The Use of Oral Washes to Diagnose *Pneumocystis carinii* Pneumonia: A Blinded Prospective Study Using a Polymerase Chain Reaction–Based Detection System

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*Pneumocystis carinii* pneumonia (PCP) can be diagnosed by direct microscopic examination of induced sputum or by bronchoalveolar lavage (BAL). However, many institutions have little diagnostic success with induced sputum, and BAL is invasive and expensive. This prospective, blinded study assessed oral washes as a more convenient specimen than either sputum or BAL fluid and used a dissociation-enhanced lanthanide fluoroimmunoassay time-resolved fluorescent hybridization polymerase chain reaction (PCR) detection system that is feasible for clinical laboratories. The study assessed 175 oral washes, each paired with either an induced sputum that was positive for *Pneumocystis* or a BAL sample. The PCR test based on the *Pneumocystis* major surface glycoprotein primers had a sensitivity of 91% and a specificity of 94%, compared with a test based on mitochondrial large subunit rRNA primers, which had a sensitivity of 75% and a specificity of 96%. These results suggest that oral washes can provide a useful sample for diagnosis of PCP when a sensitive PCR detection system is used.

Methods

**Patient population and definition of PCP.** During a 3-year period (1997–2000), patients were entered into this study if (1) they were scheduled for bronchoscopy at the National Institutes of Health (NIH) or at the Washington Hospital Center, (2) they were considered to be susceptible to PCP, and (3) they gave written informed consent under a protocol that was approved by the institutional review boards at both institutions. Immediately after providing the oral wash, each NIH patient then attempted to provide an induced sputum sample (no induced sputum samples were ordered at Washington Hospital Center). If an induced sputum sample demonstrated ≥1 cluster of *Pneumocystis* by direct fluorescent antibody staining, the patient was considered to have PCP, and no BAL was performed. If an induced sputum sample either was not obtained or was negative by direct microscopy, a BAL was encouraged. If direct microscopy of induced sputum or BAL fluid revealed ≥1 cluster of *Pneumocystis*, the patient was considered to have PCP.

**Staining methods.** *Pneumocystis* detection at NIH was performed by direct fluorescent antibody (DFA) stain that used the Monofluo Test Kit (BioRad/Sanofi Diagnostics) on cytospin slide preparations, whereas detection at the Washington Hospital Center was performed by use of a methenamine silver stain.
Sample collection. Before sputum induction or BAL, patients gargled with 50 mL of sterile saline for 10–30 s to provide the oral wash specimens.

DNA preparation. For PCR testing, each oral wash sample (5–50 mL) was centrifuged at 2800 g for 10 min. The supernatant was decanted, leaving ~1 mL of liquid, which then was centrifuged at 8160 g for 10 min in a 2-mL microfuge tube, and all but ~25 μL of this supernatant fluid was removed. A 250-μL aliquot of InstaGene Matrix (Bio-Rad) was added to the pellet and was vortexed briefly. Samples were incubated at 56°C for 20 min, were vortexed for 10 s, were incubated at 100°C for 8 min, were vortexed again for 10 s, and were centrifuged at 11,750 g for 3 min. A 5-μL sample of the supernatant was used in a 50-μL PCR. After December 1998, the NucliSens (Organon Teknika) kit was used on 0.2 mL of resuspended pellet for DNA extraction, according to the manufacturer’s instructions.

Primers and probes for Pneumocystis detection have been described elsewhere for the major surface glycoprotein [13] and for mitochondrial (mt) large subunit (LSU) rRNA [5]. All specimens were coded and evaluated by a technician who was unaware of the DFA stain result.

Internal controls. To detect false-negative results due to inhibition of PCR amplification, an internal control (mimic) was constructed for each P. carinii primer pair by attaching the 2 primer sites to the ends of a region of the pBR322 tetracycline resistance gene that was ~50 bases longer than the P. carinii target [14].

DNA amplification. A 45-μL aliquot of a mixture that contained the appropriate primers, mimic, Isopsoralen-10, and H2O was pipetted into tubes with Ready-To-Go PCR beads (Amersham Pharmacia Biotech), and 5 μL of the DNA sample were added to each reaction tube. Tubes then were incubated in a thermal cycler (DNA Engine PTC-200; MJ Research). The thermal cycler conditions used for the major surface glycoprotein (MSG) primers were initial denaturation for 5 min at 94°C, 44 cycles of 94°C for 30 s, 65°C for 1 min, and 72°C for 2 min. Conditions used for amplification with the mt LSU rRNA primers were an initial denaturation for 2 min at 94°C, then 40 cycles of 94°C for 90 s, 55°C for 90 s, and 72°C for 2 min.

Detection by dissociation-enhanced lanthanide fluoroimmunoassay time-resolved fluorescence hybridization. Probe hybridization to biotinylated product was detected in streptavidin-coated 96-well plates (Wallac Oy). Fluorescence was measured on the VICTOR 1420 Multilabel Counter (Wallac Oy).

Interpretation of assay results. Both the MSG and mt LSU rRNA P. carinii PCR assays were performed in triplicate on DNA preparations from each sample. Positive and negative controls were performed in duplicate. A fluorescent signal >12,000 (3–4 times higher than the average background signal) was defined as positive. Sets of replicates where 3 of 3 or 2 of 3 PCR tubes gave signals >12,000 were considered to be positive for that PCR assay. When only 1 tube gave a signal >12,000, the test was repeated by use of a new aliquot of the patient sample. If >1 tube of the repeat test was also positive, the sample was considered to be positive.

Statistics. For comparison of specificity and sensitivity of the 2 PCR methods, McNemar’s test was used (SPSS for Macintosh, version 6.1.1; SPSS). For comparison of positive and negative predictive values of the 2 PCR methods, comparison of 2 proportions was used (Primer of Biostatistics, version 3.0; Stanton A. Glantz McGraw-Hill Health Professions Division).

Results

Assay specificity. The MSG and mt LSU rRNA assays were negative with DNA from Saccharomyces cerevisiae, Schizosaccharomyces pombe, Candida albicans, Candida krusei, Candida tropicalis, Candida glabrata, Trichosporon beigelii,
Cryptococcus neoformans, Histoplasma capsulatum, Coccioidoides immitis, and Blastocystis hominis.

Assay sensitivity. Relative sensitivity of mt LSU rRNA and MSG assays was determined by simultaneously testing serial dilutions made from a P. carinii DFA-positive BAL sample. Both PCR assays detected P. carinii DNA at dilutions up to 1:500 prepared in either BAL fluid or saline. Both PCR assays were consistently at least 10-fold more sensitive than the DFA examination, and the MSG assay was 10-fold more sensitive than the mt LSU rRNA assay.

Assessment of clinical samples. Figure 1 and table 1 show the results of direct microscopy and PCR testing. Of the 32 patients who had PCR detected by direct microscopy, the oral wash was positive by PCR in 29 patients when the MSG primers were used (91%) and in 24 patients when the mt LSU rRNA primers were used (75%; \( P = .06 \), McNemar’s test; table 1). These results indicate that the oral wash had an excellent sensitivity and that MSG primers have a higher sensitivity than the mt LSU rRNA primers. Of 143 patients who had a negative BAL by direct microscopy, 9 patients had a positive oral wash PCR when the MSG primer was used (6.3%), and 6 had a positive oral wash PCR when the mt LSU rRNA primers were used (4.2%; \( P = .45 \), MSG vs. mt LSU rRNA, McNemar’s test).

For any test to be clinically useful, both sensitivity and specificity must be high. Did the PCR tests accurately identify Pneumocystis that was “missed” by direct microscopy in the 4 samples identified as false-positive by both the MSG and mt LSU rRNA-based PCR and the 7 samples identified as false-positive by either the mt LSU rRNA or the MSG PCR?

A review of clinical charts revealed that none of the patients with a false-positive oral wash PCR test developed PCP or was treated for PCP during the 6 months after the test. However, 8 of 11 patients with a false-positive test had a concurrent sputum or BAL specimen that was positive by PCR. These results suggest that, in a high percentage of patients with apparent false-positive oral wash PCR results based on negative direct microscopy of a BAL specimen, there is confirmation by PCR of the BAL or sputum specimen that Pneumocystis is present (i.e., that organisms are present in the patient’s respiratory secretions).

Discussion

The present study has demonstrated that a sensitive PCR assay can detect the presence of Pneumocystis in a high percentage of oral wash specimens from patients who are shown to have PCP by direct microscopy of an induced sputum or BAL specimen (table 1). Prior work by other researchers and the results of the present study have indicated that oral washes do not contain enough organisms to be detected by direct microscopy. However, 3 groups have shown that Pneumocystis can be detected in oral washes if a highly sensitive PCR detection system is used [2–5]. Oral washes would be a highly desirable diagnostic specimen, because they can be obtained quickly and noninvasively in a wide variety of health-care settings.

For PCR testing to be more widely used, the PCR testing technique should be technically appropriate for a clinical microbiology laboratory. Most systems described in the literature are too labor intensive to be ideal for clinical laboratories. We used a system with a standard 96-well plate format. This system uses europium-labeled hybridization probes and time-resolved fluorescence to detect positive amplification reactions.

It is likely that organisms are more abundant in patients with clinically apparent PCP than in patients with colonization but no disease. Thus, organisms detected by PCR in patients without PCP—that is, those who are colonized—might be present in extremely small quantities. An assay to quantitate the organisms present in the sample is currently being assessed [15]. Preliminary results suggest that false-positive samples do, in fact, contain fewer target copies than samples from patients with PCP documented by direct microscopy.

References


Table 1. Performance characteristics of 2 polymerase chain reaction (PCR) techniques to diagnose Pneumocystis carinii pneumonia using oral washes (n = 175).

<table>
<thead>
<tr>
<th>PCR technique</th>
<th>MSG</th>
<th>mt LSU rRNA</th>
<th>( P^a )</th>
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<tbody>
<tr>
<td>Sensitivity</td>
<td>29/32 (91)</td>
<td>24/32 (75)</td>
<td>.06</td>
</tr>
<tr>
<td>Specificity</td>
<td>134/143 (94)</td>
<td>137/143 (96)</td>
<td>.45</td>
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<tr>
<td>Positive predictive value</td>
<td>29/38 (76)</td>
<td>24/30 (80)</td>
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<tr>
<td>Negative predictive value</td>
<td>134/137 (98)</td>
<td>137/145 (94)</td>
<td>.16</td>
</tr>
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</table>

NOTE. Data are no. of positive specimens/total no. of specimens (%). LSU, large subunit; MSG, major surface glycoprotein; mt, mitochondrial.

\( ^a \) MSG vs. mt LSU rRNA, McNemar’s test.


