** Bronchoalveolar lavage, carriage, epidemiology, interstitial lung disease, *Pneumocystis jirovecii*

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## INTRODUCTION

*Pneumocystis jiroveci* (formerly *Pneumocystis carinii* f. sp. *hominis*) [1] is an atypical fungus with pulmonary tropism that is considered to be an opportunistic fungal pathogen causing severe pneumonia in immunocompromised individuals, principally AIDS patients. However, cases of *Pneumocystis* pneumonia have been described in individuals without predisposing illness [2,3], and an increasing number of reports have described the occurrence of asymptomatic pulmonary colonisation by *P. jirovecii* in immunocompetent hosts with underlying pulmonary disease [4–7]. Interstitial lung diseases (ILDs) account for *c.* 15% of respiratory disease, and are associated with a substantial degree of morbidity and mortality [8]. ILDs include a heterogeneous group of disorders of the lower respiratory tract, most with unknown causes, in which injury to the lung parenchyma leads to a cascade of inflammatory and immune processes that, in many cases, cause lung fibrosis. The exact mechanism by which connective tissue proliferation occurs in some cases is unknown, although infectious agents have been implicated in the pathogenesis of ILDs [9,10]. Thus, in animal models, it has been demonstrated that low numbers of *Pneumocystis* organisms induce the activation of alveolar macrophages, increase levels of pro-inflammatory interleukins, and cause changes in pulmonary surfactant components [11,12]. This pulmonary inflammation, together with the adaptive immune response triggered by alveolar injury caused by *Pneumocystis*, could play a role in the pathogenesis of ILDs and in the progression of the disease to fibrosis.

The aim of the present study was to determine the prevalence of *P. jirovecii* colonisation by using PCR to detect *P. jirovecii* DNA in bronchoalveolar lavage (BAL) fluid from a large population of patients with ILD.

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## PATIENTS AND METHODS

### Patients

Between 2001 and 2003, 120 consecutive patients attending the bronchoscopy unit with suspicion of ILDs were studied. A complete clinical, functional and radiological evaluation was made of all patients. Diagnostic bronchoscopy with BAL fluid examination was also performed. Informed consent was obtained from all patients included in the study. Exclusion criteria were underlying malignant disease, autoimmune disorders, immunosuppressive therapy (steroids and cytotoxic treatment) and a final diagnosis that did not include ILD. None of the patients was known to be positive for human immunodeficiency virus. The final study population included 80 newly diagnosed patients with untreated ILDs. The study was approved by the hospital's ethical committee.

### Diagnostic criteria

For practical reasons, the patients were divided into three groups: group A comprised patients with idiopathic interstitial pneumonias; group B comprised patients with sarcoidosis; and group C comprised patients with other ILDs. Group A patients, according to the classification proposed by the American Thoracic Society (ATS) and the European Respiratory Society (ERS) Consensus [13], comprised: patients with idiopathic pulmonary fibrosis, non-specific interstitial pneumonia, cryptogenic organising pneumonia, acute interstitial pneumonia, desquamative interstitial pneumonia and lymphoid interstitial pneumonia. In seven of the 18 patients with idiopathic pulmonary fibrosis, the diagnosis was established by evidence of interstitial pneumonia in histological lung specimens. In the absence of histology, the diagnosis of idiopathic pulmonary fibrosis was established according to the clinical diagnostic criteria proposed by the ATS/ERS Consensus Statement [14]. For all other disorders considered as interstitial idiopathic pneumonias, the diagnosis was confirmed histologically. For patients in group B, the diagnosis was based on histological evidence of non-caseating granulomas in lung specimens for 13 of 16 patients, with the diagnosis established for the remaining three patients by consistent clinical features and BAL fluid analysis, according to the ATS/ERS/World Association of Sarcoidosis and Other Granulomatous Disorders (WASOG) guidelines [15]. Patients in group C had a range of disorders, including Langerhans' cell pulmonary histiocytosis, silicosis, extrinsic allergic alveolitis, pulmonary alveoli calcification of parathyroid origin, drug-induced pneumonitis, chronic eosinophilic pneumonia, allergic bronchopulmonary aspergillosis, diffuse alveolar haemorrhage and non-classified interstitial pneumonia. Disorders with diffuse interstitial damage, but with characteristic clinical features, were included as non-classified interstitial pneumonia. For patients in group C without histological confirmation, the final diagnosis was established by criteria reported previously for each disease. In all cases, other disorders with a similar clinical picture were excluded.

### Data and specimen collection

General data, including age, gender, smoking habit and number of pack-years smoked, were recorded. Peripheral blood lymphocytes and BAL specimens were obtained from the patients.

### Bronchoalveolar lavage procedure

Three 50-mL aliquots of sterile saline (0.9% w/v) were instilled in the third-generation bronchus of the middle lobe, lingula, or the area containing most lung infiltrates, under local anaesthesia, with a fibreoptic bronchoscope (Type 40; Olympus, Tokyo, Japan). Each aliquot was recovered immediately by suction. Samples for bacterial, fungal and parasitological analysis were transported immediately to the laboratory in sterile containers. Total cells were counted with a Neubauer haemocytometer counting chamber. Differential cell counts were performed after staining with modified May–Giemsa–Grünwald (Diff-Quick) stains. Lymphocyte sub-populations were analysed by immunocytochemical techniques with monoclonal antibodies against $CD4^+$ and $CD8^+$ T-cells only for those patients whose lymphocyte percentage was >15%.

Two techniques were used to identify *P. jirovecii* in BAL samples:

1. *Conventional technique*. Microscopy was performed using a conventional stain and an immunofluorescence assay with monoclonal antibodies against *P. jirovecii* (Dako, Glostrup, Denmark).
2. *Molecular analysis.* DNA was extracted using a QIAamp tissue kit (Qiagen, Hilden, Germany), and the pneumocystis mitochondrial region (mtLSU rRNA) was amplified using nested PCR [16]. Briefly, in the first amplification round, external primers pAZ102 (5′-GATGGCTGTTTCC-AAGCCCA-3′-E) and pAZ102-H (5′-GTGTACGTT-GACAAGTACTC-3′) were used to give a 346-bp fragment. The second round of amplification utilised the internal primers pAZ102-X (5′-GTGAAATACAAATCGGACTA-GG-3′) and pAZ102-Y (5′-TCACTTAATATTAATTGGG-GAGC-3′) to give a 260-bp product. Both rounds comprised 40 cycles of amplification. The amplification products were visualised by UV light following agarose gel electrophoresis and staining with ethidium bromide. Standard precautions and controls were included to prevent false-positive reactions caused by contamination. All experiments were performed at least twice.

### Definition of a *P. jirovecii* carrier

A *P. jirovecii* carrier was defined as an individual without *P. jirovecii* pneumonia from whom a BAL specimen was obtained that contained *P. jirovecii* DNA detectable by nested PCR in two independent analyses.

### Statistical analysis

SPSS v. 12.0 (SPSS Inc., Chicago, IL, USA) was used for analysis, with $p \leq 0.05$ considered to be significant. Data were expressed as means (SD) or medians with 25–75% interquartile range in accordance with the normal or non-normal distributions. Comparisons between carriers and non-carriers were tested using chi-square or Fisher's exact test for qualitative variables, and Student's *t*-test or Mann–Whitney *U*-test for quantitative variables.

## RESULTS

PCR for *P. jirovecii* was positive for 27 (33.8%) patients (carriers) and negative for 53 (66.2%)

patients (non-carriers). In seven (8.8%) carriers, conventional techniques also yielded positive results for *P. jirovecii*. The baseline characteristics and lymphocyte counts of all patients are summarised in Table 1. The carrier group had significantly higher proportions of men and smokers than the non-carrier group (p 0.04 and p 0.02, respectively). The median total lymphocyte count was significantly lower in carriers than in non-carriers (p 0.04). Table 2 shows the numbers of colonised and non-colonised patients in groups A, B and C. There were no significant differences in the prevalence of *P. jirovecii* colonisation between the three groups (p 0.36).

No differences in gender or age were found when carriers and non-carriers of each subgroup were analysed, but more carriers were smokers, and the median of the total lymphocyte counts was lower for carriers than for non-carriers; however, these differences were only statistically significant in groups C and A (p 0.018 and p 0.03, respectively).

In the overall group, the BAL parameters did not differ significantly between carriers and non-carriers, but the percentage of $CD4^+$ T-cells and the CD4/CD8 index were lower in carriers than in non-carriers, although the differences did not reach statistical significance (p 0.13 and p 0.07, respectively; Table 3). The BAL analysis did not differ significantly between carriers and non-carriers in the subgroups.

None of the patients had received oral corticosteroids at the time of bronchoscopy. All 27 carrier patients were human immunodeficiency virus-negative, and none developed *Pneumocystis* pneumonia during a 1-year follow-up period.

**Table 1.** Baseline characteristics of total population and comparison of *Pneumocystis jirovecii* non-carriers and carriers

| | Total ($n$ = 80) | Non-carriers ($n$ = 53) | Carriers ($n$ = 27) | p |
|---|---|---|---|---|
| Median age, years (range) | 58 (42–68) | 59 (42–70) | 56 (41–70) | 0.14[a] |
| Males, $n$ (%) | 53 (66.3) | 31 (58.5) | 22 (81.5) | 0.04[b] |
| Smokers, $n$ (%) | 45 (56.3) | 25 (47.2) | 20 (74.1) | 0.02[b] |
| Median lymphocytes/mm$^3$ (range) | 2200 (1520–3000) | 2455 (1800–3500) | 1850 (1400–2402) | 0.04[a] |

[a]Student's *t*-test.
[b]Chi-square test.
p < 0.05 was considered to be statistically significant.

**Table 2.** Number of *Pneumocystis jirovecii* non-carriers and carriers according to the interstitial lung disease groups

| | Non-carriers $n$ = 53 (66.2%) | Carriers $n$ = 27 (33.8%) |
|---|---|---|
| Group A: IIPs | | |
| IPF | 13 | 5 |
| NSIP | 2 | 2 |
| COP | 5 | 5 |
| AIP | 0 | 2 |
| DIP | 2 | 0 |
| LIP | 1 | 0 |
| Total ($n$ = 37) | 23 (62.2%) | 14 (37.8%) |
| Group B: sarcoidosis ($n$ = 16) | 13 (81.3%) | 3 (18.8%) |
| Group C: other ILDs | | |
| Pulmonary Langerhans' cell histiocytosis | 1 | 2 |
| Silicosis | 1 | 1 |
| Pulmonary calcification | 1 | 0 |
| Extrinsic allergic alveolitis | 2 | 0 |
| Amiodarona-induced pneumonitis | 2 | 1 |
| Chronic eosinophilic pneumonia | 0 | 2 |
| Allergic bronchopulmonary aspergillosis | 1 | 0 |
| Diffuse alveolar haemorrhage | 1 | 0 |
| NCIPs | 8 | 4 |
| Total ($n$ = 27) | 17 (63%) | 10 (37%) |

ILD, interstitial lung disease; IIPs, idiopathic interstitial pneumonias; IPF, idiopathic pulmonary fibrosis; NSIP, non-specific interstitial pneumonia; COP, cryptogenic organising pneumonia; AIP, acute interstitial pneumonia; DIP, desquamative interstitial pneumonia; LIP, lymphoid interstitial pneumonia; NCIPs, non-classified interstitial pneumonias.

**Table 3.** Bronchoalveolar lavage differential cell counts in non-carriers and carriers

| | Non-carriers $n$ = 53/46[a] | Carriers $n$ = 27/26[a] | p |
|---|---|---|---|
| Macrophages, % (range) | 56 (28–78) | 62 (33–78) | 0.71 |
| Lymphocytes, % (range) | 29 (10–48) | 21 (7–36) | 0.09 |
| Neutrophils, % (range) | 4 (1–12) | 3 (2–16) | 0.84 |
| Eosinophils, % (range) | 1 (0–5) | 1 (0–6) | 0.75 |
| T-lymphocyte sub-populations | $n$ = 25 | $n$ = 9 | |
| CD4, % (range) | 51 (22–79) | 32 (15–48) | 0.13 |
| CD8, % (range) | 38 (16–65) | 42 (28–64) | 0.62 |
| CD4/CD8 index | 2 (0.4–4) | 0.5 (0.2–1.3) | 0.07 |

[a]Number of patients in which BAL was valid for cellular count.
Values are median (25–75% interquartile range).
p < 0.05 was considered to be statistically significant (Mann–Whitney *U*-test).

## DISCUSSION

The present study found a high percentage (33.8%) of *P. jirovecii* colonisation in patients with ILDs, with no differences in the prevalence of colonisation among different kinds of ILD. In the absence of a reliable method for *P. jirovecii* culture, the use of PCR allowed the detection of low levels of microorganisms in lung samples from asymptomatic subjects. Early studies on post-mortem lungs from immunocompetent individuals failed to detect *Pneumocystis* [17,18], although more recent studies have demonstrated that *Pneumocystis* DNA can be detected in healthy infants and healthy adults [19,20].

*Pneumocystis* carriage has been shown to be common in immunocompetent patients with

underlying pulmonary disease. In patients with chronic pulmonary disease, the apparent rate of *P. jirovecii* colonisation ranges from 7% to 41%, depending upon the precise disease studied and the diagnostic technique used [4,7,16]. A recent study has suggested that oral glucocorticoid therapy is an independent risk-factor for colonisation [21], but none of the patients included in the present study had received oral corticosteroids at the time of bronchoscopy, and only two patients had been prescribed inhaled steroids.

There are only two previous reports regarding the prevalence of *P. jirovecii* in ILDs, as a subgroup of respiratory diseases [5,6], and the present study is the first to include a relatively large number of patients with ILDs. A predominance of males was observed among *P. jirovecii* carriers, associated with a higher percentage of smokers in the carrier group. Previous studies have demonstrated that cigarette consumption is an independent risk-factor for *Pneumocystis* colonisation in subjects infected with human immunodeficiency virus [22]. The exact mechanism by which smoking facilitates the infection is unknown, but it has been demonstrated that mucociliary clearance and pulmonary surfactant are deficient in smokers [23,24]. Thus, smoking could modify the pulmonary host defence against the microorganism, thereby facilitating colonisation.

In carrier patients, the peripheral blood lymphocyte levels were lower than in non-carriers. These results differ from those in a study of patients with chronic obstructive pulmonary disease, which showed a higher total lymphocyte count in carrier patients [25]. This finding might be related to the frequent infections caused by other pathogens in patients with chronic obstructive pulmonary disease. In the present study, the reduced lymphocyte count or lymphopenia observed in colonised patients might facilitate the carrier status [26].

The differential cell counts in the BAL fluid revealed a low CD4/CD8 ratio in carrier patients, mainly because of a decreased percentage of $CD4^+$ T-cells. Previous studies have demonstrated a correlation between a specific BAL fluid cellular profile and the presence of infection caused by *Pneumocystis*. A study performed with simian immunodeficiency virus-infected macaques colonised by *Pneumocystis* found that infected animals had an increased $CD8^+$ T-cell count in BAL fluid (>90%), compared with simian immunodeficiency virus-infected animals without *Pneumocystis* colonisation [27]. In the same study, BAL fluid neutrophilia was correlated with progression of disease.

A methodological limitation of the present study was the absence of a control group, since obtaining lung samples by invasive techniques, such as fibrobronchoscopy, from healthy individuals is not ethically acceptable. Nevertheless, other studies that have evaluated the implications of sub-clinical *P. jirovecii* infection in the pathogenesis of chronic lung disease have suggested that *P. jirovecii* colonisation could accelerate the progression of the bronchial obstruction in chronic obstructive pulmonary disease [28], and that there is a relationship between asymptomatic colonisation and the presence of small-cell lung carcinomas [29].

The present study detected a high prevalence of *P. jirovecii* carriers among a large number of immunocompetent patients with ILDs, but no differences were found in the rates of colonisation according to each individual pathology. More extensive studies will be necessary to determine whether the increased inflammatory response caused by *P. jirovecii* in the lung parenchyma is linked to the development and/or the progression of these diseases.

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