High prevalence of *Pneumocystis jirovecii* colonization among HIV-positive patients in southern Brazil

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Received 4 June 2014; Revised 14 August 2014; Accepted 22 August 2014

Abstract

A high prevalence of *Pneumocystis jirovecii* colonization was observed in patients positive for the human immunodeficiency virus (HIV) admitted to a tertiary hospital in southern Brazil between August 2012 and December 2012. Amplification of the mitochondrial large subunit ribosomal RNA gene in oropharyngeal samples through nested polymerase chain reaction identified *P. jirovecii* colonization in 26 of 58 (44.8%) HIV-positive patients admitted for causes other than *Pneumocystis* pneumonia. Colonization was more frequent among patients with an absolute CD4 count ≤200 cells/µl. These findings suggest that the HIV-infected population is a major reservoir and source of *P. jirovecii* infection and that identification of such individuals may contribute to future strategies for improving management of HIV-infected patients.

Key words: *Pneumocystis jirovecii*, colonization, HIV.

Introduction

*Pneumocystis jirovecii* is an atypical fungus that causes *Pneumocystis* pneumonia (PcP), an opportunistic infection associated with high morbidity and mortality in patients infected with the human immunodeficiency virus (HIV) [1].

The advent of trimethoprim-sulfamethoxazole (TMP-SMX) prophylaxis and antiretroviral therapy (ART) has resulted in a major reduction in the incidence of PcP among HIV-positive patients. However, PcP remains frequently associated with acquired immunodeficiency syndrome (AIDS) in patients who are unaware of their HIV status and/or lack access to ART. Recent data indicate that PcP remains common in developing countries. For example, autopsy studies of AIDS patients in Brazil in the post-ART era show a PcP prevalence ranging from 17.3% to 27.0% [2].
Polymerase chain reaction (PCR) methods have been used to demonstrate that humans can be colonized by P. jiroveci. This technique can detect this microorganism in respiratory specimens such as bronchoalveolar lavage (BAL), sputum, oropharyngeal washings, and nasopharyngeal swabs from patients with no clinical or radiographic manifestations of PCP. Nested PCR amplification of the mitochondrial large subunit ribosomal RNA (mtLSUrRNA) gene is the most sensitive technique for detecting P. jiroveci colonization in respiratory samples [3].

The prevalence of P. jiroveci colonization has been reported in several populations, for example, 40.5% in patients with chronic obstructive pulmonary disease (COPD), 37.8% in patients with idiopathic interstitial pneumonias, 28.8% in individuals with cystic fibrosis, 15.5% in pregnant women, and 29% in patients with rheumatologic diseases treated with infliximab, an antitumor necrosis factor agent [4–8].

To date, few studies have described P. jiroveci colonization in HIV-positive patients, and estimates of the colonization rate vary considerably from 10% to 68.8% [9]. A landmark study in which BAL samples from 50 patients were analyzed showed colonization in 16%, but the prevalence increased to 40% when analysis was confined to patients with a CD4 count <60 cells/µl [10]. Another study described colonization in 68% of 172 patients through the use of nested PCR in BAL or induced-sputum samples. These individuals had CD4 counts <50 cells/µl and TMP-SMX prophylaxis was absent, both of which have been reported as risk factors for the presence of P. jiroveci [11]. In a multicenter AIDS cohort study, colonization was detected in autopsy specimens of 46% of HIV-infected men dying of non-PCP, and only smoking was associated with an increased risk of Pneumocystis colonization [12].

Regarding developing countries, researchers reported a low prevalence (6%) of P. jiroveci colonization in 124 HIV-positive patients undergoing BAL in Uganda, sub-Saharan Africa [13]. In another study conducted in Cameroon among patients without pulmonary symptoms treated at a regional hospital, the Pneumocystis colonization rate in HIV-positive individuals was 42.9% but ranged from 20% in patients with CD4 counts >500 cells/µl to 57.1% in patients with CD4 counts <200 cells/µl [14].

Colonized individuals are possibly at risk of developing PCP. Infections in immunosuppressed rats with Pneumocystis were shown to progress from colonization to pneumonia [15]. In a human study, Leigh et al. found that 2 of 50 colonized patients developed PCP within 6 weeks [10]. Conversely, Davis et al. found that none of 172 colonized patients developed PCP during a similar follow-up period [11]. However, further studies are required to ascertain the real risk of progression from colonization to PCP in HIV-positive patients.

Additionally, evidence that P. jiroveci colonization could itself be harmful has been described. Several authors have demonstrated that Pneumocystis colonization is associated with airway and systemic inflammation. In addition, it was found in a recent study of nonhuman primates infected with the simian immunodeficiency virus that persistent Pneumocystis colonization led to the development of COPD [3,16,17]. A potential association between P. jiroveci colonization and chronic lung disease in HIV-positive patients has been debated by some authors and warrants further investigation [18].

No reports on P. jiroveci colonization among HIV-infected individuals in Latin America have been published [2,19]. In the present study, we investigate the prevalence and factors associated with P. jiroveci colonization in HIV-infected patients treated at Hospital de Clínicas de Porto Alegre, a tertiary hospital in Southern Brazil.

Materials and methods

In this cross-sectional study, we assessed all adult patients (aged ≥18 years) diagnosed with HIV infection who were admitted for various reasons to Hospital de Clínicas de Porto Alegre (Rio Grande do Sul, Brazil) between August 2012 and December 2012.

Oropharyngeal wash samples were obtained from each patient on the first day of hospitalization. The following clinical and demographic data were collected by means of specific questionnaires: age; gender; current ART—chemoprophylaxis for PCP or corticosteroid therapy; current smoking habits; COPD; previous episodes of PCP; and absolute CD4 cell count (cells per microliter).

Patients were classified as having COPD if such a diagnosis was present in their medical records. ART was defined as the use of a combination of highly active antiviral drugs for the treatment of HIV infection [20]. PCP chemoprophylaxis was considered to be the use of a drug with anti-Pneumocystis efficacy to prevent the development of PCP, for which TMP-SMX is the most widely used agent for this purpose [21]. The CD4 count considered for analysis was either the measurement obtained at study admission or any measurement obtained within the last 6 months if the patient was stable with regard to HIV treatment. Patients were stratified into the following CD4 count (cells per microliter) groups: <100, 101–200, 201–350, and >350.

HIV-positive patients with suspected PCP, as determined clinically by the attending physician at the time of hospital admission, were excluded from the study. Patients unable to provide specimens due to altered levels of consciousness (eg, somnolence) were also not included. The hospital’s ethics committee approved the study protocol.
committee approved the investigation, and all patients provided written informed consent for participation.

Oropharyngeal washing is a simple, noninvasive sample collection method that consists of gargling with 10 ml of normal saline solution for 1 min and collecting the resulting expectorate in a sterile container [22].

Pneumocystis jirovecii was detected by analyzing oropharyngeal samples with nested PCR amplification of the Pneumocystis mtLSUrRNA gene. After sample digestion with proteinase K at 56°C, DNA was extracted from the samples using a commercial kit (QIamp DNA mini kit; Qiagen, Hilden, Germany), and the gene encoding mtLSUrRNA was amplified. A two-step protocol was used for nested PCR [22]. In brief, this consisted of an initial amplification round, for which 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, yielding a 346-bp fragment. 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') were used in a second round of amplification to yield a 260-bp product. Both amplification rounds included 40 cycles of amplification. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel that contained ethidium bromide, and the bands were visualized under ultraviolet light. To prevent false-positive results due to contamination, pipette tips with filters were used at all stages. DNA extraction, reaction mixture preparation, PCR amplification, and detection were performed in different areas of the laboratory. In addition, a positive control was included in each reaction. To detect any cross-contamination, all PCR steps were performed with a negative control of sterile water. All experiments were repeated at least twice.

Statistical analysis was performed using SPSS software (version 20.0; SPSS, Chicago, IL, USA). The χ² test or Fisher exact test was used for qualitative variables. The Student t test or Mann-Whitney U test was applied for quantitative variables, and the variance test was used to confirm a normal distribution. Pearson χ² test was also used to compare the different CD4 count groups according to colonization. A P value < 0.05 was considered statistically significant.

Results

Sixty-seven HIV-positive adults were admitted to Hospital de Clínicas de Porto Alegre for different reasons between August 2012 and December 2012. Of these patients, five were excluded due to clinical presentation suggestive of PcP and four due to altered levels of consciousness that prevented oropharyngeal sample collection. In all patients with clinical suspicion of PcP, the diagnosis was later confirmed by detection of P. jirovecii in BAL fluid by microscopic examination of Grocott methenamine-silver-stained material.

Thus, 58 patients were examined for P. jirovecii colonization, of which 10 with AIDS had opportunistic infections other than PcP, 8 had tuberculosis, 5 were diagnosed as having bacterial pneumonia, 9 had various other major infections, 7 had cancer, 6 had cardiovascular diseases, 2 had anemia, and the remaining 11 had other medical conditions.

The mean (standard deviation) age of the patients included in the study was 41.7 ± 12.9 years, and 28 patients (48.2%) were male. Thirty-nine patients (67.2%) reported current use of corticosteroids, seven (12%) experienced one or more past episodes of PcP, and a diagnosis of COPD was identified in two patients (3.4%).

Pneumocystis jirovecii colonization was detected by nested PCR in oropharyngeal samples in 26 (44.8%) of the 58 patients tested. Table 1 presents the clinical and demographic characteristics of patients according to colonization status.

We identified a strong association between CD4 count ≤ 200 cells/µl and P. jirovecii colonization (P < 0.001) using the Pearson χ² test. Figure 1 presents the distribution of individuals according to stratified CD4 count and the frequency of colonized individuals in each CD4 cell count group.

Discussion

In this study, we identified a high prevalence of P. jirovecii colonization (44.8%) among HIV-infected patients admitted to a hospital in southern Brazil. Furthermore, colonization was more frequent among individuals with a CD4 count ≤ 200 cells/µl. These findings are similar to those from previous studies and indicate that severely immunosuppressed patients, for example, HIV-positive individuals, are more susceptible to colonization [10,11,14].

Our knowledge of the epidemiology of P. jirovecii infections has expanded greatly with the advent of molecular biology methods in recent years. Several studies using PCR-based techniques suggest that P. jirovecii is a Pneumocystis species that multiplies only in the human lung, that is, humans serve as the P. jirovecii reservoir. Therefore, the reservoir and source of infection for this microorganism appear to be composed of PcP patients and individuals colonized by the fungus [3,19].

Advances in molecular research have also shown that P. jirovecii is very likely transmitted from person to person via the airborne route [23]. This form of transmission...
Table 1. Characteristics of 58 human immunodeficiency virus–infected patients according to *Pneumocystis jirovecii* colonization status.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Colonized patients</th>
<th>Noncolonized patients</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex, no. (%)</td>
<td>13 (50)</td>
<td>15 (46.8)</td>
<td>0.999&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Age in years, mean ± standard deviation</td>
<td>41.2 ± 14.5</td>
<td>42.2 ± 11.7</td>
<td>0.776&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Antiretroviral therapy, no. (%)</td>
<td>15 (57.7)</td>
<td>24 (75.0)</td>
<td>0.260&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD4 count, median cells/µl (range)</td>
<td>85 (2–355)</td>
<td>256 (6–1048)</td>
<td>&lt;0.001&lt;sup&gt;d&lt;/sup&gt; *</td>
</tr>
<tr>
<td>Anti-<em>Pneumocystis</em> prophylaxis, no. (%)</td>
<td>12 (46.1)</td>
<td>11 (34.3)</td>
<td>0.415&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Previous <em>Pneumocystis</em> pneumonia, no. (%)</td>
<td>3 (11.5)</td>
<td>4 (12.5)</td>
<td>0.999&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Corticosteroid use, no. (%)</td>
<td>0 (0.0)</td>
<td>2 (6.2)</td>
<td>0.495&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Chronic obstructive pulmonary disease diagnosis, no. (%)</td>
<td>0 (0.0)</td>
<td>2 (6.2)</td>
<td>0.497&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Active smoker, no. (%)</td>
<td>9 (34.6)</td>
<td>14 (43.7)</td>
<td>0.588&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>χ² test with Yates correction.
<sup>b</sup>Student t test.
<sup>c</sup>Fisher exact test.
<sup>d</sup>Mann-Whitney U test.
<sup>*</sup>Statistically significant.

Figure 1. *Pneumocystis jirovecii* colonization in 58 human immunodeficiency virus–infected patients, stratified by CD4 cell count (cells per microliter).

was documented in a report of a PcP cluster occurring in renal transplant patients. Molecular analysis indicates that patients in this cluster were infected with microorganisms that shared similar genotypic features [24].

Recent evidence supports the hypothesis, demonstrated in animal models, that individuals who are merely colonized by *P. jirovecii* can transmit it to other individuals, some of whom may be susceptible to developing PcP [13]. In humans, molecular evidence of colonized individuals as potential sources of *P. jirovecii* infections has been described. One report discussed the case of a six-month-old infant who developed PcP after contact with her grandparents who had COPD and rheumatoid arthritis and were colonized by the fungus [25]. Another study analyzed colonized individuals who came into contact with patients in a PcP cluster in which the genotypic characterization and analysis of the timing of contact suggest that the colonized individuals were responsible for transmission of the organism among patients of this PcP cluster [26].

The results of the present study show a high frequency of *P. jirovecii* colonization among hospitalized HIV-positive individuals, mainly in patients with severe immunosuppression. However, from an epidemiological point of view, it is important to note that HIV-positive patients who have a CD4 count >350 are able to harbor *P. jirovecii*.

Therefore, HIV-positive patients, irrespective of CD4 count, may constitute a significant portion of the reservoir and source of *P. jirovecii* infection in the hospital setting. Currently, the US Centers for Disease Control and Prevention do not recommend respiratory isolation for inpatients with PcP and do not provide any guidance regarding
P. jirovecii colonization [27]. However, recent evidence on the transmission of this pathogen may lead experts to consider introducing strategies to prevent the spread of P. jirovecii in the nosocomial environment [28].

In the present study, we used oropharyngeal washing, a noninvasive clinical sampling method, to assess P. jirovecii colonization in HIV-positive patients. In immunocompetent patients, nested PCR is a highly sensitive (95.6%) technique for detecting colonization in oropharyngeal washings as compared with BAL fluids and induced sputum samples [22]. Other investigators have used oropharyngeal washings to assess colonization in several populations, including patients with cystic fibrosis, those receiving immunosuppressants, and healthy individuals [6,8,29]. Our results demonstrate the utility of this type of clinical specimen for identifying P. jirovecii colonization among HIV-infected patients.

A previous study of P. jirovecii colonization in a developing country showed a very low prevalence rate, that is, only 6% of HIV-positive subjects living in Uganda were colonized. According to the authors, these data suggest a limited reservoir for P. jirovecii in this population, potentially explaining the low incidence of PcP in sub-Saharan Africa [13]. However, more recent studies carried out in Cameroon show a rate of P. jirovecii colonization that is comparable to that found in our study [14]. Further investigation is needed to better define the epidemiology of Pneumocystis colonization in developing countries.

In conclusion, we reveal a high prevalence of P. jirovecii colonization among HIV-positive patients admitted to a tertiary referral hospital in southern Brazil. Colonization was most frequent among patients with severe immunosuppression (eg, CD4 count ≤200 cells/ul), which is consistent with previous reports. These findings suggest that HIV-infected individuals constitute a major reservoir and source of infection for this microorganism.

Acknowledgments
This work was supported by the Hospital de Clinicas de Porto Alegre (Fundão de Incentivo à Pesquisa, 12-0282) and the Red Iberoamericana sobre Pneumocystosis (212RT0450) in the framework of the Ibero-American Programme for Science, Technology and Development (Ciencia y Tecnología para el Desarrollo).

Declaration of interest
The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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