

## Development of a Rapid Real-Time PCR Assay for Quantitation of *Pneumocystis carinii* f. sp. *carinii*

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**A method for reliable quantification of *Pneumocystis carinii* in research models of *P. carinii* pneumonia (PCP) that is more convenient and reproducible than microscopic enumeration of organisms would greatly facilitate investigations of this organism. We developed a rapid quantitative touchdown (QTD) PCR assay for detecting *P. carinii* f. sp. *carinii*, the subspecies of *P. carinii* commonly used in research models of PCP. The assay was based on the single-copy dihydrofolate reductase gene and was able to detect <5 copies of a plasmid standard per tube. It was reproducibly quantitative ( $r = 0.99$ ) over 6 log values for standards containing  $\geq 5$  copies/tube. Application of the assay to a series of 10-fold dilutions of *P. carinii* organisms isolated from rat lung demonstrated that it was reproducibly quantitative over 5 log values ( $r = 0.99$ ). The assay was applied to a recently reported in vitro axenic cultivation system for *P. carinii* and confirmed our microscopy findings that no organism multiplication had occurred during culture. For all cultures analyzed, QTD PCR assays showed a decrease in *P. carinii* DNA that exceeded the expected decrease due to dilution of the inoculum upon transfer. In conclusion, a rapid, sensitive, and reproducible quantitative PCR assay for *P. carinii* f. sp. *carinii* has been developed and is applicable to in vivo as well as in vitro systems. The assay should prove useful for conducting studies in which quantification of organism burden or growth assessment is critical, such as in vitro antimicrobial susceptibility testing or in vivo immunopathological experiments.**

Accurate quantification of *Pneumocystis carinii* is essential for the correct interpretation of many research experiments of this organism. Since the organism cannot be readily grown in vitro, the traditional quantification method for *P. carinii* is enumeration of organisms by microscopic examination, which is difficult and cumbersome due to the organism's growth in clusters and the presence of different morphological forms (14, 19). Different stains vary in their sensitivities of detection as well as in their abilities to detect the different forms of *P. carinii*. Definitive diagnosis of *P. carinii* pneumonia (PCP) often relies on identification of cysts, which frequently occur within clusters of trophozoites. However, quantification of cysts is not an optimal method for measurement of organism burden, since the cyst-to-trophozoite ratio may change under different conditions and stages of PCP (21, 22).

Quantification by a molecular method would eliminate difficulties in visual enumeration of organisms and would also be independent of the observer. Assessment of organism burden is important in animal models of immunopathogenesis, and accurate quantification is the cornerstone of a number of in vivo and in vitro studies, for example, those pertaining to antimicrobial susceptibilities. Various molecular approaches involving total DNA measurement or semiquantitative PCR methods using end point detection have previously been reported but are currently not broadly utilized (9, 14, 16).

We undertook the development of a real-time PCR assay to

quantify *P. carinii* f. sp. *carinii*, the common subspecies infecting the immunosuppressed laboratory rat, which is the most commonly used in vivo model of PCP (2, 19). We then assessed this new assay during studies attempting to establish the continuous axenic culture system recently described by Merali et al. (14).

### MATERIALS AND METHODS

**Cloned template.** The *P. carinii* f. sp. *carinii* dihydrofolate reductase (DHFR) gene (665 bp; GenBank accession no. AF322061) (3, 12) was cloned into the pCRII vector (Invitrogen) and used as a standard. After propagation and purification of the plasmid, the concentration of DHFR gene copies (number of copies per microliter) was derived from the  $A_{260}$  optical density measurement and the plasmid molecular weight. Tenfold serial dilutions ( $10^{-2}$  to  $10^5$  DHFR gene copies/ $\mu$ l) of the linearized plasmid in water were prepared with glycogen (33.3  $\mu$ g/ml) as the DNA carrier.

***P. carinii*.** *P. carinii* organisms from the lungs of corticosteroid-treated Sprague-Dawley rats (obtained by contract from Indiana University) were partially purified by Ficoll-Hypaque density gradient centrifugation as previously described (7). *P. carinii* pellets were inoculated immediately into the culture medium for in vitro cultivation purposes or stored at  $-70^{\circ}\text{C}$  for subsequent quantitative touchdown (QTD) PCR analysis. For analysis by QTD PCR and microscopy, the pellet (0.7 ml) was thawed and resuspended 20-fold in phosphate-buffered saline (PBS), and 10-fold serial dilutions in PBS were prepared. The number of DHFR gene copies per tube (QTD PCR results) was converted to the number of whole organisms per milliliter of original sample by multiplying by a factor of 30 (because of the concentration of the 200- $\mu$ l sample used during DNA extraction and the utilization of 5  $\mu$ l of DNA extract per PCR tube).

Organisms cultivated and frozen in dimethyl sulfoxide as previously described (14) were kindly provided by A. B. Clarkson, Jr., and S. Merali for inoculation of in vitro cultures.

**In vitro cultivation.** Either freshly isolated organisms from two rats or two frozen aliquots were used for initiation of primary cultures. Axenic in vitro cultures were performed as previously described (14). Briefly, the cultures were maintained in collagen-coated Transwell inserts held in appropriate multiwell

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plates (Costar) and incubated at 31°C in ambient air. The volumes of the media in the insert and below the insert were as previously described (14). The basic medium was minimal essential medium with Earle's salts (Gibco) supplemented as previously described (14). *S*-adenosyl-L-methionine (SAM; Sigma or Research Biochemical International) was added twice daily. In initial experiments, stock solutions of medium containing SAM (500 µg/ml) were freshly prepared, and the medium below the inserts was exchanged twice daily as initially reported (14). In later experiments, freshly prepared SAM stock solution (10 mM) was added separately twice daily to the culture wells to yield a final concentration of 500 µM as subsequently reported (15), and the medium below the inserts was exchanged daily.

When the wells were harvested, the organisms were suspended by agitation and the volume in the insert was removed. Subcultures were immediately performed, and slides were prepared for microscopy. The remaining volume was stored at -70°C for later analysis.

**Microscopy.** For assessing *in vitro* growth by direct microscopy, sets of two replicate slides were prepared. One set was prepared with 2 µl of the harvested material, and additional sets were prepared with diluted material corresponding to the inoculated density when passaged into new subcultures. Each set of replicate slides was stained with Diff-Quik (Dade Behring) and by an indirect immunofluorescent-antibody assay using a previously described monoclonal antibody, 7D7, known to stain rat-derived *Pneumocystis* (10).

**DNA extraction and amplification.** DNA was extracted by using the NucliSens kit (Organon Teknika) according to the manufacturer's recommendations. A 200-µl volume of each specimen was used per extraction. Primers RDHFR11 (5'-GTT GCA CTT ACA ACT TCT TAT GG-3') and RDHFR12 (5'-TAG ATC CAG AGA TTC ATT TCG AG-3') were designed to amplify a 234-bp segment of the single-copy DHFR gene (3, 12). The primers were commercially synthesized (Operon Technology). For PCR, all reactions were performed in glass capillaries (Roche) with a final reaction volume of 20 µl of 1× LightCycler-FastStart DNA Master Hybridization Probes reaction mixture (Roche) containing FastStart *Taq*, reaction buffer, deoxynucleoside triphosphate, a 0.5 µM concentration of each primer, a 0.2 µM concentration of each fluorescence resonance energy transfer (FRET) probe, 5.0 mM MgCl<sub>2</sub>, and 1 U of heat-labile uracil-DNA glycosylase (UNG) (from BMTU 3346; Roche). The FastStart *Taq* ensures hot start. In the reaction mixture, dUTP was substituted for dTTP. Five microliters of extracted DNA or cloned template per tube was analyzed. All samples were kept on ice during preparation. Tubes were incubated at room temperature (20.0 to 25.0°C) for 10 min to allow UNG activity to take place. Thermocycling and detection were performed on the LightCycler (Roche). An initial preheating step of 10 min at 95°C was used to activate the DNA polymerase, inactivate UNG, and melt double-stranded DNA. Then a touchdown procedure was utilized, consisting of 11 cycles as follows: 5 s at 95°C; annealing for 10 s at temperatures decreasing from 65 to 55°C, with a decrease of 1°C per cycle; and an extension step of 20 s at 72°C. An additional 34 cycles with annealing at 55°C were performed. At least two negative controls were included in each PCR experiment.

**Amplicon detection.** FRET detection probes were designed to anneal with a 1-base gap between the probes. The following probes were commercially synthesized (Idaho Technology) and labeled with Red 640 as the reporter: RDHFR13 (5'-CCT CTT TTG TAC CAA CTT TTG ATT CAT T-fluorescein-3') and RDHFR14 (5'-Red 640-GAA TCG ATG AAT GTT GTA TTG ATG GG-phosphate-3').

**Data analysis.** All acquired fluorescence data were analyzed by using LightCycler software. In theory, true quantification is achieved with real-time PCR since the cycle number in which amplicons become detectable is proportional to the logarithm of the initial number of templates. In each experiment, at least three standards of cloned template (10<sup>5</sup>, 10<sup>3</sup>, and 10 copies/µl of specimen) were included to generate a standard curve for quantification of positive specimens.

## RESULTS

We utilized a real-time PCR format that is quantitative, rapid, and highly reproducible (5) (Fig. 1). We chose the single-copy DHFR gene as the PCR target since this would allow direct quantitation of the number of organisms from the number of DHFR gene copies detected by the assay. The goal was to develop an assay that would be applicable to *in vivo* as well as *in vitro* studies of PCP.

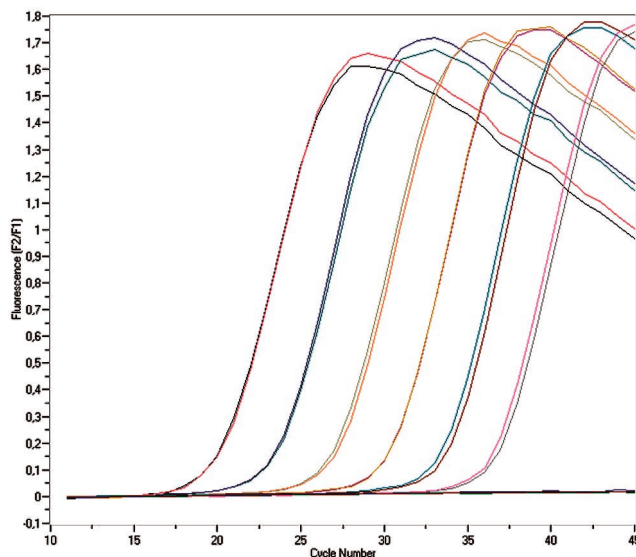


FIG. 1. Real-time detection of PCR products: fluorescence data acquired during an experiment testing cloned standards in duplicate. Standard concentrations of  $5 \times 10^0$  to  $5 \times 10^5$  copies/tube were positive. The standards became positive approximately 3.3 cycles apart, corresponding to the 10-fold increase in concentration.

**Cloned template.** The performance of the QTD PCR assay was evaluated by analyzing 10-fold serial dilutions of the cloned DHFR gene template in 20 separate experiments. Standard curves (cycle number versus log concentration) were constructed by linear regression, and the calculated values for standards were recorded. All of the 9 standards with 0.05 copies/tube were negative, 4 of the 11 standards with 0.5 copies/tube were positive (mean, 1.9), and 12 of the 13 standards with 5 copies/tube were positive, indicating a sensitivity of <5 copies per tube. Plotting all standards with  $\geq 5$  copies/tube together ( $n = 94$ ), the correlation coefficient ( $r$ ) was found to be 0.99 (Fig. 2). Thus, the assay was quantitative over 6 log values down to 5 copies per tube and had the potential to be useful for quantitation of *P. carinii* organisms.

***P. carinii* organisms isolated from *in vivo* rat model.** To evaluate the assay's ability to quantitate whole organisms, DNA was extracted from 10-fold serial dilutions of rat-derived *P. carinii* organisms and assayed. In two separate experiments, five replicates were tested at dilutions of  $10^{-1}$  to  $10^{-8}$ , and two replicates were tested at dilutions of  $10^{-9}$  to  $10^{-11}$ . All PCR tubes with dilutions of  $10^{-7}$  or more ( $n = 16$ ) were negative, and one of the five tubes with dilutions of  $10^{-6}$  (0.7 copies/tube) was positive. All tubes diluted  $10^{-1}$  to  $10^{-5}$  were positive ( $n = 25$ ) and, as shown in Fig. 3, yielded an  $r$  of 0.99. Thus, the assay was quantitative over at least 5 log values, down to 12 copies/tube, when applied to whole organisms. By extrapolation, the original pellet of organisms had a concentration of  $2.9 \times 10^9$  organisms per ml. Uninfected healthy rat DNA was negative by this assay.

***In vitro* cultures of *P. carinii*.** We attempted to reproduce a culture system recently reported to be successful in axenically culturing *P. carinii* (14), with assessment of organism growth by light microscopy. Culture growth was further assessed by QTD PCR. To determine the optimal inoculation density for pri-

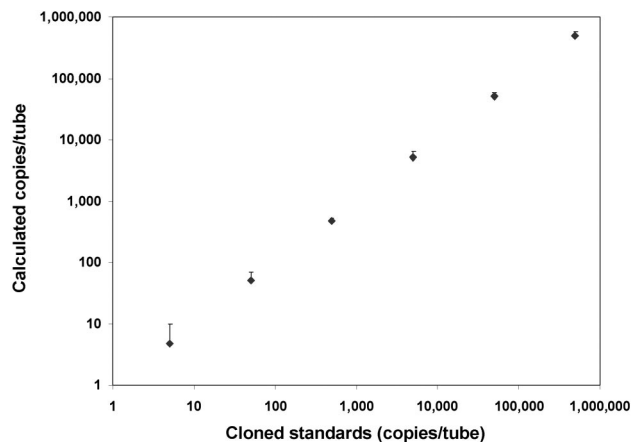


FIG. 2. QTD PCR applied to cloned template: correlation between measured and calculated concentrations of *P. carinii* DHFR gene copies using serial dilutions of plasmid DNA. Values represent the mean (+1.96 standard deviations [SD]) calculated concentrations (◆) of standards (5 to 500,000 copies/tube) assayed by QTD PCR ( $r = 0.99$ ;  $n = 94$ ).

mary cultures, the density of the inoculum for initiation of cultures was varied (with differences in density of as much as  $10^4$ -fold). The intervals between subculturing ranged from 3 to 51 days. Dilutions of 1:10 and/or 1:100 were performed at each passage with up to six passages per culture. In one experiment, replicate subcultures were initiated simultaneously and then harvested at different time points for evaluation of the growth rate over time. A total of 95 cultures (primary cultures or subcultures) were performed.

Only semiquantitative counting of *P. carinii* organisms was found to be possible by microscopy. With this method, growth was not observed at any point in any of the cultures, whether they were derived from frozen samples or from freshly isolated organisms from rat lung (data not shown). The QTD PCR findings were consistent with this observation; in three experiments, harvested material from 39 cultures showed a median

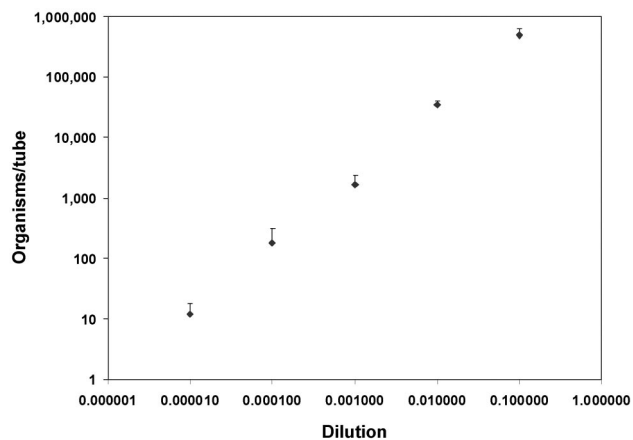


FIG. 3. QTD PCR applied to whole organisms: correlation between the calculated number of DHFR gene copies and the dilution factor of whole organisms. Values represent the mean (+1.96 SD) calculated concentrations (◆) of 10-fold dilutions of resuspended pelleted *P. carinii* organisms isolated from rat lung ( $r = 0.99$ ;  $n = 25$ ).

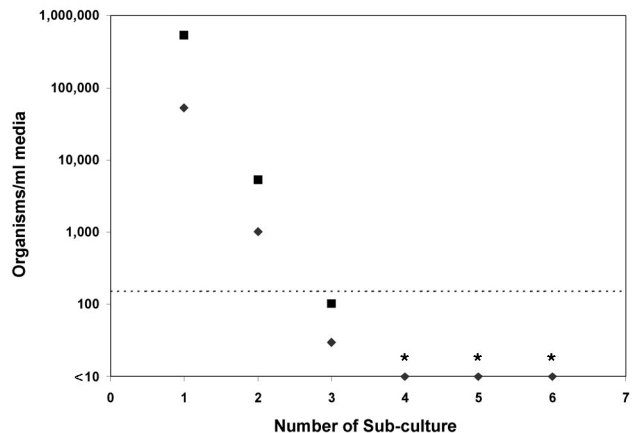


FIG. 4. Quantitation of *P. carinii* organisms passed serially (◆). For each subculture, a 1:10 dilution was performed. For reference, the calculated inoculated amount of DNA for each subculture is graphed to show the expected value if there were no replication and no loss of DNA during culture (■). The dashed line indicates the level of sensitivity and lower limit of quantitation (5 copies/tube equal to 150 organisms/ml). The first subculture was derived from a primary culture inoculated to a density of  $5.4 \times 10^6$  copies/ml by passage after 10 days of incubation. \*, subcultures 4, 5, and 6 were negative by QTD PCR.

decrease in the number of *P. carinii* DHFR gene copies of 84% (range, 19 to 100%), with no increase over time in any culture. Data from one experiment are shown in Fig. 4. In this experiment, the primary culture was inoculated with  $5.4 \times 10^6$  organisms/ml, as quantitated by QTD PCR, and the first subculture was passaged (1:10) 10 days later. Passages 4, 5, and 6 were negative by QTD PCR, indicating  $<150$  organisms/ml of medium. In another experiment, replicate subcultures initiated simultaneously at day 0 and harvested at different time points also showed a decrease in *P. carinii* DNA at all time points (Fig. 5). This sampling method circumvented any potentially negative effects caused by repetitive sampling and disruption of organisms in a single well (14).

DISCUSSION

This QTD PCR assay is able to detect fewer than 5 copies of the rat-derived *P. carinii* DHFR gene per tube, which correlates to fewer than 150 organisms/ml of specimen. Using cloned templates (Fig. 2) and organisms isolated from infected rat lung (Fig. 3), the assay yielded reproducible results over several log values. The slightly higher correlation for cloned templates compared to that for whole organisms (Fig. 2 and 3) was possibly due to heavy organism clumping, which may have compromised the accuracy of the serial dilutions. The unusual heavy clumping was possibly due to the use of pelleted frozen organisms for the dilution series. We compared quantitation by light microscopy with that by QTD PCR but found that clumping and the difficulty in identifying organisms permitted only a semiquantitative estimation by Diff-Quik staining. Investigators should find the QTD PCR assay to be an easier yet more reliable and accurate method for quantification of the number of organisms in rats, in culture, or in other research studies.

We undertook axenic cultivation of *P. carinii* by using a previously reported method (14) but were unsuccessful. A drop

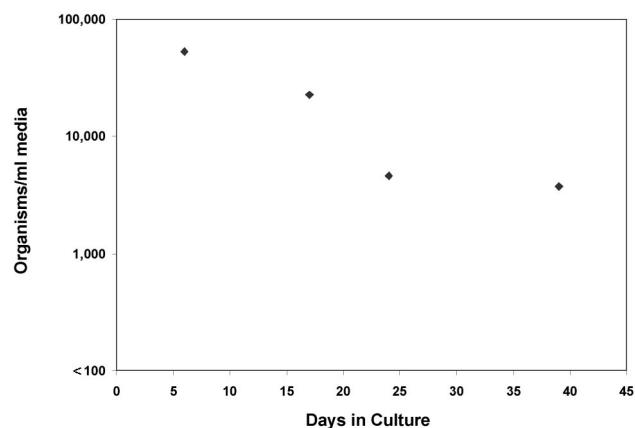


FIG. 5. *P. carinii* culture over time: quantitative results from four replicate subcultures (◆). A primary culture was inoculated to a density of  $5.4 \times 10^6$  copies/ml and subsequently passaged (1:10) 10 days later (day 0) into four replicate subcultures, which were then assayed at the indicated time intervals.

in organism density was estimated by microscopy for all cultures. With QTD PCR, a steady decrease in *P. carinii* DNA during culture was observed and no increase in DNA was demonstrated at any time in any of the cultures. The decrease in DNA was likely due to degradation of DNA from dead organisms. There are several potential explanations for our inability to reproduce the continuous axenic cultivation previously reported (14, 15). First, although the fresh rat lung-derived organisms used for inoculation of primary cultures were morphologically intact by microscopy, they were not assessed for viability. We also used frozen samples reported to retain viability (14), but it is possible that these organisms were also not viable. A second explanation involves the stability of SAM, which has been reported to be a key factor in the successful cultivation of *P. carinii* (14). *P. carinii* is reported to be a SAM auxotroph (15). SAM is a key intermediate metabolite (e.g., as a methyl-group donor and precursor of polyamines), and to our knowledge, no other organism has been reported to be a SAM auxotroph (other than SAM-requiring mutants [1, 15]). SAM is a very unstable compound, so methodological differences in preparation, storage, or administration may have affected our culture attempts.

Our QTD PCR method has a number of advantages over standard PCR assays. A touchdown protocol can increase the specificity and sensitivity of assays (4). Initial high annealing temperatures reduce formation of spurious PCR products that might interfere with amplification of the specific target in low-copy-number samples. The annealing temperature is then gradually lowered to a level that increases yield. We have recently reported that a touchdown procedure can be incorporated into a real-time PCR assay without compromising detection by FRET probes, which ensures efficient and specific amplification in low-copy-number samples, thereby increasing the sensitivity (8). No manipulation of amplicons is required at any step during real-time PCR, lessening the possibility of contamination problems. To further reduce contamination risks, UNG and dUTP were used for prevention of amplicon carryover (20). The entire assay has a turnaround time of <math><4</math> h, including

DNA extraction, which further increases the potential utility of this procedure.

A recent study utilized real-time PCR with the LightCycler to quantitate rat- and human-derived organisms but used SYBR green rather than specific probes for detection of the amplified product (6). We feel that a gene-specific confirmatory probe is essential for maximization of the specificity of this method. The authors of the latter study (6) noted that rat-derived DHFR primers (18) amplified human-derived *P. carinii* RNA. However, we and others have recently shown that those primers amplify rat-derived but not human-derived *P. carinii* DHFR genes (11, 17). Molecular viability assays targeting *P. carinii* f. sp. *carinii* mRNA have also been described recently (6, 13). However, such assays are not suitable for quantifying the numbers of organisms, since the number of mRNA molecules per organism is unknown and since one cannot assume that the amount of mRNA per organism is the same under all conditions.

In conclusion, we have developed a rapid, sensitive, and reproducible method for quantitating *P. carinii* f. sp. *carinii* by PCR that has the potential to replace the tedious and cumbersome quantitative methods currently used in animal studies and that provides a reliable and accurate method for other research efforts, such as in vitro cultivation and antimicrobial drug efficacy testing.

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