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Real time quantitative PCR and RT–PCR for analysis of *Pneumocystis carinii hominis*

Karine Kaiser *, Meja Rabodonirina, Stéphane Picot

Laboratoire de Parasitologie, Mycologie Médicale et Maladies Tropicales–Hospices Civils de Lyon-EA 3087-Université C. Bernard Lyon I-8, avenue Rockefeller 69373, Lyon Cedex 08, France

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Abstract

Pneumocystis carinii hominis is a common cause of pneumonia in immunocompromised patients and particularly in those infected by HIV. Giemsa- and Gomori–Grocott-stained smears are widely used for detection and quantification of this opportunistic fungus obtained from biological samples or from in vitro culture. But these methods are fastidious and time-consuming. Thus, instead of performing a count of organisms, we focused our attention on the level of specific DNA by a quantitative PCR technique. This procedure has the advantage of greater precision and more objectivity. To verify the presence of organisms, quantitative RT–PCR based on DHFR and a cell cycle mRNA have been developed. In this current study, we present a detailed description of these methods and their applications for analysis of *P. carinii hominis*.

Keywords: *Pneumocystis carinii hominis*; Quantification assay; Viability assay; Quantitative PCR; Quantitative RT–PCR

1. Introduction

Pneumocystis carinii hominis is a common cause of pneumonia in immunocompromised patients, particularly those infected with HIV. Diagnosis of pneumocystosis depends on the detection of the fungal pathogen in bronchoalveolar lavage (BAL) samples obtained by pulmonary bronchoscopy. Giemsa- and Gomori–Groccot-stained smears are widely used for that purpose, but these methods provide only low sensitivity and reliability. Giemsa stains help visualize these small (< 10 μm) organisms, and silver stains help distinguish the cystic stages. However, proper training is needed to accurately identify *P. carinii* from the other cells and microorganisms in BAL samples. Furthermore, *P. carinii* are found in thick mucoid human BAL samples as clumps of varying sizes, these clumps are very difficult to disperse. Thus, pathogen load is commonly estimated by counting *P. carinii* clumps (Baughman and Liming, 1998). The current clinical situation makes quantitation of human-derived *P. carinii* questionable.

Recent progress has been made in the establishment of axenic cultures of *P. carinii* (Merali et al., 1999), thus it is much more feasible to develop methods for reliable quantification of organism proliferation. Also, viability assays of organisms in

* Corresponding author. Tel.: +33-47877-7218; fax: +33-47875-1772.

E-mail address: kaiser@rockefeller.univ-lyon1.fr (K. Kaiser).

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culture can be assessed. Thus, instead of performing microscopic enumeration of organisms, quantitation of *P. carinii*-specific DNA can provide greater precision and avoid identifications of other organisms in the sample.

Two methods have been described for that purpose. One approach is by using DNA amplification of *P. carinii* nucleotide sequences by PCR, described as semiquantitative PCR (O'Leary et al., 1995). The other is by using quantitative image analysis with a fluorescence microscope or fluorescence-activated cell sorter employing *P. carinii*-specific probes in conjunction with fluorescent dyes. Both methods present serious limitations in the ability to monitor replication and proliferation of *Pneumocystis* organisms in situ, as well as in axenic or monoxenic cultures. These techniques can provide only semiquantitative data compared to a standard template. Other investigators have recently described the use of Hoescht and other fluorescent dyes to detect DNA from *P. carinii carinii* (isolated from rat or culture system), and used those values to estimate the amount of DNA by the fluorescence of a single data point for each sample.

As an alternative improved method for monitoring *P. carinii* organism numbers, we measured the amount of *P. carinii* DNA by quantitative PCR using LightCycler™ (Roche Molecular Biochemicals, Mannheim, Germany) technology. Furthermore, to verify the viability of organisms in the samples, methods were developed for quantitative RT–PCR of a DHFR mRNA plus *cdc*2 cell cycle regulation mRNA. In this report, we present a detailed description of these methods and their applications to monitoring of in vitro cultivation of *P. carinii*.

2. Materials and methods

2.1. P. carinii hominis samples

P. carinii hominis were obtained from BAL samples described from patients with AIDS or from HIV negative individuals submitted to immunodepressing treatments for neoplasia, blood malignancies, organ transplantation or chronic inflammatory diseases. Twenty-three of these samples were maintained in culture, according to previously described methods (Kaiser et al., 1999a,b), several days prior to these experiments using DNA amplification.

For quantification of DNA and cDNA, standards were prepared from *P. carinii* organisms isolated from the lungs of rats immunosuppressed by dexamethasone treatment, as previously described (Bartlett et al., 1988). Serial dilutions of these standard nucleic acid samples were frozen in small aliquots, then used in these experiments.

2.2. Preparation of nucleic acids

DNA of *P. carinii hominis* from BAL samples and in vitro cultures, and the standard rat-derived *P. carinii* DNA were extracted with Qiamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Total RNA was extracted using a High Pure RNA Isolation Kit (Roche Molecular Biochemicals) including DNAse treatment. The mRNA was subjected to reverse transcription into cDNA by the following protocol: 20 μl of reverse transcription reaction mixture containing total cellular RNA, 5 μM of oligo$(dT)_{15}$, 5 × RT buffer (260 mM Tris–HCl, 200 mM KCl, 25 mM $MgCl_2$, 2.5% Tween 20), 100 mM dithiothreitol, 10 mM dNTP, 40 U/ml of RNase Inhibitor and 50 units of Expand™ Reverse Transcriptase (Roche Molecular Biochemicals) were incubated at 42°C for 60 min, heated to 95°C for 5 min, and then quick-chilled on ice.

2.3. Primers pairs

The sequence of the primers (Paz 102-E 5′-GATGGCTGTTTCCAAGCCCA-3′, Paz 102-H 5′-GTGTCGTTGCAAAGTACTC-3′) used for the amplification of the *P. carinii* mitochondrial small subunit rRNA (mtLSUrRNA) gene was designed according to Wakefield et al. (1990). The *P. carinii hominis cdc*2 gene was amplified using the primers (5′-TATTCCAAAAGACATGATGC-3′, 5′-CAGAATCGCCTGGAAATAAC-3′) previously designed (Kaiser et al., 1999a,b) by analogy with the *cdc*2 gene reported from rat-specific *P. carinii* (Thomas et al., 1998). These primers were checked using Oligo 5 software (MedProbe, Oslo, Norway) and were controlled for the absence of base mispairs between the primer and non-*P. carinii* sequence.

2.4. Real time PCR

The real time PCR assay involves LightCycler™ technology, which combines rapid thermocycling with on-line fluorescence detection of the PCR products. The reactions were performed in a volume of 20 μl of a mixture containing 0.6 mM of each oligonucleotide primer and 2 μl of DNA Master SYBR green (Roche Molecular Biochemicals) containing Taq DNA polymerase, reaction buffer, dNTP mix and the double stranded DNA (dsDNA)-specific fluorescent dye SYBR green I. The final concentration of $MgCl_2$ was adjusted to 5 mM. Samples were placed into glass capillary, capped, centrifuged for a few seconds in a micro-centrifuge using appropriate adapters, and then placed into the LightCycler™ rotor. Amplification occurred in a three-step procedure: denaturation at 95°C for 2 min, 45 cycles of denaturation at 95°C for 5 s, annealing at 56°C for 10 s and extension at 72°C for 20 s. The transition rate of temperature was set at 20°C/s for denaturation to annealing, 20°C/s from annealing to extension and 10°C/s from extension to denaturation. Our different primer pairs have the identical optimal PCR annealing temperature. Acquisition of the fluorescent signal from the samples was carried out at the end of the elongation step. To detect the specific product from non-specific products and primer dimers, melting analysis was done immediately after amplification. The PCR product was heated to 95°C, annealed at 66°C (annealing temperature +10°C), and then slowly heated from 66°C to 95°C at 0.2°C/s to obtain the melting curve. The PCR products were subjected to analysis by electrophoresis on a 1.5% agarose gel, to confirm the efficiency of the melting curve analysis.

3. Results and discussion

The aim of this study was to provide a reliable method to measure the amount of *P. carinii hominis* cells in samples obtained from the lungs of immunocompromised patients or from in vitro cultures. Since the enumeration of this organism by microscopic examination of stained smears is not simple, accurate or highly sensitive for this purpose, we focused our attention on quantifying the level of *P. carinii*-specific DNA using a quantitative PCR method for DNA amplification.

It was found that an accurate quantitation of the sample DNA could only be obtained during the log-linear phase of the amplification procedure. Real time PCR with dsDNA SYBR green I incorporation enabled the monitoring of the PCR product formation once during each cycle. Moreover, at the end of the reaction, the fluorescence can be measured continuously during a slow rise in temperature to monitor the dissociation of the PCR product

The fluorescence signal (F) was plotted in real time against temperature (T) to produce melting curves for each sample (F vs. T). A mathematical transformation of the data is obtained with conversion of melting curves to melting peaks by plotting the negative derivative of the fluorescence against temperature ($-\mathrm{d}F/\mathrm{d}T$ vs. T). The turning point of the curve is a peak that represents the melting point (T_m). Each dsDNA product has its own specific T_m, which is defined as the temperature at which 50% of the DNA becomes single stranded, and 50% remains double stranded. Profiles of the amplification reflect the dynamics of the reaction (Fig. 1). The cycle number (crossing point) at which the fluorescence rises above the defined threshold is the most reliable point for the quantification.

Non-specific products can be formed by non-specific amplification or primer-dimerization in the absence of an adequate amount of template DNA. Non-specific products and primer dimers were not observed in these experiments, demonstrating that the designed primers are appropriate for LightCycler™ quantification with a T_m of 79.6°C (Fig. 2). The PCR products of the expected size, 346 bp, were observed in the agarose gel analyses (data not shown). The T_m analysis demonstrated that artifacts were not generated. In the event artifacts were observed, one can go further and eliminate their formation by using TaqStart antibody (ClonTech, Palo Alto, USA) or FastStart DNA Master SYBR green I (Roche Molecular Biochemicals). Using primers dedicated to mtLSUrRNA, *P. carinii hominis* has been detected from samples containing a specific amount of DNA as little as that of BAL from patients with AIDS. Similar data have been obtained when samples were collected from dishes of a culture assay in development in the laboratory.

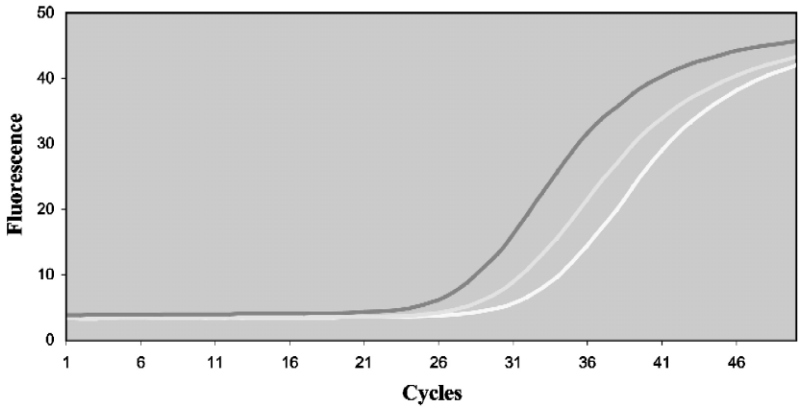

Fig. 1. Fluorescence emitted in the presence of SYBR green I during the amplification of three different standard dilutions of *P. carinii* DNA.

The fluorescence signal was plotted against the cycle numbers for all *P. carinii* samples and the external standards. These standards, made of serial dilutions of rat-derived *P. carinii* DNA (34,000–3.4 pg DNA), have been treated in dedicated capillaries for each amplification cycle.

A standard curve for each run was constructed by plotting the crossing point against the log of the concentration of the *P. carinii carinii* DNA used as the standard in this study (Fig. 3). The quantity of the target molecules in each sample has been calculated by the software provided with the LightCycler™ kit, entering data used to generate this curve. Samples from in vitro cultures of *P. carinii hominis* (from day 3 to day 28) were analyzed. These analyses demonstrated the efficiency of this method for precise evaluation of the amount of *P. carinii hominis* in these samples (Fig. 4).

P. carinii DNA has been detected by various authors in BAL specimens and in air samples collected from various environments but this does not provide information about its viability. However, this information could help to define the potential source of infection. For this reason, and to test whether the *P. carinii* DNA quantified represents viable organisms, we have developed a molecular viability assay for *P. carinii*. Our method is based upon the detection of *P. carinii* mRNA by a quantitative RT–PCR.

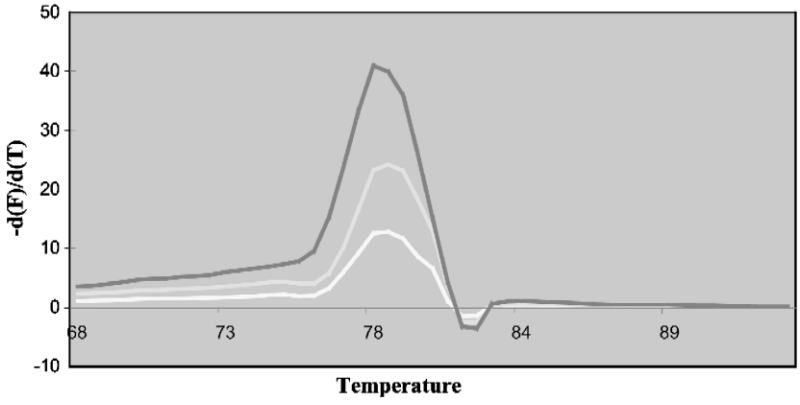

Fig. 2. Melting curves analysis of serial dilutions of *P. carinii* DNA. mtLSUrRNA specific product was generated by LightCycler™ amplification using SYBR green I assay. The T_m of the specific amplified product was 79.6°C.

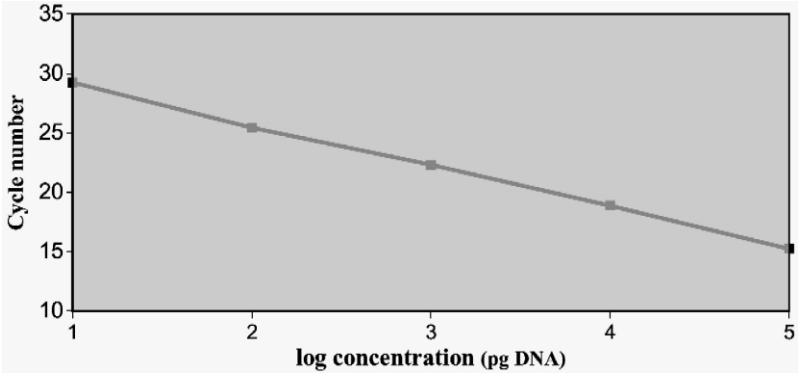

Fig. 3. *P. carinii* DNA (34,000 to 3.4 pg) amplified by LightCycler™ real time PCR. The crossing points (cycle numbers) and the logarithm of the DNA concentration showed a linear standard curve.

Indeed, mRNA molecules are produced only by viable cells.

Primer pairs amplifying DHFR gene (Charry-Reddy and Graves, 1996) was also checked for dimerization and specificity. The quantification of mRNA transcripts were also performed using a DNA standard from rat-derived *P. carinii*. We observed that the DHFR gene is transcribed in vitro (data not shown), thereby suggesting that *P. carinii hominis* DNA represents live *P. carinii* organisms.

Since we have demonstrated the existence of the *cdc*2 gene in *P. carinii hominis* (Kaiser et al., 1999a,b), and its role in the growth of the fungus, we have followed-up the expression of this proliferative cell-cycle protein. The quantification of *cdc*2 transcripts has shown that this gene is transcribed at high levels during in vitro cultivation of the organisms, suggesting that fungi are viable and in the proliferating phase.

Our purpose was to test whether real time PCR could be useful for the detection and the quantification of *P. carinii hominis* under various biological conditions. The data obtained clearly demonstrated that this goal could be achieved using LightCycler™ tools, which combines the advantage of rapidity (half an hour to obtain complete data), no need for isotope or migrations in gels, high specificity for the organism, and accurate quantification of DNA in the

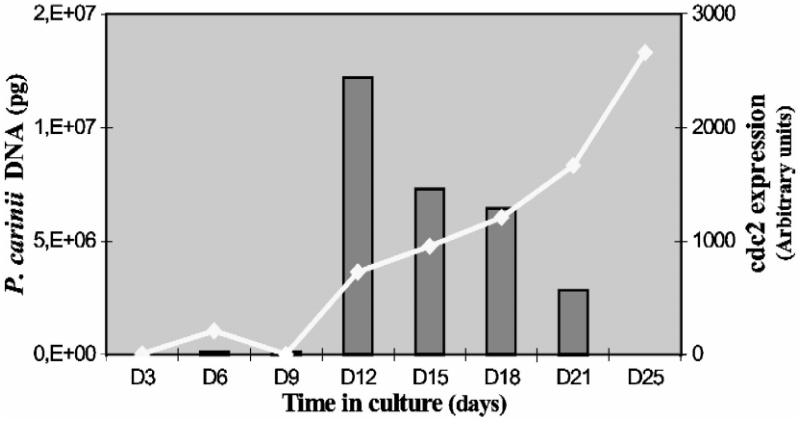

Fig. 4. Follow-up of *P. carinii hominis* culture from day 3 to day 25. The curve represents quantification of *P. carinii* DNA (pg of DNA). Histograms show expression of $p34^{cdc2}$ (Arbitrary units). The ability of human-derived *P.carinii* to transcript *cdc*2 mRNA in vitro, confirms the viability of proliferative organisms.

sample. Since this method is sensitive and easy to perform, it could be used for detecting putative reservoirs of fungus in the outdoor and indoor environments. This could help clinicians to determine if patients are colonized by low amounts of organisms in their lungs and hence, could also serve as a reservoir of the organism. It is well-known that after treatment, examination of stained smears do not readily allow the differentiation between viable cysts from dead "ghosts" or excysted spore cases of *P. carinii*. In that case, the RT–PCR with LightCycler™ that can analyze low numbers of organisms in biological samples is able to distinguish between proliferative and non-viable *P. carinii*. Real time PCR should allow the routine analysis of various samples from patients, including BAL, blood or other biological fluids. This method can provide important information concerning the potential dissemination of the fungus to other sites of the body, and the physiopathology of *P. carinii* pneumonia. Moreover, this method could be applied to other opportunistic pathogens, e.g. *Aspergillus* that may also be in the BAL or blood samples. We now have the capability to detect various microbes in the same sample and thus, can rapidly get data to guide the diagnosis and treatment of patients with respiratory diseases. Another new technology, pan-fungal real time PCR with several pairs of primers, is currently being developed in the laboratory, using similar protocols described in the present report.

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