Development and Evaluation of a Molecular Viability Assay for *Pneumocystis carinii*

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Despite recent declines in incidence, *Pneumocystis carinii* pneumonia (PCP) remains the most commonly occurring opportunistic illness among persons with AIDS in the United States. While *P. carinii* DNA has been detected in patient respiratory specimens and in air samples collected from various indoor environments housing PCP patients, the viability of these organisms is unknown. For this reason, we have developed and evaluated a molecular viability assay for *P. carinii*. This method is based upon the detection of *P. carinii* mRNA by a reverse transcription-PCR that employs specific primers from a member of the heat shock protein 70 family. Under optimal assay conditions, these primers were capable of detecting as few as 100 viable trophozoites as determined by ethidium bromide staining, while no signal was obtained from 10^6 trophozoites killed by heat, desiccation, or UV radiation. This assay was also capable of distinguishing *P. carinii* from other common fungi present in the air. Therefore, this molecular viability assay may be useful in conjunction with standard bioaerosol collection devices and procedures for the detection of viable *P. carinii* collected from various indoor environments. It may also be useful in confirming the presence of viable trophozoites in respiratory specimens collected by noninvasive techniques from putatively infected individuals.

Despite the recent declines in incidence due to widespread chemoprophylaxis and antiretroviral therapy in immunosuppressed human immunodeficiency virus (HIV)-infected individuals, *Pneumocystis carinii* pneumonia (PCP) is still the most common opportunistic infection during the course of AIDS in the United States (7). However, for those persons who cannot tolerate antipneumocystis therapy, or who are unaware of their HIV status, the risk of PCP remains high (41).

Basic knowledge of *P. carinii* ecology and epidemiology is still lacking. The once widely accepted theory of PCP reaction in persons with severe immunosuppression has come into question with recent studies providing evidence to support the hypothesis that most episodes of PCP result from a de novo acquisition. Support for this new hypothesis comes from studies in which both human and rat *P. carinii* DNAs have been detected in large-volume air samples collected from the outdoor air (40) and in which the seroprevalence of antibodies against *P. carinii* found in young children has been high (32).

The method of choice for the diagnosis of PCP involves collection of specimens by either induction of sputum or bronchoalveolar lavage (BAL), followed by microscopic visualization of *P. carinii* trophozoites and/or cysts. The sensitivity of microscopic examination of induced sputum has been shown to be variable. For this reason, it has been recommended that BAL be performed on patients presenting with symptoms of PCP but who exhibit a negative induced-sputum sample (41). On the other hand, because of the intrusiveness of these techniques, many clinicians empirically treat suspected PCP cases with antipneumocystis drugs, although this has been demonstrated to be a highly nonspecific approach (6).

Recent studies have demonstrated that *P. carinii* DNA can be detected in oral wash samples by PCR (2, 15, 37). This technique represents a less invasive method for diagnosing the presence of *P. carinii* organisms in presumptively infected individuals, providing a strong incentive to demonstrate organism viability in these samples.

Although it is possible to detect *P. carinii* DNA by PCR in environmental and clinical samples, DNA detection gives no indication of organism viability. It is therefore unclear what the
above-mentioned results mean in terms of potential risk for susceptible individuals, potential exposure control measures, or the ecology of *P. carinii*. In order to evaluate the viability of *P. carinii* organisms collected from environmental and clinical sources, we have developed a molecular viability assay to be used in conjunction with standard bioaerosol collection techniques as well as noninvasive clinical specimen collection methods.

**MATERIALS AND METHODS**

*P. carinii* sp. *f. carinii* culture. *P. carinii* sp. *f. carinii* trophozoites and cysts were harvested from preparations derived from spinner-flask cultures prepared as previously described (22). Briefly, mixed cultures of *P. carinii* trophozoites and cysts isolated from infected rat lungs were grown in a spinner-flask culture containing human embryonic cells sheeted to Cytodex microcarrier previously described (22). Briefly, mixed cultures of *P. carinii* harvested from preparations derived from spinner-flask cultures prepared as described above were initially annealed in a solution containing a 4 **-CTGCTGCAGTAGGCTCATTG-3** gene sequence. Sequences prepared from one aliquot (10⁴ to 10² trophozoites), while the other three aliquots of trophozoites were subjected either to heat (autoclave), desiccation (left on bench top for 1 week), or UV light exposure (left under a germicidal lamp for 24 h). Loss of viability in these treated samples was confirmed by microscopy, as described in Materials and Methods. Total RNA was then extracted from each of the treated samples in the same manner as for the untreated sample. A positive signal was obtained from the aliquot containing 10² viable trophozoites, while no signal was obtained from any of the treated samples (Fig. 3). Thus, the assay was capable of distinguishing 10² viable *P. carinii* trophozoites from 10⁴ nonviable *P. carinii* trophozoites.

**RESULTS**

RNA-specific primers from a member of the *P. carinii* sp. *f. carinii* HS70 multigene family (PeSA1) were designed for use in an RT-PCR assay to assess organism viability. While the majority of the HS70 genes in *P. carinii* are constitutively expressed, expression of PeSA1 is upregulated by brief exposure to moderate heat (35). The 5′ (coding orientation) primer was designed from the eDNA sequence so that the sequence of the primer included the second intron splice site in the PeSA1 sequence (GenBank accession number U80967) (Fig. 1A). The 3′ (noncoding) primer was derived from the third exon (Fig. 1A). Use of these primers in an RT-PCR assay employing total RNA extracted from roughly 10⁴ trophozoites isolated from a spinner-flask culture resulted in the production of a 530-bp PCR product, equivalent in size to that predicted from analysis of the mature mRNA (Fig. 1B). DNA sequence analysis of this product confirmed that it was derived from the mature PeSA1 transcript. Furthermore, the 530-bp product was not obtained from mRNA extracted from uninfected rat lungs, providing additional evidence that this product was derived from *P. carinii* and not host cell mRNA (data not shown). As expected, the 530-bp product was also absent from reaction mixtures lacking reverse transcriptase, as the design of the coding primer across the intron splice site prevented the amplification of this product from contaminating *P. carinii* genomic DNA (Fig. 1B). In order to determine the sensitivity of the assay, RNAs extracted from serial dilutions of spinner-flask-cultured organisms were employed as templates in the RT-PCR assay. The assay was found to detect as few as 10² trophozoites as determined by EB staining (Fig. 2). To examine the specificity of the RT-PCR for the detection of viable organisms (i.e., the ability of the assay to distinguish viable from nonviable organisms), four aliquots, each containing 10⁹ viable trophozoites, were prepared. Total RNA was extracted from an untreated dilution series prepared from one aliquot (10⁴ to 10² trophozoites), while the other three aliquots of trophozoites were subjected either to heat (autoclave), desiccation (left on bench top for 1 week), or UV light exposure (left under a germicidal lamp for 24 h). RRNA was then extracted from each of the treated samples in the same manner as for the untreated sample.
One major application of a molecular viability assay for *P. carinii* will be to detect the presence of viable organisms in the environment. It was therefore of interest to explore the level of species specificity of the RT-PCR. To accomplish this, the *P. carinii* primers were employed to attempt to amplify a related sequence from RNAs prepared from three different species of ascomycetous fungi as described in Materials and Methods. The PcSA1 primers amplified a small fragment from *Penicillum chrysogenum* and a fragment derived from *S. cerevisiae* of approximately the same size as that amplified from *P. carinii* (Fig. 4A). However, the band derived from *S. cerevisiae* was easily distinguished from that of *P. carinii* on the basis of hybridization with the bona fide *P. carinii* PCR product (Fig. 4B).

Since spinner-flask cultures consisted primarily of trophozoites (95 to 99%), it was unlikely that the small number of cysts in the harvest preparations contributed much to the results obtained from the spinner-flask cultures. Therefore, it was necessary to determine if the heat shock RT-PCR assay was capable of detecting a preparation primarily composed of cysts. To accomplish this, *P. carinii* organisms were isolated from rat...
lungs with overt *P. carinii* infection. The mixed-life cycle preparation was then treated in order to isolate the cysts from the trophozoites, as described in Materials and Methods. Bright-field microscopic examination of the cyst-enriched preparation revealed no intact trophozoites in 20 fields, while the cyst concentration was estimated to be at least $10^7$/ml. Together, these data suggested that the enriched-cyst preparation contained less than 0.1% trophozoite contamination. Epifluorescence microscopic examination of the preparation suggested that greater than 80% of the cysts in the enriched preparation were viable.

Total RNA was isolated from the enriched-cyst preparation as described in Materials and Methods, and poly(A)$^+$ RNAs purified from the total RNA preparations were used as templates in the RT-PCR. The additional purification step [poly(A)$^+$ RNA isolation] was found to be necessary to eliminate inhibitors of the RT reaction found to be present in the total RNA preparations prepared from the enriched cysts (data not shown). Following RT-PCR, the poly(A)$^+$ RNA preparation from the enriched-cyst preparation resulted in material that supported the amplification of the PcSA1 product (Fig. 5).

**FIG. 4.** Species specificity of the PcSA1 RT-PCR. RT-PCRs were carried out on fungal RNA preparations prepared as described in Materials and Methods. (A) EB-stained gel of PCR products; (B) Southern blot of the gel shown in panel A probed with the labeled PcSA1 PCR product. In each panel, lane 1 contains RNA extracted from *P. carinii*, lane 2 contains RNA extracted from *S. cerevisiae*, lane 3 contains RNA extracted from *Penicillium chrysogenum*, and lane 4 contains RNA extracted from *A. niger.*

**FIG. 5.** Detection of *P. carinii* cysts by the PcSA1 RT-PCR. Lane 1, poly(A)$^+$ mRNA extracted from a cyst-enriched preparation; lane 2, RNA derived from a spinner-flask culture; lane 3, cyst-enriched preparation of RNA amplified in the absence of reverse transcriptase; lane 4, PCR negative control.

**DISCUSSION**

The rationale for the viability assay described above was that mRNA molecules, as opposed to DNA or rRNA, are usually unstable following the death of an organism. By designing primers that span an intron splice site in the PcSA1 sequence, we anticipated that only intact mRNA molecules would serve as a template in the RT-PCR and that these molecules would be labile following the death of the organism. The data described above suggest that this was the case. The RT-PCR assay was capable of detecting as few as $10^2$ viable trophozoites as determined by EB staining. In contrast, $10^4$ nonviable trophozoites killed by either high heat, desiccation, or UV exposure produced no signal in the RT-PCR assay. This is significant, as the last two methods of killing represent the two most important mechanisms resulting in the loss of viability of airborne microorganisms (10). Apart from its ability to distinguish viable and nonviable *P. carinii*, this RT-PCR assay can distinguish between viable *P. carinii* and other fungi that commonly occur in the indoor environment. Both *Aspergillus* and *Penicillium* sp. are commonly present in the indoor environment (14), a finding confirmed with our own air sampling of an urban indoor residence, in which moderate levels of viable *A. niger*, *Aspergillus fumigatus*, and *Penicillium* were found (data not shown).

Although most of the experiments carried out in the work described above involved trophozoites obtained from spinner-flask-cultured organisms, the assay was also capable of detecting the PcSA1 transcript in a cyst-enriched preparation obtained directly from an infected rat lung. Although this preparation was highly enriched for cysts, we cannot rule out the possibility that the positive signal obtained from this preparation resulted from contamination of the cyst preparation, either with intact trophozoites or with residual mRNA released from the lysed trophozoites.

While the life cycle stage involved in airborne transmission is unknown, the likely candidate would be the *P. carinii* cyst due to the structure of its cell wall, which possibly allows for protection against environmental stressors such as desiccation. Thus, determining how well this RT-PCR assay assesses cyst viability is key to its use with environmental samples. There are precedents for use of the RT-PCR in assessing the viability of both *Giardia* cysts (1, 19) and *Cryptosporidium* oocysts collected from environmental samples (19). These assays all exploited the heat shock responses of the two organisms, distinguishing viable from nonviable cysts by detecting the heat shock transcript by RT-PCR after heat treatment.

Culturing of air samples collected from the environment is standard procedure in the field of aerobiology when the presence of viable biological agents is assessed (11). Thus, one possible approach for viable *P. carinii* cyst detection might involve short-term (<24 h) culture of cysts collected from the environment by bioaerosol collection methods such as filtration or liquid impingement. This would allow for maturation of the eight intracystic bodies to trophozoites. RNAs extracted from the cultures containing the newly emerged trophozoites could then be used as templates in the RT-PCR assay.

When bacteria are selectively sampled, a fungicide is added to the medium, and when fungal spores are selectively sampled, antibiotics are added to the medium. Unlike any other known species of fungus, *P. carinii* lacks the steroid ergosterol in its cell wall. Thus, it is not susceptible to the class of fungicidal drugs that inhibit ergosterol biosynthesis (4). Therefore, antibiotics and antifungals such as the imidazole drugs can be added to a short-term culture of *P. carinii*, allowing for its
growth while preventing the growth of other fungi and bacteria collected in the sample.

One additional application of this viability assay may be in clinical diagnosis of PCP. It is possible to envision a two-step system to utilize the RT-PCR in combination with the DNA-based PCR for this purpose. The DNA-based PCR might first be used to detect P. carinii DNA in patient oral wash samples. If evidence for the presence of P. carinii DNA is obtained, positive individuals can be restated by employing the RT-PCR assay to confirm the presence of viable trophozoites, which are the most prevalent life cycle stage found in an infected host (24). This strategy might represent a sensitive and specific diagnostic protocol that is much less invasive than BAL or induction of sputum. This approach might also prove useful in detecting low levels of viable organisms in the face of highly active therapies where organism burdens may be lower than in untreated disease.

Additional work on this assay will be needed before it can be used as a reliable tool in a field study of the occurrence of viable P. carinii sp. f. hominis in the environment. Issues such as organism recovery efficiency from bioaerosol collection devices and potential environmental interference must be worked out. Finally, the system must be adapted for use with the human-specific variant of P. carinii. With the recent successes in the development of a human P. carinii culture system (27), this should be relatively straightforward.

Determination of organism viability does not prove infectiousness. However, determining where, when, and under what circumstances viable P. carinii occurs in the environment will help to narrow down the list of potential sources of infection. If the theory of PCP patients contaminating indoor environments with infectious organisms holds true, then there are many infection control approaches that can be used to limit exposure to at-risk individuals to the organism. Given the potential for antibiotic resistance (25) and the toxicity associated with P. carinii prophylaxis among HIV patients (33), exposure prevention may be preferable if person-to-person transmission can be confirmed as an important mode of infection in studies employing the molecular viability assay described herein.

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REFERENCES


