High prevalence of *Pneumocystis jirovecii* colonization in Brazilian cystic fibrosis patients

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A high rate of *Pneumocystis jirovecii* colonization was observed in Brazilian cystic fibrosis (CF) patients (13 out of 34; 38.2%) who underwent bronchoscopy between March 2006 and August 2009 at the Hospital de Clínicas de Porto Alegre, Brazil. Bronchoalveolar lavage samples were collected from these patients and studied by nested PCR amplification of the mitochondrial gene coding for the large subunit ribosomal RNA (mtLSUrDNA). The observed rate of colonization was higher than that reported in European populations. Genotypic characterization of the mtLSU rDNA locus revealed a predominance of the polymorphisms 85C/248C (genotype 1) and 85T/248C (genotype 3), with all samples possessing the wild-type genotype of dihydropteroate synthase. These findings suggest that cystic fibrosis patients could be an important reservoir and source of *P. jirovecii* infection. Further studies are required to elucidate the role of this common fungal colonization in the evolution of CF patients.

**Keywords:** *Pneumocystis jirovecii*, cystic fibrosis, colonization

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**Introduction**

Cystic fibrosis (CF) is the most common life-shortening autosomal recessive disorder in Caucasian populations. A recent study found that CF occurs approximately once in every 1,587 live births in southern Brazil [1]. Understanding the microbial flora of the CF respiratory tract is of considerable importance, as the morbidity and death of CF patients are primarily caused by chronic respiratory infections. However, chronically colonized airways of these patients represent a surprisingly complex and diverse ecosystem [2].

*Pneumocystis jirovecii* is an atypical opportunistic fungus with apparent lung tropism and worldwide distribution that causes pneumonia in immunosuppressed persons. *Pneumocystis* colonization has recently been described in subjects with various lung diseases, and accumulating evidence suggests that this colonization may be an important clinical phenomenon [3]. Only a few European studies have evaluated the prevalence of *Pneumocystis* colonization in patients with CF, reporting ranges from 1.3–21.5% [4–6] and there is no information about *Pneumocystis* colonization in CF patients outside of Europe. In this investigation, we examined the prevalence of *P. jirovecii* colonization and the distribution of the gene(s) encoding the mitochondrial large subunit ribosomal RNA (mtLSU rDNA) genotypes of *P. jirovecii* among CF patients in Brazil.

**Materials and methods**

**Patients**

This study included 34 CF patients who were admitted consecutively between March 2006 and August 2009 to the bronchoscopy unit for evaluation of their disease at
Hospital de Clínicas de Porto Alegre, southern Brazil. In all cases, informed consent was obtained from the patients or their guardians. The study was approved by the hospital’s ethics committee. All patients underwent clinical, functional, and radiological evaluations. For each patient, epidemiological information was obtained by review of medical charts and included age, sex, height, weight, bacterial colonization in the last three months, pancreatic enzyme use, diabetes mellitus diagnosis, percentage from predicted forced expiratory volume in one second (FEV1-%), and the number of hospital admissions over the previous year. We noted any prior treatment with sulfadiazines or azithromycin, which was defined as the prescription of any of these agents, regardless of duration of use, during the six months before the bronchoscopy study. The weight-for-age Z-score was used to identify cases of moderate to severe malnutrition [7]. We also performed diagnostic bronchoscopy with bronchoalveolar lavage (BAL) fluid examination.

Detection of \textit{P. jirovecii}

\textit{Pneumocystis jirovecii} detection was carried out by analyzing BAL samples with nested PCR amplification of the mtlSUrDNA gene of \textit{Pneumocystis}. After samples were digested with proteinase K at 56°C, DNA was extracted from the BAL samples using a commercial kit (QIAamp DNA mini kit; Qiagen, Hilden, Germany) and the gene encoding the mtlSUrRNA was amplified. A two-step protocol was used for nested PCR which included in the first amplification round, the use of 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’) to obtain a 346 bp fragment. The second round of amplification utilized the primers pAZ102-X (5’-GTG AAA TCT AAT TTA AAT C-3’) and pAZ102-Y (5’-TCA CTT AAT ATT TAC TGG GGA GC-3’) to amplify a 370 bp fragment. The PCR product was divided into three aliquots. One was used to confirm the presence of a 370-bp fragment from the DHPS gene. The other two aliquots were used to identify mutations in codons 55 and 57 by RFLP with the restriction enzymes AccI and HaeIII (Roche Diagnostics, Mannheim, Germany), respectively.

Statistical analysis

Statistical analysis was performed using SPSS software, version 15.0 (SPSS, Chicago, IL, USA). Student’s \( t \)-test, the Chi-square test with Yates’ correction, Fisher’s exact test or the Mann-Whitney U-test were used to compare the characteristics of colonized and non-colonized cases, and \( P < 0.05 \) was regarded as statistically significant. The variance test was used to confirm a normal distribution.

Results

The study included 34 patients (17 males and 17 females; median age 11.0 years, range 1–35 years) from whom BAL samples were collected. Seven patients (20.5%) had a diagnosis of diabetes mellitus, and 27 patients (79.4%) suffered from pancreatic insufficiency. Moderate or severe malnutrition was found in 10 patients (29.4%). Eight patients (23.5%) had had three or more CF-related hospitalizations
in the previous year. Five patients (14.7%) had received azithromycin or co-trimoxazole in the last six months, and bacterial colonization was identified in 29 patients (85.2%) during the previous three months. The patients enrolled in the study had a mean of FEV1-% of 69.3 ± 15.9%.

*P. jirovecii* colonization was detected in 13 out of 34 CF patients (38.2%; CI 95%: 22–56%). Standard cytological staining was negative in all cases and none of the CF patients who tested positive for *P. jirovecii* DNA suffered from overt *Pneumocystis* pneumonia.

Twelve of the positive samples yielded typing results for positions 85 and 248 of the mtLSU-rDNA gene. Genotype 1 (85C/248C) of the mtLSU-rDNA gene was observed in five cases (41.6%; CI 95%: 15–72%), genotype 2 (85A/248C) in two patients (16.6%; CI 95%: 2–48%), genotype 3 (85T/248C) in three cases (25.0%; CI 95%: 5–57%), and mixed genotype 1 and 3 in two cases (16.6%; CI 95%: 2–48%) (Fig. 1). The wild-type DHPS genotype (threonine55/proline57) was detected in all samples.

The clinical and demographic data of the patients included in the study are showed in Table 1 according to *P. jirovecii* colonization status. Patients with CF who were colonized by *P. jirovecii* had a higher frequency of *P. aeruginosa* infection than did non-colonized subjects, and this difference was statistically significant (*P* = 0.02). None of the other prognostic indicators, e.g., number of CF-related hospitalizations and nutritional characteristics, was associated with *P. jirovecii* colonization in this study. Patients with poor nutritional status had a higher prevalence of colonization, but this difference was not statistically significant.

**Discussion**

The results of our study reveal, for the first time, the high prevalence of *P. jirovecii* colonization in Brazilian patients with CF and show an association between *P. jirovecii* colonization and *P. aeruginosa* infection in patients with this disease, suggesting a possible interaction between both pathogens, which could be of important medical significance in CF progression.

In our study, we found a prevalence of *P. jirovecii* colonization of 38.2% among CF-patients, whereas studies in Spain, Germany and France showed rates of 21.5%, 7.4% and 1.3%, respectively [4–6].

Prevalence of *P. jirovecii* colonization among CF patients is significantly higher in Brazil than in Germany (7.4% vs. 38.2%; *P* = 0.0001) or France (1.3% vs. 38.2%; *P* < 0.00001), but similar to prevalence among Spanish patients (21.6% vs. 38.2%; *P* = 0.1).

There are several factors that could explain the high rate of *P. jirovecii* colonization observed in Brazilian CF patients. The incidence of acquired immunodeficiency syndrome (AIDS)-related *P. jirovecii* pneumonia (PcP) in a particular geographic region could contribute to the rate of colonization by this fungus in CF patients [6]. Currently there is evidence that the transmission of *P. jirovecii* occurs by airborne route from person to person [10] and that encounters between infected and non-infected persons could be more likely in areas with high incidence of AIDS-related PcP [11]. Between 1980 and June 2010, 592,914 AIDS cases were reported in Brazil [Epidemiological Bulletin, Ministry of Health of Brazil, 2010]. Moreover, in Brazil, PcP was reported to be the second most common AIDS-defining condition after the introduction of combined antiretroviral therapy [12].

Several authors have shown seasonal changes in PcP incidence that seem to be associated with climate factors [13–16]. While previous studies showed PcP incidence to be maximal during the winter months [13–15], a recent study revealed the opposite pattern, with PcP incidence being maximal in the summer months [16]. Southern Brazil is a subtropical region, with an average yearly temperature of 18°C (similar to Seville, 18.6°C), and both areas had a high prevalence of *P. jirovecii* colonization suggesting that this situation could be more frequent in countries with warmer climates. This idea is consistent with results from a previous study that showed a higher rate of colonization in chronic obstructive pulmonary disease (COPD) patients from Sevilla (southern Spain) than from Amiens (northern France) [17]. Further studies are needed to define the relationship between climate and the prevalence of colonization by *P. jirovecii*.

Differences in colonization rates might also be related to the different types of clinical specimens that were analyzed. While previous authors described results from analyses of sputum or oropharyngeal washes (OW), the presented study used BAL samples. BAL samples provide the highest sensitivity for the diagnosis of PcP by

![Fig. 1. Rates of genotypes at the mtLSU-rDNA *Pneumocystis jirovecii* locus from 34 Brazilian cystic fibrosis patients.](image-url)
<p>molecular methods [18]. Therefore, BAL specimens probably have the greatest capability to detect <em>P. jirovecii</em> colonization using PCR.</p>

It has been reported that the patient’s age may influence colonization by <em>P. jirovecii</em> in CF patients. Patients aged less than 18 years had a higher rate of colonization than patients over this age, as reported by Respaldiza et al. [4]. The median age of the cases studied was lower in Brazilian patients (11.0 y, this study) than in the Spanish (15.8 y), German (18.5 y) and French (23.2 y) studies. It is known that the spectrum of pathogens in CF varies with the age of the patient. In children, the most frequent bacterial colonization is by pathogens such as <em>Staphylococcus aureus</em> and <em>Haemophilus influenzae</em>. Later, there is frequent colonization by <em>Pseudomonas aeruginosa</em> [2]. The findings of our study might indicate that colonization by the fungus <em>P. jirovecii</em> is more frequent in younger CF patients. However, further studies are needed to clarify this issue.</p>

In addition, variations in the rates of colonization could be related to differences in prior exposure to co-trimoxazole or azithromycin to prevent bacterial infections among the CF patients studied [11]. Among the Brazilian patients in this study, only 14.7% (5/34) had used these drugs in the six months prior to bronchoscopy. The high rate of <em>P. jirovecii</em> colonization could also be an outcome of the fact that most patients did not use anti-<em>Pneumocystis</em> drugs.</p>

This study also presented the results of genotyping of two DNA regions of the fungus that are commonly used in molecular epidemiology studies of <em>P. jirovecii</em>. The mtLSU rDNA genotype distribution among Brazilian CF patients was similar to that described in a population of Spanish children with CF [19]; polymorphisms 85C/248C (genotype 1) and 85T/248C (genotype 3) were the most frequent. A recent report suggested that underlying disease may determine the presence of a specific mtLSU rDNA genotype of <em>P. jirovecii</em>, supporting the hypothesis that the polymorphism 85T/248C might be the best adapted to patients with CF [20]. The absence of mutations associated with sulfa resistance in the <em>P. jirovecii</em> DHPS gene from Brazilian CF patients is consistent with the same prior observation in Brazilian AIDS patients and is probably related to the low use of sulfa drugs in this population [21].</p>

Recent studies have proposed that <em>P. jirovecii</em> colonization may play a role in the pathophysiology of COPD by inducing inflammatory changes [22]. Moreover, there is an association between <em>P. jirovecii</em> colonization and the severity of airflow obstruction in smokers [23]. In this series of 34 CF patients, <em>P. jirovecii</em> colonization was statistically associated with <em>P. aeruginosa</em> colonization, a respiratory infection that is a major predictor of morbidity and mortality in CF [24]. None of the other prognostic indicators, e.g., number of CF-related hospitalizations and nutritional characteristics, was associated with <em>P. jirovecii</em> colonization in this study. Further studies should be conducted with larger numbers of patients to verify and further explore this finding.</p>

In an animal model, molecular and histological analyses have demonstrated that immunocompetent mice that are colonized by <em>Pneumocystis</em> act as a reservoir and source of infection [25]. Additionally, there is molecular evidence that the transmission of <em>P. jirovecii</em> from colonized immunocompetent carrier hosts to susceptible subjects may occur in humans [26]. It has been speculated that CF

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**Table 1** Demographic and clinical data from 34 Brazilian cystic fibrosis patients according to <em>Pneumocystis jirovecii</em> colonization.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patients with &lt;em&gt;P. jirovecii&lt;/em&gt; colonization (n = 13)</th>
<th>Patients without &lt;em&gt;P. jirovecii&lt;/em&gt; colonization (n = 21)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, median (range)</td>
<td>12 (1–35)</td>
<td>8 (1–22)</td>
<td>0.12*</td>
</tr>
<tr>
<td>Male sex, no. (%)</td>
<td>5 (38.7)</td>
<td>12 (57.1)</td>
<td>0.29*</td>
</tr>
<tr>
<td>Bacterial colonization, no. (%) ‡</td>
<td>12 (92.3)</td>
<td>17 (80.9)</td>
<td>0.63*</td>
</tr>
<tr>
<td>&lt;em&gt;Pseudomonas aeruginosa&lt;/em&gt; colonization, no. (%) ‡</td>
<td>12 (92.3)</td>
<td>11 (52.4)</td>
<td>0.02**</td>
</tr>
<tr>
<td>&lt;em&gt;Staphylococcus aureus&lt;/em&gt; colonization, no. (%) ‡</td>
<td>11 (84.6)</td>
<td>16 (76.2)</td>
<td>0.68*</td>
</tr>
<tr>
<td>&lt;em&gt;Haemophilus influenzae&lt;/em&gt; colonization, no. (%) ‡</td>
<td>1 (7.6)</td>
<td>3 (14.2)</td>
<td>0.99*</td>
</tr>
<tr>
<td>&lt;em&gt;Burkholderia cepacia&lt;/em&gt; Complex colonization, no. (%) ‡</td>
<td>1 (7.6)</td>
<td>3 (14.2)</td>
<td>0.99*</td>
</tr>
<tr>
<td>&lt;em&gt;Stenotrophomonas maltophilia&lt;/em&gt; colonization, no. (%) ‡</td>
<td>2 (15.4)</td>
<td>2 (9.5)</td>
<td>0.63*</td>
</tr>
<tr>
<td>Sulfa or azithromycin use, no. (%) ‡</td>
<td>1 (7.7)</td>
<td>4 (19.0)</td>
<td>0.35*</td>
</tr>
<tr>
<td>FEV-1% (mean ± SD)</td>
<td>70.9 ± 16.1</td>
<td>67.9 ± 16.3</td>
<td>0.78*</td>
</tr>
<tr>
<td>Diabetes mellitus, no. (%)</td>
<td>2 (15.4)</td>
<td>5 (23.8)</td>
<td>0.44*</td>
</tr>
<tr>
<td>Pancreatic enzyme use, no. (%)</td>
<td>10 (76.9)</td>
<td>17 (81.0)</td>
<td>0.55*</td>
</tr>
<tr>
<td>Moderate to severe malnutrition, no. (%) ‡</td>
<td>5 (38.4)</td>
<td>5 (23.8)</td>
<td>0.45*</td>
</tr>
<tr>
<td>Three or more CF-related number of hospitalizations/year, no. (%) ‡</td>
<td>4 (30.7)</td>
<td>4 (19.1)</td>
<td>0.68*</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mann-Whitney U-test; <sup>b</sup>Chi-square test with Yates’ correction; <sup>c</sup>Fisher’s exact test; <sup>d</sup>Student’s t-test; <sup>e</sup>Colonization in the last three months; <sup>f</sup>Use in the last six months; <sup>g</sup>Percentage from predicted forced expiratory volume in 1 s; <sup>h</sup>weight-for-age Z-score; <sup>i</sup>hospitalizations in the last year; *statistically significant.
patients colonized by *P. jirovecii* can be significant reservoirs of this atypical fungus, serving as infective sources of this microorganism to susceptible persons.

In summary, this study revealed a high prevalence (38.2%) of *P. jirovecii* colonization in Brazilian CF patients. Together with previous data from European populations, our data suggest that *P. jirovecii* colonization in CF is a frequent, possibly worldwide, occurrence. Because these patients could potentially be sources of infection for other susceptible persons, further research is warranted to assess the true scope of the problem and to design rational preventive strategies. Moreover, it is necessary to elucidate the role of *P. jirovecii* infection in the natural history of CF to improve the clinical management of this disease.

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