A Prospective, Blinded Study of Quantitative Touch-Down Polymerase Chain Reaction Using Oral-Wash Samples for Diagnosis of Pneumocystis Pneumonia in HIV-Infected Patients

Hans Henrik Larsen,^1,^5 Laurence Huang,^2 Joseph A. Kovacs,^2 Kristina Crothers,^3 Victoria A. Silcott,^1 Alison Morris,^3 Joan R. Turner,^3 Charles B. Beard,^4 Henry Masur,^2 and Steven H. Fischer^1

Departments of ^1^Laboratory Medicine and ^2^Critical Care, Clinical Center, National Institutes of Health, Bethesda, Maryland; ^3^Department of Medicine, San Francisco General Hospital, University of California, San Francisco; ^4^Division of Parasitic Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia; ^5^Copenhagen HIV Programme and ^6^Department of Clinical Microbiology, Hvidovre University Hospital, Hvidovre, Denmark

Oral-wash samples obtained during 113 episodes of suspected Pneumocystis pneumonia (PCP) in human immunodeficiency virus–infected patients were tested by use of a quantitative touch-down PCR (QTD PCR) assay. QTD PCR had a sensitivity of 88% and a specificity of 85%. Treatment for PCP prior to oral wash collection had an impact on the sensitivity, and PCR-positive oral-wash samples obtained within ≤1 day of treatment from patients without PCP had significantly fewer copies per tube than did those from patients with PCP; thus, application of a post hoc cut-off value of 50 copies/tube increased the specificity to 100%. QTD PCR of oral-wash samples can be an accurate and noninvasive method for diagnosis of PCP.

Pneumocystis pneumonia (PCP), which is caused by the opportunistic fungus *Pneumocystis jiroveci* [1], continues to occur in immunosuppressed patients, despite the widespread use of anti-*Pneumocystis* prophylaxis, and in patients with AIDS, despite the use of highly active antiretroviral therapy [2]. A major impediment to reducing morbidity and mortality due to PCP has been the difficulty of establishing a definitive diagnosis.

The standard method for diagnosis of PCP is direct microscopic examination of lower respiratory–tract specimens obtained by bronchoscopy, with bronchoalveolar lavage (BAL), or by induction of sputum. However, bronchoscopy is invasive, uncomfortable for the patient, and expensive. In addition, a considerable number of institutions have been unsuccessful in achieving adequate sensitivity with induced sputum.

In an effort to develop a noninvasive diagnostic test, examination of oral-wash samples has been investigated. Oral-wash samples are easy to obtain, but a more sensitive technique, such as polymerase chain reaction (PCR), is required for detection [3–7]. However, some of the PCR techniques previously used have been too complicated for routine use in a clinical laboratory. Although PCR methods have the potential to be very sensitive, specimens have been found to be positive by PCR for patients without PCP, who are presumed to be colonized with *P. jiroveci* [4–6, 8–10].

The present prospective, blinded study evaluated PCR analysis of oral-wash samples obtained from HIV-infected patients with suspected PCP, by use of a rapid, closed-tube, quantitative touch-down (QTD) PCR assay format that would likely be feasible for routine use in a clinical molecular laboratory, as described elsewhere [11]. We sought to determine whether quanti-
fication and the use of a cut-off value could improve specificity by distinguishing between infection and colonization.

PATIENTS, MATERIALS, AND METHODS

During a 3-year period (April 1999–July 2002), HIV-infected patients admitted to San Francisco General Hospital were prospectively enrolled in the present study if they were suspected of having PCP, were scheduled to undergo diagnostic testing, and agreed to provide an oral-wash sample. Informed consent was obtained from patients, and institutional guidelines were followed. The standard laboratory approach for diagnosis of PCP was direct microscopy of induced sputum or BAL by use of Diff-Quik (Dade Behring) [12]. For the purposes of the present study, PCP was considered to be the correct diagnosis if Pneumocystis organisms were visualized in a sample by this method. For a patient to be evaluated, a definite diagnosis of either PCP or non-PCP had to have been established by either an induced sputum sample that was positive for Pneumocystis (i.e., PCP) by Diff-Quik or by a bronchoscopy with BAL (i.e., PCP or non-PCP).

Investigators analyzing the oral-wash samples were blinded to the patient-related data, and investigators collecting the clinical data were blinded to the PCR-related results. The study was designed to be unblinded after the first year of enrollment, to establish a cut-off level for PCR positivity to be imposed on successive study samples.

Oral-wash samples were obtained at San Francisco General Hospital by having the patient gargle 10 mL of sterile saline for ∼60 s. Oral-wash samples were obtained before either induction of sputum or bronchoscopy. Clinical data were obtained from a standardized medical chart abstraction by use of uniform data abstraction forms. All samples were extracted and assayed in the Clinical Microbiology Laboratory of the National Institutes of Health (Bethesda, MD). DNA was extracted from the oral-wash samples by use of the NucliSens kit (Organon Teknika), as reported elsewhere [11].

A QTD PCR method that targets the multicopy major surface glycoprotein (MSG) gene of P. jiroveci, with associated internal control, and that uses fluorescent probes for detection was used, as described elsewhere [11]. A patent for use of the MSG gene in diagnosing PCP has been applied for. P. jiroveci–positive and –negative BAL samples were included with each DNA extraction and in each PCR run as external controls. The internal control, which is amplifiable by the P. jiroveci primers, was included to detect PCR inhibitors in the samples. All patient samples were analyzed in duplicate, with 1 tube containing the internal control. Ten-fold serial dilutions (10^-2–10^9 copies/μL) of a plasmid containing a P. jiroveci MSG gene insert were prepared, and 3 standards were included in each PCR run, to generate a standard curve and to allow quantification.

A sample was regarded as positive by QTD PCR if both tubes tested positive. If only 1 tube tested positive, a new aliquot of the sample was retested, and, if at least 1 more tube tested positive, the sample was considered to be positive for P. jiroveci. If PCR inhibitors were detected, an additional aliquot of the original sample was retested.

Acquired fluorescence data were analyzed by use of LightCycler software (Roche). For quantification of PCR-positive samples, the results of tubes not containing internal control were recorded, since the coamplification and dual-color detection interfere with the accuracy of the quantification. Statistics were performed by use of the SAS system (version 8.02 for Windows; SAS Institute).

Wilcoxon’s 2-sample test was used to compare quantitative results (copies per tube). A 2-sided P<.05 was considered to be significant. Data are presented as median (range).

RESULTS

After the first year of inclusion, data on 47 episodes were unblinded, in accordance with the study protocol. Only 1 episode of non-PCP was found to be positive by QTD PCR; hence, it was decided not to impose a cut-off value for interpretation of the QTD PCR results on the successive study samples. All samples were included for the final single analysis.

One hundred thirty-one episodes were enrolled in the present study. Eighteen episodes (14%) met criteria for exclusion from data analysis because the diagnosis of PCP or non-PCP was not definitively established. Thus, oral-wash samples from 108 HIV-infected patients with 113 episodes of suspected PCP were included for analysis. At least 6 weeks separated individual episodes in patients with >1 episode.

A diagnosis of PCP was established by direct microscopy (Diff-Quik) for 93 (82%) of 113 episodes. This unusually high prevalence of PCP indicates that the study population is highly select, on the basis of the initial screening done at the clinic.

Overall, the QTD PCR had a sensitivity of 88% and a specificity of 85%. Eighty-two of 93 samples from PCP episodes and 3 of 20 samples from non-PCP episodes were positive by QTD PCR. A positive QTD PCR result was 5.9 times more likely to occur in PCP episodes than in non-PCP episodes, and a negative QTD PCR result was 7.2 times more likely to occur in non-PCP episodes than in PCP episodes.

The duration of treatment for PCP before oral-wash samples were obtained had an effect on the sensitivity of the assay. Oral-wash samples from patients with PCP had higher copy numbers if the patient had received ≤1 day of treatment before oral-wash samples were obtained, compared with >1 day of treatment (median, 417 copies/tube [range, 0–21,290 copies/tube; n = 53] vs. 7.0 copies/tube [range, 0–3673 copies/tube; n = 40]; P<.0001; figure 1A).
Figure 1.  A, Copies per tube detected in oral-wash samples from episodes of *Pneumocystis pneumonia* (PCP) in which the patients had received ≤1 or >1 day of treatment before samples were obtained ($P<.0001$).  B, Copies per tube detected in polymerase chain reaction–positive oral-wash samples obtained within 1 day of treatment from episodes of non-PCP and PCP ($P<.05$). The tentative cut-off of 50 copies/tube is marked for reference. *Below lower limit of detection, previously determined to be 1–5 copies/tube, equaling 150 copies/mL of concentrated sample to be extracted [11]. IQR, interquartile range.

A post hoc analysis limited to the 65 episodes (53 PCP and 12 non-PCP) in which the patients had received ≤1 day of treatment before oral-wash samples were obtained revealed that the QTD PCR had a sensitivity of 92% and a specificity of 75%. Forty-nine of 53 samples from PCP episodes and 3 of 12 samples from non-PCP episodes were positive by QTD PCR. A positive QTD PCR result was 3.7 times more likely to occur in PCP episodes than in non-PCP episodes, and a negative QTD PCR result was 9.9 times more likely to occur in non-PCP episodes than in PCP episodes.

For the 47 episodes in which the patients had received >1 day of treatment, the sensitivity was 83%, and the specificity was 100%. Thirty-three of 40 samples from PCP episodes and 0 of 7 samples from non-PCP episodes were positive by QTD PCR.

Oral-wash samples from 3 (15%) of 20 episodes in patients without PCP were positive by this assay, and all 3 were from patients who had received ≤1 day of treatment before oral-wash samples were obtained. One of these 3 patients was placed on empirical treatment for presumptive PCP because of results of a chest computed tomography study and the presence of pneumothorax. The patient was discharged after 3 days of hospitalization.

The copy numbers detected in these 3 oral-wash samples were lower than the copy numbers detected in PCR-positive oral-wash samples from patients with PCP obtained within 1 day of treatment (median, 32 copies/tube [range, 4.5–35 copies/tube] vs. 575 copies/tube [range, 0.4–21,290 copies/tube]; $P<.05$; figure 1B).

**DISCUSSION**

The present study has demonstrated that the use of this QTD PCR assay on oral-wash samples is a sensitive and specific method for diagnosing PCP in HIV-infected patients. The sensitivity of QTD PCR may be improved by obtaining the oral-wash sample within the first day of treatment for PCP.

Oral-wash samples are noninvasive and easily obtained, and, in the present study, the sensitivity (88%) and specificity (85%) of QTD PCR analysis of oral-wash samples compared well with the previously reported performance of microscopy of induced sputum samples, which, using 3 different stains, had sensitivities of 76%–92% and specificity of 100% [13]. Furthermore, this rapid, quantitative PCR assay yields qualitative results comparable to those of previously reported PCR assays of oral-wash samples in HIV-infected patients, in which sensitivity and specificity have been reported to be 50%–89% and 92%–100%, respectively [3, 4, 6, 7].

What is the significance of finding *Pneumocystis* DNA in samples from patients who are negative for *Pneumocystis* by direct microscopy? One possibility is that the assay has yielded a biologic false-positive result. However, we believe that cross-contamination is highly unlikely, since, in addition to standard laboratory precautions, this assay uses a closed-tube format, and uracil-DNA glycosylase and dUTP were used for additional prevention of amplicon carry over. The second possibility is that the organisms represent colonization. The rate of colo-
Pneumocystis drugs causes a decrease of exposure to therapy for detected by this assay if the oral-wash sample is obtained before possible. However, cases of colonization are more likely to be present in the respiratory tract. To increase the sensitivity of the assay, oral-wash samples should be obtained as early as possible. The third possibility is that Pneumocystis is the cause of concurrent pulmonary dysfunction or fever and that the direct microscopy was incorrect.

We have previously shown that the quantitative results of a QTD PCR assay format correlates well with the number of whole Pneumocystis organisms present in the tested material [14] and that this assay has a high reproducibility of quantitative results [11]. Figure 1A demonstrates that oral-wash samples from patients with PCP had fewer copy numbers if the patient had been receiving treatment for >1 day, rather than ≤1 day, before sampling, which suggests that exposure to anti-Pneumocystis drugs causes a decrease of Pneumocystis organisms present in the respiratory tract. To increase the sensitivity of the assay, oral-wash samples should be obtained as early as possible. However, cases of colonization are more likely to be detected by this assay if the oral-wash sample is obtained before exposure to therapy for >1 day. Figure 1B demonstrates that the PCR-positive oral-wash samples obtained within 1 day of initiation of therapy from colonized patients had fewer copy numbers than those from patients with PCP, suggesting that a cut-off could be useful.

Figure 2 demonstrates, in a post hoc analysis, that, by imposing a cut-off of 50 copies/tube to the samples acquired within the first day of therapy, a specificity of 100% is achieved with a concurrent loss of sensitivity to 70%. Thus, detecting a high copy number (>50 copies/tube) is diagnostic for PCP, whereas detecting a positive QTD PCR result with a low copy number (<50 copies/tube) supports the diagnosis, but further diagnostic procedures would be warranted to achieve sufficient diagnostic precision. A negative QTD PCR result is 9.9 times more likely to occur in non-PCP than PCP, but further testing could be appropriate in a highly suspect case.

One potential weakness of the present study is the high pre-test probability of PCP. The low rate of non-PCP cases in this series (20/113 [18%]) attests to a highly efficient screening procedure at the clinic. Because of the unusually high prevalence of PCP in the study population, we have chosen to calculate likelihood ratios instead of predictive values, because the latter are highly dependent on the overall prevalence of the disease [15]. Positive [sensitivity/(1 − specificity)] and reciprocal negative [specificity/(1 − sensitivity)] likelihood ratios are cited above.

The results of the present study suggest that oral-wash samples may be of value in screening for PCP. Sensitivity is improved when the oral-wash sample is obtained within 1 day of initiation of treatment. Furthermore, the results suggest, but do not prove, that, by using this QTD PCR assay, it may be possible to establish an interpretative cut-off value for PCR-positive samples obtained within 1 day of initiation of treatment to distinguish infection from colonization.

References


