CONCISE COMMUNICATION

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

Steven Fischer,[1] Vee J. Gill,[1] Joseph Kovacs,[2] Peter Miele,[3] Jodie Keary,[1] Victoria Silcott,[1] Sheng Huang,[2] Luciana Borio,[2] Frida Stock,[1] Gary Fahle,[1] Dennis Brown,[2] Barbara Hahn,[2] Ellen Townley,[3] Daniel Lucey,[3] and Henry Masur[2]

Departments of [1]Laboratory Medicine and [2]Critical Care Medicine, Clinical Center, National Institutes of Health and Division of Infectious Diseases, and [3]Washington Hospital Center, Washington, DC

***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.**

Pneumocystis carinii pneumonia (PCP) continues to occur in immunosuppressed patients, despite widespread use of anti-pneumocystis prophylaxis [1]. Examination of induced sputum or bronchoalveolar lavage (BAL) fluid by direct microscopy that uses an immunofluorescent or tinctorial staining technique has provided a highly sensitive and specific diagnostic method for identifying the presence of *Pneumocystis* organisms, thereby determining whether anti-pneumocystis therapy should be initiated. However, bronchoscopy is invasive, uncomfortable for the patient, and expensive. Induced sputum tests have had very high sensitivity at some institutions, but a considerable number of institutions have been unsuccessful in achieving adequate sensitivity with this technique. Examination of oral washes by PCR has been reported to be a promising technique to diagnose PCP [2–5], and other researchers have used a variety of PCR techniques to detect *Pneumocystis* in BAL fluid or sputum [6–12]. This project was initiated to develop and evaluate a polymerase chain reaction (PCR) assay that would allow PCP to be diagnosed from an easily obtained specimen.

Received 5 April 2001; revised 15 August 2001; electronically published 13 November 2001.

Reprints or correspondence: Dr. Henry Masur, National Institutes of Health, Dept. of Critical Care Medicine, Bldg. 10, Rm. 7D43, Bethesda, MD 20892 (hmasur@cc.nih.gov).

The Journal of Infectious Diseases 2001;184:1485–8

0022-1899/2001/18411-0019$02.00

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. Figure 1 shows the number of samples obtained, as well as smear and PCR results. All sputum and BAL samples were obtained within 24 h of collecting the oral wash. Analysis excluded 147 patients who provided an oral wash and had a sputum sample that was negative for *Pneumocystis* because they did not have a follow-up BAL; thus, the presence or absence of PCP could not be definitively substantiated.

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.

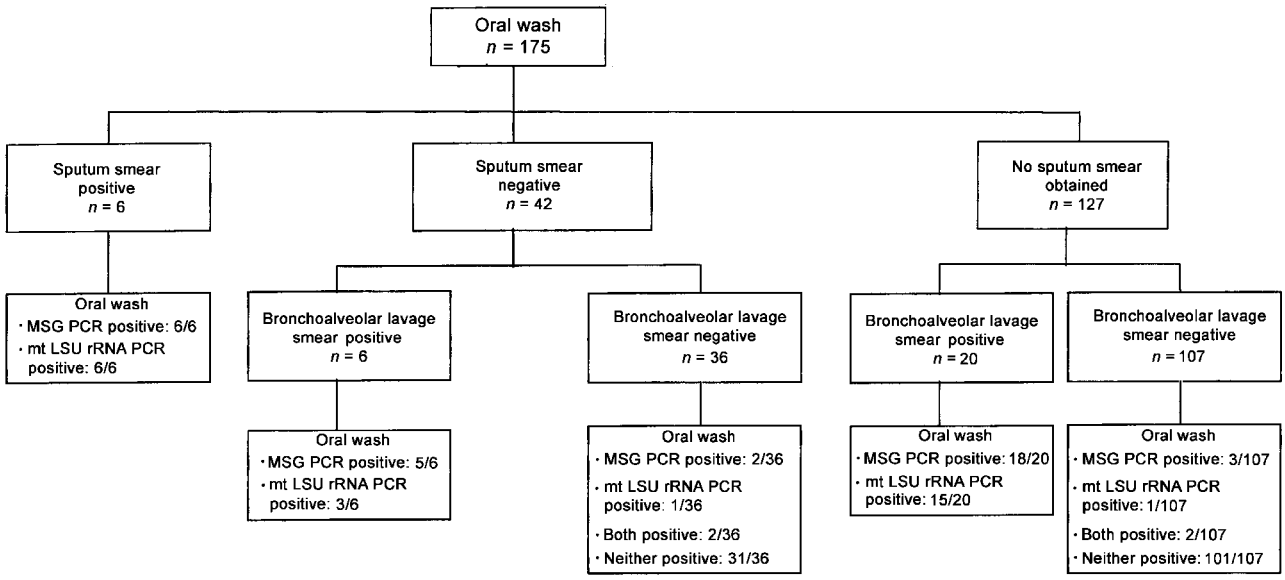

Figure 1. Flow diagram of specimens obtained, bronchoalveolar lavage or sputum smear results, and oral wash polymerase chain reaction (PCR) results. LSU, large subunit; MSG, major surface glycoprotein; mt, mitochondrial.

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 H_20 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 sensitivity and specificity 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 cervisiae, Schizosaccharomyces pombe, Candida albicans, Candida krusei, Candida tropicalis, Candida glabrata, Trichosporon beigelii,*

Cryptococcus neoformans, Histoplasma capsulatum, Coccidioides 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 PCP 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).

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

	PCR technique		
Performance characteristic	MSG	mt LSU rRNA	P[a]
Sensitivity	29/32 (91)	24/32 (75)	.06
Specificity	134/143 (94)	137/143 (96)	.45
Positive predictive value	29/38 (76)	24/30 (80)	.92
Negative predictive value	134/137 (98)	137/145 (94)	.16

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.

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

1. Kaplan JE, Hanson D, Dworkin MS, et al. Epidemiology of human immunodeficiency virus–associated opportunistic infections in the United States in the era of highly active antiretroviral therapy. Clin Infect Dis **2000**; 30(Suppl 1):S5–14.
2. Helwig-Larsen J, Jensen JS, Benfield T, Svendsen UG, Lundgren JD, Lundgren B. Diagnostic use of PCR for detection of *Pneumocystis carinii* in oral wash samples. J Clin Microbiol **1998**;36:2068–72.
3. Helwig-Larsen J, Jensen JS, Lundgren B. Non-invasive diagnosis of *Pneumocystis carinii* pneumonia in hematological patients using PCR on oral washes. J Eukaryot Microbiol **1997**;44:59S.
4. Helweg-Larsen J, Jensen JS, Lundgren B. Non-invasive diagnosis of *Pneumocystis carinii* pneumonia by PCR on oral washes. Lancet **1997**; 350:1363.
5. Oz HS, Hughes WT. Search for *Pneumocystis carinii* DNA in upper and lower respiratory tract of humans. Diagn Microbiol Infect Dis **2000**; 37:161–4.
6. Wakefield AE, Guiver L, Miller RF, Hopkin JM. DNA amplification on induced sputum samples for diagnosis of *Pneumocystis carinii* pneumonia. Lancet **1991**;337:1378–9.
7. Tamburrini E, Ortona E, Visconti E, et al. Detection of *Pneumocystis carinii* in oropharyngeal washings by PCR-SHELA and nested PCR. J Eukaryot Microbiol **1997**;44:48S.

8. Lu JJ, Chen CH, Bartlett MS, Smith JW, Lee CH. Comparison of six different PCR methods for detection of *Pneumocystis carinii.* J Clin Microbiol **1995**;33:2785–8.
9. Sandhu GS, Kline BC, Espy MJ, Stockman L, Smith TF, Limper AH. Laboratory diagnosis of *Pneumocystis carinii* infections by PCR directed to genes encoding for mitochondrial 5S and 28S ribosomal RNA. Diagn Microbiol Infect Dis **1999**;33:157–62.
10. Sing A, Trebesius K, Roggenkamp A, et al. Evaluation of diagnostic value and epidemiological implications of PCR for *Pneumocystis carinii* in different immunosuppressed and immunocompetent patient groups. J Clin Microbiol **2000**;38:1461–7.
11. Huang SN, Fischer SH, O'Shaughnessy E, Gill VJ, Masur H, Kovacs JA. Development of a PCR assay for diagnosis of *Pneumocystis carinii* pneumonia based on amplification of the multicopy major surface glycoprotein gene family. Diagn Microbiol Infect Dis **1999**;35:27–32.
12. Torres J, Goldman M, Wheat LJ, et al. Diagnosis of *Pneumocystis carinii* pneumonia in human immunodeficiency virus infected patients with polymerase chain reaction: a blinded compassion to standard methods. Clin Infect Dis **2000**;30:141–5.
13. Mei Q, Turner RE, Sorial V, Klivington D, Angus CW, Kovacs JA. Characterization of major surface glycoprotein genes of human *Pneumocystis carinii* and high level expression of a conserved region. Infect Immun **1998**;66:4268–73.
14. Fahle GF, Fischer SH. Compassion of six commercial DNA extraction kits for the recovery of CMV DNA from spiked human specimens. J Clin Microbiol **2000**;38:3860–3.
15. Larsen HH, Masur H, Kovacs JA, Gill VJ, Silcott VA, Fischer SH. A rapid and quantitative real time PCR assay for *Pneumocystis carinii.* In: Program and abstracts of 38th annual meeting of the Infectious Diseases Society of America (New Orleans). Alexandria, VA: Infectious Diseases Society of America, **2000**;87:238.