Diagnostic value of direct fluorescence antibody staining for detecting *Pneumocystis jirovecii* in expectorated sputum from patients with HIV infection

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Abstract

Direct fluorescent antibody (DFA) staining of induced sputum is frequently used to diagnose *Pneumocystis* pneumonia (PCP) in patients infected with human immunodeficiency virus, although induction can provoke nausea and bronchospasm. Since the diagnostic value of expectorated sputum examined with DFA stain has not been well evaluated, we reviewed the medical records of HIV-infected patients who were clinically diagnosed as having PCP between 1999 and 2011. Over this 13-year period, we found 76 patients whose records included the results of DFA staining of expectorated sputum and noted that 42 (55.3%) were positive. Polymerase chain reaction to detect *Pneumocystis* in the sputum of 65 of the patients resulted in the finding of 43 (66.2%) who were positive. Our findings suggest that DFA staining of expectorated sputum could be a useful initial diagnostic method in HIV-infected patients with PCP.

Key words: HIV, pneumocystis, sputum, fluorescent antibody technique.

Introduction

*Pneumocystis jirovecii* is the cause of *Pneumocystis* pneumonia (PCP) [1]. However, because *P. jirovecii* cannot be isolated in culture, the diagnosis of PCP is made by demonstrating organisms that are morphologically similar to *Pneumocystis* in respiratory specimens. A variety of such samples, including expectorated sputum, induced sputum, bronchoalveolar lavage (BAL) fluid, and lung tissue, can be used to detect *P. jirovecii* [2].

Staining methods to detect *P. jirovecii* can be cytological (CYT) staining, for example, Wright-Giemsa, Gomori methenamine silver, and toluidine blue O, or immunofluorescence (IF) staining, which includes direct fluorescent
antibody (DFA) and use of a monoclonal antibody to detect Pneumocystis antigen. DFA stains have higher sensitivity and specificity for detecting Pneumocystis in induced sputum than CYT stains, but this difference is less significant with BAL fluid [3,4] specimens.

There is no “gold standard” for confirming PCP, but staining of lung tissue or BAL fluid has been considered the most sensitive diagnostic test [5]. In the mid-1980s it was reported that examination of sputum induced by inhalation of hypertonic saline solution was frequently diagnostic for PCP [6], especially in human immunodeficiency virus (HIV)–infected patients because of the higher concentration of Pneumocystis in these samples. Although the diagnostic yield from induced sputum ranges from 50% to 90% at different medical centers, it has emerged as a noninvasive technique for diagnosing PCP [7,8]. In recent years polymerase chain reaction (PCR) has proven to have greater sensitivity for detecting Pneumocystis in respiratory specimens than either CYT or IF staining [9]. However, PCR can detect colonization in asymptomatic patients, and differentiation between infection and colonization requires an additional diagnostic test [10].

In contrast to induced sputum, expectorated sputum is considered to have poor sensitivity [11,12] for detecting Pneumocystis, even in HIV-infected patients. Although it has been reported that the introduction of DFA staining improved the sensitivity of expectorated sputum [3,4], its diagnostic value in HIV-infected patients is not well known. Here, we assess the value of DFA stain for detecting Pneumocystis in expectorated sputum from HIV-infected patients with clinically diagnosed PCP.

Materials and methods

We retrospectively reviewed the medical records of HIV-infected patients with clinically diagnosed PCP who had been admitted to Seoul National University Hospital, a 1600-bed teaching hospital, from January 1999 to December 2011. We selected for evaluation only HIV-infected patients with available results for DFA staining and 2 others whose result of DFA staining was negative if at least two characteristic cysts were observed. PCR for detecting Pneumocystis was carried out using oligonucleotide primers pAZ102-E (5′-GATGGCTGTTTCCAGCCA-3′) and pAZ102-H (5′-GTGTACGTTGCAAAGTACTC-3′) specific to the large subunit of the mitochondrial ribosomal RNA gene, as previously described [13]. The following information was collected: age, gender, CD4 lymphocyte counts, HIV-1 viral load, alveolar-arterial oxygen difference (A-aDO_2), care in the intensive care unit (ICU) during admission, and 30-day mortality.

Proportions were compared using the χ² test or Fisher exact test. Continuous variables were compared using Student t test. Statistical significance was accepted at P < 0.05.

The Seoul National University Hospital Institutional Review Board approved the study protocol, and the board waived the requirement for informed consent.

Results

Over a 13-year study period, 1288 HIV-infected patients were treated, with a total follow-up duration of 5982 person-years. Among these patients, we identified 92 (7.1%) cases of clinically diagnosed pneumocystis, yielding a crude incidence rate of 1.53/100 person-years. After excluding 14 patients whose expectorated sputum was not examined using DFA staining and 2 others whose result of DFA staining of BAL fluid was negative, 76 patients were evaluated. At the time of PCP diagnosis, the mean CD4 lymphocyte count was 45.6 cells/mm³ and the mean HIV-1 viral load was 4.7 log_{10} copies/ml (Table 1).

Of the 76 patients whose expectorated sputum was examined, 42 (55.3%) had positive results with DFA staining. PCR for detection of Pneumocystis in expectorated sputum was performed for 65 patients, of which positive results were found for 43 (66.2%). The concordance rate between DFA staining and PCR examination was 56.9% (Table 2). Of four patients whose BAL fluid specimens were DFA positive, one showed positive DFA staining of expectorated sputum and three did not.
Table 1. Demographic and clinical characteristics of human immunodeficiency virus–infected patients with clinically diagnosed *Pneumocystis* pneumonia according to the result of direct fluorescent antibody staining of expectorated sputum.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Total (n = 76)</th>
<th>Positive (n = 42)</th>
<th>Negative (n = 34)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>40.7(±10.4)</td>
<td>41.4±12.0</td>
<td>39.8±8.0</td>
<td>NS</td>
</tr>
<tr>
<td>Male (%)</td>
<td>71(93.4)</td>
<td>41(97.6)</td>
<td>30(88.2)</td>
<td>NS</td>
</tr>
<tr>
<td>CD4 lymphocyte counts (cells/mm&lt;sup&gt;3&lt;/sup&gt;)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>45.6±61.5</td>
<td>48.9±65.3</td>
<td>41.6±57.2</td>
<td>NS</td>
</tr>
<tr>
<td>Human immunodeficiency virus–1 viral load (log&lt;sub&gt;10&lt;/sub&gt; copies/mL)&lt;sup&gt;†&lt;/sup&gt;</td>
<td>4.7±1.3</td>
<td>4.5±1.6</td>
<td>5.1±0.9</td>
<td>NS</td>
</tr>
<tr>
<td>A-aDO₂ (mmHg)&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>109.4±123.3</td>
<td>108.8±136.3</td>
<td>110.0±104.1</td>
<td>NS</td>
</tr>
<tr>
<td>Intensive care unit stay</td>
<td>12(15.8%)</td>
<td>6(14.3%)</td>
<td>6(17.6%)</td>
<td>NS</td>
</tr>
<tr>
<td>30 day mortality</td>
<td>14(18.4%)</td>
<td>7(16.7%)</td>
<td>7(20.6%)</td>
<td>NS</td>
</tr>
</tbody>
</table>

A-aDO₂, alveolar-arterial oxygen difference; DFA, direct fluorescent antibody; NS, not significant; PCP, *Pneumocystis* pneumonia.

<sup>*</sup>CD4 lymphocyte count at the time of diagnosis of PCP.

<sup>†</sup>Viral loads were tested in 38 DFA-positive and 34 DFA-negative patients at the time of diagnosis of PCP.

<sup>‡</sup>A-aDO₂ was tested in 40 DFA-positive and 31 DFA-negative patients at the time of diagnosis of PCP.

Table 2. Relationship between direct fluorescent antibody staining and polymerase chain reaction results for detecting *Pneumocystis* in expectorated sputum from human immunodeficiency virus–infected patients with clinically diagnosed *Pneumocystis* pneumonia.

<table>
<thead>
<tr>
<th></th>
<th>Positive</th>
<th>Negative</th>
<th>Sum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct fluorescent antibody staining</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>25</td>
<td>18</td>
<td>43</td>
</tr>
<tr>
<td>Negative</td>
<td>10</td>
<td>12</td>
<td>22</td>
</tr>
</tbody>
</table>

Sum               | 35       | 30       | 65  |

Age, sex, CD4 lymphocyte count, HIV-1 viral load, A-aDO₂, and 30-day mortality rate did not differ significantly between DFA-positive and DFA-negative patients (Table 1). The proportion of the expectorated sputa that stained positive with DFA was 71.4% (15/21) for patients with A-aDO₂ ≤ 45 mmHg and 50.0% (25/50) for patients with A-aDO₂ > 45 mmHg, which was not significantly different (P = 0.15).

**Discussion**

Because we do not examine BAL fluid routinely, we were unable to evaluate the exact sensitivity of DFA staining for detecting *Pneumocystis* in expectorated sputum. However, in the present study, the expectorated sputum of 55.3% of the patients with clinically diagnosed PCP was DFA positive, which means that PCP could be confirmed in 55.3% of patients by examination of expectorated sputum. Hence, we suggest that DFA staining of expectorated sputum could be used as a first-line diagnostic step for HIV-infected patients, especially in hospitals where sputum induction is not available.

*Pneumocystis* primarily affects the alveoli, and deep pulmonary samples are necessary for appropriate microscopic examination [2]. For many years, bronchoscopic examination was considered essential for the diagnosis of PCP, but bronchoscopy is not available in some resource-limited countries. Patients with HIV infection and PCP have a significantly increased number of *Pneumocystis* organisms in their lungs, with fewer neutrophils, than do patients with PCP in the absence of HIV infection [14]. This greater organism burden results in a higher diagnostic yield of respiratory specimens that confirm PCP in HIV-infected patients than for other conditions associated with immunosuppression. For this reason, sputum induced by hypertonic saline was accepted as the initial step in diagnosis. However, trained personnel and care in the processing of specimens are required to maintain the quality of induced sputum. In addition, sputum induction can be uncomfortable for the patient and can provoke nausea, vomiting, dyspnea, and bronchospasm [7].

The sensitivity of CYT staining of expectorated sputum for the diagnosis of PCP in HIV-infected patients has been reported to be low. The introduction of the DFA
stain has increased the sensitivity for the diagnosis of PCP from respiratory specimens. In one comparative study of the diagnostic performance of different microscopic techniques for the diagnosis of PCP, DFA stain was superior in sensitivity (91%) to staining with Calcofluor white (74%) and silver (77%), both of which were more sensitive than Diff-Quik (Baxter Scientific, McGraw Park, IL, USA) (48%) [4]. The sensitivity of expectorated sputum is expected to be greater if a DFA stain is used instead of a CYT stain. However, to date, there has been little data on the sensitivity of DFA staining of expectorated sputum in HIV-infected patients. Metersky et al. reported a sensitivity of DFA staining of expectorated sputum for *Pneumocystis* in patients with clinically diagnosed PCP of between 50% and 55%; however, a small number of patients were evaluated [15,16].

In this study, the rate of positive DFA staining did not differ according to A-aDO2. That means that DFA staining of expectorated sputum should be useful regardless of the severity of PCP.

Investigators have detected *Pneumocystis* colonization using DFA staining of BAL fluid [17], PCR of BAL fluid in patients with chronic obstructive pulmonary disease[18], and PCR of oropharyngeal washes in nonimmunosuppressed patients [19]. There has been no report of the detection of *Pneumocystis* colonization using DFA staining of expectorated sputum in nonimmunosuppressed patients. This might be due to the low organism burden associated with colonization [20]. In this study, all patients with positive DFA staining of expectorated sputum had compatible clinical and radiological features for PCP.

Recently, PCR has offered a sensitive and specific tool for detecting *Pneumocystis* in clinical specimens [9]. When the performance of DFA staining for expectorated sputum was evaluated relative to PCR as the reference technique, the sensitivity was 58.1%, specificity was 54.4%, the positive predictive value was 71.4%, the negative predictive value was 40%, the positive likelihood ratio was 1.28, and the negative likelihood ratio was 0.76. However, PCR can detect *Pneumocystis* colonization in asymptomatic patients, and differentiation between infection and colonization requires an additional diagnostic test [10].

This study has several limitations. Although there is no gold standard for confirming PCP, staining of BAL fluid has generally been used as the reference method. However, because BAL fluid was examined in a small number of patients in this study, we were unable to calculate the exact sensitivity, specificity, positive predictive value, and negative predictive value of DFA staining of expectorated sputum. Second, the number of evaluated patients was relatively small, although we have evaluated the largest number of patients to date.

When expectorated sputum was stained with DFA, *Pneumocystis* was detected in 55.3% of HIV-infected patients with clinically diagnosed PCP. This suggests that examination of expectorated sputum using DFA staining could be a useful initial diagnostic method.

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**Declaration of interest**

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

**References**

1. Cushion MT. Are members of the fungal genus pneumocystis (a) commensals; (b) opportunists; (c) pathogens; or (d) all of the above? PLoS Pathog 2010; 6: e1001009.