Development and Characterization of a Molecular Viability Assay for *Pneumocystis carinii* f sp *hominis*

Nancy H. Maher,¹ Sten H. Vermund,² David A. Welsh,¹ H. Kenneth Dillon,¹ Abeer Awooda,² and Thomas R. Unnasch³

*Pneumocystis carinii* pneumonia (PCP) remains the most common opportunistic infection among human immunodeficiency virus–infected persons. Despite this, little is known concerning the transmission dynamics of this infection. In the absence of a reliable method to isolate and culture *P. carinii* from environmental samples, it has not been possible to assess the importance of person-to-person transmission in the epidemiology of PCP. A molecular viability assay was developed for the human form of *P. carinii* (*P. carinii* f sp *hominis*) that is applicable to both clinical specimens and environmental samples. This assay will enable the evaluation of the spread and persistence of viable human *P. carinii* in the environment.

Pneumocystis carinii pneumonia (PCP) remains the most common opportunistic illness among human immunodeficiency virus–infected persons in the United States, because many persons who develop PCP do not meet current criteria for initiation of prophylaxis, are unaware of their HIV status, or are not receiving medical care [1]. Little is known about the transmission dynamics of this infection. In the absence of a reliable method to isolate and culture *P. carinii* from environmental samples, it has not been possible to assess the importance of person-to-person transmission in the epidemiology of PCP. A molecular viability assay was developed for the human form of *P. carinii* (*P. carinii* f sp *hominis*) that is applicable to both clinical specimens and environmental samples. This assay will enable the evaluation of the spread and persistence of viable human *P. carinii* in the environment.

Materials and Methods

Subjects enrolled in this study were inpatients diagnosed with putative PCP at the Louisiana State University Health Science Center. The diagnosis was microscopically confirmed for 5 of 9 patients; no laboratory confirmation was made for 4 patients. The population comprised whites and blacks (8 men and 1 woman) with an age range of 31–52 years. All patients were treated for PCP (with TMP-SMZ or dapsone) before study enrollment.

Sputum and bronchoalveolar lavage (BAL) samples collected from study subjects were centrifuged at 4200 g for 20 min. Pellets were resuspended in 250 μL of PBS, and 25 μL of 12,000 U/mL of lyticase in PBS (Sigma Chemical) was added. Samples were incubated at 37°C for 30 min, and 1 mL of a commercial guanidinium isothiocyanate solution (RNAzol B; Teltest) was added. Total nucleic acid was isolated from the samples by following the manufacturer’s instructions, except that the samples were mixed vigorously to release genomic DNA.

Indoor air samples were collected from the operatory of a dental clinic associated with the outpatient clinic for HIV-positive persons at the University of Alabama at Birmingham (UAB) and from a UAB dormitory. Outdoor air samples were collected outside the clinic and dormitory by filtering air through 25-mm diameter 0.8-μm pore size polyvinylidene difluoride filters at 1.0 L/min for 4 h. Each filter was diced and hydrated with 500 μL of PBS. Lyticase (25 μL of 12,000 U/mL in PBS) was added, and the samples were...
incubated at 37°C for 30 min. Total nucleic acid was extracted from the filters, as described above.

Human _P. carinii_ DNA was detected by amplification of 18S mitochondrial ribosomal DNA sequences, as described elsewhere [10]. The RT-PCR viability assay was based on detection of the Phsb1 transcript of human _P. carinii_ (GenBank accession no. U80970). The coding primer (Phsb1-161C, 5'-TGTTAAAAAG-ACATGAAATG-3') and noncoding primer (Phsb1 566nc, 5'-CAGCAGTGGCTTTAACTGAA-3') were designed to span the boundaries of the third and fifth introns of the Phsb1 gene, permitting amplification from cDNA but not genomic templates. The Phsb1 566nc primer was added to RNA preparations, to a final concentration of 4.3 μM in a volume of 5.75 μL. The mixture was incubated at 70°C for 15 min and was cooled to 0°C for 5 min. The solution was adjusted to 10 μL so that it contained 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 10 mM dithiothreitol, 1 mM each of dATP, dCTP, dGTP, and dTTP, 100 U of RT (Superscript II; Life Technologies), and 10 U of pancreatic RNase inhibitor (RNASin; Promega). The reaction was incubated at 45°C for 50 min and then at 70°C for 10 min. The reaction was adjusted to 50 μL by the addition of 40 μL of a solution containing 60 mM Tris-HCl (pH 8.5), 15 mM (NH4)2SO4, 3.5 mM MgCl2, 200 μM each of dATP, dCTP, dGTP and dTTP, 0.5 μM of each primer, and 2.5 U of AmpliTaq DNA polymerase (Applied Biosystems). PCR conditions consisted of 40 cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C. RT-PCR products were analyzed by gel electrophoresis and then were stained with ethidium bromide.

**Results**

To adapt the RT-PCR viability assay developed for human _P. carinii_, primers were designed to target a fragment of the Phsb1 mRNA [11]. Like the gene targeted in the rat _P. carinii_ assay, this gene is a member of the hsp70 family. The primers were designed so that the amplification could proceed only from mRNA-derived sequences. Use of these primers in RT-PCR resulted in an amplicon of the expected size of 425 bp when total nucleic acid extracted from a fresh BAL sample was used as a template (figure 1A). A product of the expected size was not seen in reactions lacking RT, which confirms that the reaction was specific for mRNA. DNA sequence analysis of the expected product confirmed that it was derived from Phsb1 mRNA (data not shown).

The assay was applied to 3 samples—a fresh BAL sample, an aliquot from the same BAL that was heat-killed by autoclaving, and an oral wash sample obtained before collection of the BAL fluid—collected from a patient with confirmed PCP. Strong amplification was obtained from the nucleic acid extracted from the viable BAL sample, whereas a weak signal was obtained from the oral wash sample (figure 1B). No signal was obtained from the heat-killed BAL sample, which confirms that the assay was capable of distinguishing viable from nonviable organisms.

To gain insight into the ability of the Phsb1 RT-PCR to detect _P. carinii_ mRNA in clinical specimens, nucleic acids were isolated from BAL fluid from 9 persons with putative PCP. All had received antipneumocystis treatment for varying periods of time before sample collection. PCR-detectable human _P. carinii_ DNA was detected in 6 of 9 samples and in 5 of 5 of those with diagnoses confirmed by microscopic examination. Of the samples positive in the DNA PCR, 4 of 6 overall and 4 of 5 with microscopically confirmed diagnoses contained mRNA detectable by the Phsb1 RT-PCR (table 1). Previous studies have demonstrated that it is possible to detect DNA derived from _P. carinii_ in air samples collected from areas inhabited by persons at risk for and infected with _P. carinii_ [4]. To determine whether similar samples contained detectable _P. carinii_ mRNA, nucleic acid was extracted from air samples collected as described in Materials and Methods. These samples were assayed first for the presence of PCR-detectable human _P. carinii_ DNA, and positive samples were tested further for that organism’s mRNA by using the Phsb1 RT-PCR. None of the samples collected from environments not inhibited by per-
PCR assays reflect differences in the persistence of RNA and DNA in organisms that are killed by drug treatment. More research will be needed to determine whether this is the case.

Our data show that the Phsb1 RT-PCR can be applied to detect viable human *P. carinii* in air samples. However, there is currently no reference standard assay for detecting *P. carinii* in air samples. Furthermore, in the current study, RT-PCR analysis was done only on air samples that were found to be positive by *P. carinii* DNA PCR. Thus, more work is needed to assess the relative sensitivity and specificity of the Phsb1 RT-PCR assay for the detection of viable *P. carinii* in environmental samples. Despite this, the assay should prove to be useful in detecting and estimating the persistence of viable human *P. carinii* in the environment. A PCP prevention strategy that incorporates exposure prevention might permit the more judicious use of prophylaxis. Determining whether or not such an exposure prevention strategy is feasible is directly related to determining relative importance of the potential sources of infectious *P. carinii*. If specific sources such as a particular environmental niche or infected persons are found to be important sources of viable organisms, then infection control practices might prove to be useful. The molecular viability assay described here will be useful in assessing the prevalence and persistence of viable human *P. carinii* in the environment, allowing these questions to be addressed.

### Table 1. Analysis of clinical and environmental samples for the presence of *Pneumocystis carinii f* sp *hominis* by 18S rRNA polymerase chain reaction (PCR) and Phsb1 reverse-transcriptase (RT)-PCR.

<table>
<thead>
<tr>
<th>Sample type</th>
<th>No. <em>Phsb1</em> PCR positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bronchoalveolar lavage</td>
<td></td>
</tr>
<tr>
<td>6 Positive</td>
<td>4</td>
</tr>
<tr>
<td>3 Negative</td>
<td>0</td>
</tr>
<tr>
<td>Non-HIV–positive environment</td>
<td></td>
</tr>
<tr>
<td>0 Positive</td>
<td>ND</td>
</tr>
<tr>
<td>18 Negative</td>
<td>ND</td>
</tr>
<tr>
<td>HIV outpatient clinic</td>
<td></td>
</tr>
<tr>
<td>27 Positive</td>
<td>7</td>
</tr>
<tr>
<td>56 Negative</td>
<td>ND</td>
</tr>
</tbody>
</table>

NOTE. HIV, human immunodeficiency virus; ND, not determined.

Discussion

Our data demonstrate that an RT-PCR assay that targets Phsb1 mRNA is capable of distinguishing viable from heat-killed human *P. carinii*. This assay was based on a previous report that described a molecular viability assay for the *P. carinii* that infects rats and which could distinguish viable organisms from those killed by a variety of methods (e.g., heat, desiccation, and UV irradiation) [9]. Because of the difficulty in culturing and quantitating the human *P. carinii*, it has not been possible to determine the limit of detection of the Phsb1 RT-PCR assay. Since the parallel assay developed for the rat *P. carinii* was capable of detecting as few as 10^5 viable organisms [9], it is likely that the assay reported above will exhibit a roughly similar limit of detection.

When the Phsb1 RT-PCR was evaluated on BAL samples collected from persons with putative PCP, none of the samples negative in the DNA PCR was positive in the RT-PCR, whereas 4 of 6 samples positive in the DNA PCR were positive by RT-PCR. These results suggest that the RT-PCR is specific, but only ~60% sensitive, compared with the DNA PCR. There are 2 possible explanations for this. First, it is possible that the limit of detection of the RT-PCR is higher than that of the DNA PCR, leading to a relative lack of sensitivity in the RT-PCR, compared with that in the DNA-based assay. However, all the study subjects were treated for PCP before the samples used in this study were collected. Thus, it is possible that the differences in the results obtained with the RT-PCR and DNA PCR assays reflect differences in the persistence of RNA and DNA in organisms that are killed by drug treatment. More research will be needed to determine whether this is the case.

Our data show that the Phsb1 RT-PCR can be applied to detect viable human *P. carinii* in air samples. However, there is currently no reference standard assay for detecting *P. carinii* in air samples. Furthermore, in the current study, RT-PCR analysis was done only on air samples that were found to be positive by *P. carinii* DNA PCR. Thus, more work is needed to assess the relative sensitivity and specificity of the Phsb1 RT-PCR assay for the detection of viable *P. carinii* in environmental samples. Despite this, the assay should prove to be useful in detecting and estimating the persistence of viable human *P. carinii* in the environment. A PCP prevention strategy that incorporates exposure prevention might permit the more judicious use of prophylaxis. Determining whether or not such an exposure prevention strategy is feasible is directly related to determining relative importance of the potential sources of infectious *P. carinii*. If specific sources such as a particular environmental niche or infected persons are found to be important sources of viable organisms, then infection control practices might prove to be useful. The molecular viability assay described here will be useful in assessing the prevalence and persistence of viable human *P. carinii* in the environment, allowing these questions to be addressed.

### References