Comparative Study of Antipneumocystis Agents in Rats by Using a *Pneumocystis carinii*-Specific DNA Probe To Quantitate Infection

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A repetitive genomic DNA clone (B12-2) that specifically hybridizes to *Pneumocystis carinii* DNA has been identified. No cross-hybridization to genomic DNA prepared from bacteria, other fungi, protozoa, or mammals was observed. Clone B12-2 is multiply represented in the *P. carinii* genome. By direct hybridization to DNA prepared from the lungs of immunosuppressed rats, the probe can detect the equivalent of fewer than 1,000 *P. carinii* organisms. A hybridization assay employing clone B12-2 has been developed to quantitate organism load in the rat model for *P. carinii*. Application of the assay to track the accumulation of organisms during the immunosuppression regimen as well as to monitor the efficacy of two drug therapies used clinically for the treatment of *P. carinii* pneumonia is described here. The clone B12-2 hybridization assay for the determination of *P. carinii* organism load possesses several advantageous features and thus should serve to complement conventional staining and immunohistochemical methods.

*Pneumocystis carinii* pneumonia (PCP) is the most common opportunistic infection in AIDS patients in the United States (17). Current treatments for this disease have major limitations because of a high incidence of adverse reactions. As a result, there is a need for new, safer therapeutic agents. Evaluation of potential antipneumocystis agents is hindered by the lack of dependable methods of culturing *P. carinii* in vitro, although some limited progress has been made in this area (4, 7). As a result, most laboratories evaluate potential drugs in an immunosuppressed rat model for PCP similar to that originally described by Frenkel et al. (12). Rats from colonies known to have latent *P. carinii* infections or rats inoculated with *P. carinii*-infected lung tissue (3) are treated with immunosuppressive drugs for 6 to 8 weeks to induce acute PCP, after which they are treated with candidate compounds with continued immunosuppression. At the completion of therapy, the extent of disease is determined either by examination of histological sections of the lung or by microscopic examination of stained slides of processed lung tissue or impression smears.

There are two known developmental stages of *P. carinii*. The cyst form is 5 to 8 μm in diameter and contains up to eight intracystic bodies, sometimes referred to as sporozoites. The trophozoite form of the life cycle varies markedly in size, ranging from 2 to 5 μm. Usually the degree of *P. carinii* infection is determined by examining slides of histological sections or stained cysts, and a qualitative score from 0 to 5+ is assigned (2, 16, 31). Only rarely is the actual cyst load determined. The number of trophozoites forms is determined even less frequently because of both their small size and the fact that they commonly aggregate in large clumps. Evaluation of the trophozoite load in rat lung tissue is important, since this stage accounts for a substantial component of the *P. carinii* infection. Organism load assignments based merely on microscopic cyst counts are potentially tremendously misleading. For example, novel antipneumocystis therapies which selectively target trophozoites might go unnoticed if only cyst load is monitored. Quantitation of both cyst and trophozoite forms may also lead to a clearer understanding of how these stages relate to each other during the course of a *P. carinii* infection.

There are numerous reports in the literature of pathogen-specific DNA probes being used to quantitate organism load in various tissues (1, 29). In the present paper, a repetitive genomic DNA clone (33) is demonstrated to be specific in its ability to hybridize to *P. carinii* DNA. An assay with the probe has been developed to quantitate the progression of the *P. carinii* infection in the immunosuppressed rat model. To further test the utility of this probe, immunosuppressed rats treated with trimethoprim-sulfamethoxazole (TMP-SMZ) or pentamidine to clear the *P. carinii* infection were assayed visually and by DNA hybridization. The results of these studies indicate that the antipneumocystis activity of drug treatments can be effectively monitored in this model with a DNA probe.

MATERIALS AND METHODS

Animal model and evaluation of compounds. A rat model similar to that originally described by Frenkel et al. (12) and Hughes et al. (15) was used. Briefly, male Sprague-Dawley rats weighing approximately 250 g each, obtained from Sasco, Inc. (Omaha, Nebr.), were maintained on a low-protein diet (8%) and immunosuppressed with dexamethasone (Phoenix Pharmaceuticals, St. Joseph, Mo.) in the drinking water (2 mg/liter) to induce *P. carinii* pneumonia. Tetracycline (1 g/liter; Vedco, Inc., St. Joseph, Mo.) was also added to the drinking water to prevent bacterial infections. In the time course study to monitor the onset of the *P. carinii* infection, six rats were sacrificed each week for 9 weeks.

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Generally, after 6 weeks of steroid therapy, the rats had a significant organism load. In the drug evaluation studies, several animals were sacrificed at this point to confirm the presence of *P. carinii*. The remaining animals were divided into six to eight animals per treatment group. Steroid pressure was continued during the treatment period, which continued for as long as 21 days. A long-term therapy study was conducted to compare the efficacies of the two major treatments for PCP in humans. Four treatment periods (4, 7, 14, and 21 days) were compared to determine the relative rates of cyst clearance and the overall reduction of *P. carinii* organisms. TMP-SMZ (Interchem Corporation, Paramus, N.J.) was administered continuously in the drinking water at a dose equivalent to at least 2.5 times the recommended human dose (50 mg of TMP and 250 mg of SMZ per kg of body weight). Pentamidine (Sigma Chemical Company) was administered intravenously daily at a dose of 10 mg/kg (6 mg/ml in water). This was equivalent to 2.5 times the recommended dose for humans. Parallel groups of control animals were similarly immunosuppressed, treated with the appropriate vehicle, and sacrificed at each time point. At the completion of the respective treatments, all rats were sacrificed by exposure to carbon dioxide gas, and the lungs were removed and homogenized with a Brinkmann homogenizer (Brinkmann Instruments, Westbury, N.Y.) in 10 ml of saline and processed for quantitation as described below and in Schmatz et al. (22). In some experiments, tissue homogenates from heart, intestine, and kidney were similarly prepared.

**Visual evaluation of infected lung tissues.** The lung homogenates were washed once with 10 ml of saline by centrifugation at 1,700 × g for 10 min. The erythrocytes were lysed by resuspending the pellet in 5 ml of 0.85% ammonium chloride and incubated for 5 min at 37°C. The samples were then washed two additional times in saline, and the final pellets were suspended in 2 ml of saline. A 5-μl aliquot was taken from each sample and dried onto Teflon-coated microscope slides with a fixed surface area (11-mm circles; Carlson Scientific, Peotone, Ill.). The extent of disease for each animal was determined by microscopic analysis of stained slides. The total numbers of cysts per animal lung were determined by counting the numbers of cysts in 50 microscope fields (magnification, ×1,000) of homogenized lung tissue on slides fixed with ether-sulfuric acid and stained with toluidine blue (6). Numbers of total nuclei were determined from duplicate slides stained with Diff-Quik (American Scientific Products, McGaw Park, Ill.). Briefly, the slides were sequentially dipped five times in Diff-Quik Fixative, eight times in Diff-Quik Solution I, and five times in Diff-Quik Solution II. The slides were then rinsed in distilled water and allowed to air dry. The total numbers of organisms per rat lung were determined as a function of the surface area on the slide, the volume of the applied sample, and the total volume of the processed homogenate.

**Quantitation of nuclei with the *P. carinii*-specific DNA probe.** Cloning and initial characterization of genomic clone B12-2 from *P. carinii* have been described elsewhere (33). Clone B12-2 was one of a number of clones which specifically hybridized to multiple preparations of *P. carinii* genomic DNA and not to genomic DNA purified from either rat liver or the rat GH3 tissue culture cell line. All experiments which utilized B12-2 as a hybridization probe have been done after twice gel purifying the insert away from vector sequences. Other hybridization probes, including a highly repetitive rat satellite DNA clone (20) and a clone corresponding to the intervening sequence of *P. carinii* small subunit rRNA (ssrRNA) genes (24), were similarly twice gel purified.

Whole rat lungs were homogenized with a Brinkmann homogenizer as described earlier. The portion of the homogenate that was not used for microscopic evaluation of cysts was mixed with an equal volume of 2× proteinase K digestion buffer (1× proteinase K digestion buffer is 200 mM Tris-HCl [pH 7.6], 50 mM EDTA, and 0.5% sodium dodecyl sulfate [SDS]). Two to three aliquots of proteinase K (at a final concentration of at least 50 μg of homogenate per ml) were added over the course of a 24- to 36-h digestion period at 55°C. A 600-μl aliquot of the digested homogenate was extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1) and then once with chloroform-isoamyl alcohol (24:1). The DNA content of the final aqueous phase was determined by a fluorescence bisbenzimide assay (18). Treatment of the lung homogenate with Novozyme 234 (28) or lysing enzymes (Sigma L-2265) prior to proteinase K did not increase either the yield of genomic DNA or the intensity of the *P. carinii*-specific hybridization signal.

An equivalent amount of DNA (usually 50 μg) from each experimental animal was diluted to 500 μl with TE (10 mM Tris-HCl [pH 7.6], 1 mM EDTA). Three serial dilutions were similarly prepared in TE. Each sample was then adjusted to 0.3 N NaOH. The RNA was hydrolyzed and the genomic DNA was denatured by incubation for 30 to 60 min at 70°C. The samples were then cooled to room temperature and neutralized by the addition of an equal volume of 2 M ammonium acetate (pH 7.0). The samples were then immobilized on duplicate nylon filters (Nytran; Schleicher and Schuell, Keene, N.H.) in a Minifold II slot blot apparatus. The filters were air dried, baked for 2 h at 80°C under vacuum, wet in 6× SSPE (1× SSPE is 180 mM NaCl, 10 mM NaH2PO4 [pH 7.4], and 1 mM EDTA), and prehybridized at 44°C for at least 4 h in 6× SSPE–1% SDS–10× Denhardt’s solution (1× Denhardt’s solution is Ficoll, polyvinylpyrrolidone, and bovine serum albumin each at 0.02%)-100 μg of denatured salmon sperm DNA per ml. The prehybridization solution was removed and replaced with a buffer whose composition was 6× SSPE–1% SDS–50% deionized formamide–10% dextran sulfate. Hybridization probes were radioactively labeled with [α-32P]dCTP (3,000 Ci/mmol; Amersham Corporation) by random primer labeling (10, 11). The specific activity of the probes was routinely greater than 108 cpm/μg. The radioactive probe was denatured along with salmon sperm DNA (equivalent to 100 μg/ml of hybridization solution) and mixed with the hybridization buffer. Hybridization was conducted at 44°C for 16 to 20 h. The filters were then washed to a final stringency of 0.1× SSPE–0.1% SDS at 65°C, rinsed several times in 2× SSPE at room temperature, air dried, and exposed to Kodak XR-4 X-ray film.

After an adequate exposure, the filters were cut into blocks corresponding to the slots in the manifold and the amount of radioactive probe hybridizing to each slot was quantitated by liquid scintillation counting. Alternatively (and always prior to liquid scintillation counting for comparison), signals were quantitated directly from the filters with the Betascope 603 Biot Analyzer (Betagen, Waltham, Mass.) or the PhosphorImager 400E (Molecular Dynamics, Sunnyvale, Calif.). In our hands, the data collected both by liquid scintillation counting and with the Betascope 603 typically exhibited very narrow linear ranges for standardization purposes. Because of this, at least three serial dilutions (from 25 μg to 25 ng of genomic DNA) of each experimental sample were spotted and hybridized. On the other hand, the PhosphorImager 400E displayed a much broader linear range.

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**References**

1. Vol. 30, 1992 DNA HYBRIDIZATION ASSAY FOR QUANTITATION OF *P. carinii* 2969

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and therefore required fewer dilutions. Radioactive emissions were converted to organism number with standards included in each hybridization reaction mixture.

Organisms destined for use as standards were prepared by modification (33) of the stomacher process (7). This included collagenase, hyaluronidase, and DNase I digestions to remove as much debris as possible so as to allow for accurate microscopic quantitation. Following visual quantitation, genomic DNA was extracted from known numbers of organisms. We assumed a quantitative recovery of nucleic acids from the organism preparation when we assembled the standard curve. Since this is not likely, the experimental numbers presented here are probably overestimates.

**Genomic Southern zoologs**. The following organisms and/or tissue culture cell lines were kindly provided to us: rat GH3 cells, **Trypanosoma brucei**, Toxoplasma gondii, Cryptococcus neoformans, Corynebacterium kutscheri, Pasteurella multocida, Pseudomonas aeruginosa, Streptococcus pneumoniae, Aspergillus fumigatus, Aspergillus nidulans, and cytomegalovirus-infected human foreskin fibroblasts. Unless stated otherwise, genomic DNA was isolated by proteinase K digestion and organic solvent extractions as described earlier for *P. carinii*-infected tissue. C. *neoformans* spheroplasts were first prepared with lysing enzymes (Sigma L-2265) as described by Edman and Kwon-Chung (9) and then handled in a similar fashion. *Aspergillus* genomic DNA was isolated by the method of Raeder and Broda (21). Genomic DNA was prepared from *C. kutscheri*, *P. multocida*, *P. aeruginosa*, *S. pneumoniae*, and *Escherichia coli* by the following protocol. Cells were suspended in 50 mM glucose–25 mM Tris (pH 8.0)–10 mM EDTA–50 mg of lysozyme ml⁻¹ and incubated for 60 min at 37°C with shaking. After the suspension was adjusted to 0.5% SDS, digestion with proteinase K was carried out as described earlier. The suspension was then mixed with an equal volume of 5 M guanidinium thiocyanate–0.1 M EDTA–0.5% SDS and incubated on ice for 5 min. An equal volume of cold 7.5 M ammonium acetate was added, and the mixture was incubated on ice for 10 min. Samples were extracted once with 4 volumes of cold phenol–chloroform–isoamyl alcohol (25:24:1) and then extracted as described earlier for *P. carinii*-infected tissue. Preparations of genomic DNA from *Saccharomyces cerevisiae* and *Candida albicans* were kindly provided to us. Genomic DNA was restricted with EcoRI, fractionated by agarose gel electrophoresis, transferred to a nylon membrane, and hybridized with ³²P labeled B12-2. Genomic DNA was from chicken (lane 1), rat GH3 cells (lane 2), *P. carinii* (lane 3), *T. brucei* (lane 4), *T. gondii* (lane 5), *Eimeria tenella* (lane 6), *S. cerevisiae* (lane 7), *C. albicans* (lane 8), *C. neoformans* (lane 9), *A. nidulans* (lane 10), heart (lane 11), intestine (lane 12), kidney (lane 13), and lung tissues (lane 14) from an immunosuppressed rat, *E. coli* (lane 15), *C. kutscheri* (lane 16), *P. multocida* (lane 17), *P. aeruginosa* (lane 18), *S. pneumoniae* (lane 19), *A. fumigatus* (lane 20), and cytomegalovirus-infected human foreskin fibroblasts (lane 21). Relative migrations of molecular weight markers (in kilobases) are indicated between the two panels.

**RESULTS**

Specific hybridization of clone B12-2 to *P. carinii* genomic DNA. Steroid-induced immunosuppression renders the rat host susceptible to a plethora of opportunistic organisms including viruses, bacteria, fungi, and protozoa. In order to validate a DNA probe assay to quantitate organism load in this model system, it is imperative to demonstrate specific hybridization to *P. carinii* genomic DNA. The isolation of clone B12-2 from a *P. carinii*-enriched genomic library has been described elsewhere (33). The clone is approximately 5 kb in length and does not hybridize to rat tissue culture cell genomic DNA (33) (Fig. 1, lane 2). To extend the test for specificity, genomic DNAs from a wide variety of organisms were probed with clone B12-2. Hybridization was entirely restricted to *P. carinii* genomic DNA (Fig. 1). In particular, there was no cross-hybridization to *A. fumigatus*, *C. albicans*, *C. neoformans*, *C. kutscheri*, *P. multocida*, *P. aeruginosa*, or *S. pneumoniae*. Each of these organisms is associated with pulmonary pathology in humans or rats.

In light of the fact that the B12-2 hybridization probe is prepared as an EcoRI fragment, the hybridization profiles in Fig. 1 indicate that the clone is multiply represented in the *P. carinii* genome. The majority of the EcoRI-generated genomic fragments which hybridize with clone B12-2 fall within the 1.4- to 12-kb size range. The repetitive nature of this piece of DNA affords us increased sensitivity when it is used as a hybridization probe.

**Limits of detection for clone B12-2**. The results of an experiment designed to define the limits of detection of this hybridization probe are shown in Fig. 2. Enriched preparations of *P. carinii* (7, 33) from the lungs of heavily immunosuppressed rats were quantitated by microscopic analysis of stained slides. Total nucleic acids were extracted from 5 ×
10^8 organisms. The final aqueous phase was serially diluted and applied to duplicate nylon sheets in slots containing 10^7 to 10^8 organism equivalents (Fig. 2, columns A). Alternatively, in columns C, organisms were serially diluted prior to nucleic acid extraction, and then, following extraction, nucleic acids were immobilized on the membrane. Hybridization of this dilution with B12-2 (Fig. 2, left panel) indicates that as few as 1,000 organisms may be detected. This assumes the quantitative recovery of genomic DNA during the extraction process. Samples in Fig. 2, column B, are identical to those in columns A, except for the fact that they are spiked with 15 μg of genomic DNA prepared from the rat GH3 tissue culture cell line. The inclusion of rat genomic DNA to more closely approximate the composition of nucleic acids extracted from a lung homogenate did not substantially decrease the signal-to-noise ratio and therefore still allows for the detection of approximately 1,000 organisms. Extraction of nucleic acids from organisms after serial dilution does appear to result in less-efficient recovery (compare Fig. 2, columns A and B with column C).

A duplicate membrane was hybridized in Fig. 2 (right panel) with a highly repetitive rat satellite DNA probe (20). This piece of DNA has been estimated to be present in at least 10,000 copies in the rat genome. In spite of the fact that this preparation was enriched for *P. carinii*, it is apparent that a considerable amount of rat genomic material copurified during the purification procedure.

**Accumulation of *P. carinii* in the rat model.** Microscopic detection of *P. carinii* in the rat model is not reproducibly seen until after 4 weeks of steroid treatment (20a). The accumulation of *P. carinii* in groups of rats treated with 0, 2, or 4 mg of dexamethasone per liter of drinking water was monitored by hybridization with clone B12-2. Animals were sacrificed after 3, 6, and 9 weeks of treatment, and nucleic acids were prepared from lung homogenates. No *P. carinii* could be detected in rats which did not have steroid in their drinking water (Fig. 3), even after being housed for 9 weeks in the same rooms with the rats from the treatment groups. Steroid treatment at either 2 or 4 mg/liter did result in the accumulation of *P. carinii* over time, as detected by the hybridization probe. By this assay approach, organisms were obvious at 3 weeks but were not reproducibly detected any time sooner. Polymerase chain reaction analysis with amplification primers located within clone B12-2 (5) was able to specifically detect *P. carinii* earlier during the immunosuppression regimen (data not shown). The intensity of the hybridization signal indicated that organisms progressively accumulated at each of the three time points assayed in this experiment. After 6 weeks of treatment, animals dosed with 4 mg of steroid per liter had a greater organism load than those treated with 2 mg/liter. However, at 9 weeks both levels of steroid resulted in a similar *P. carinii* burden.

The sensitivity of genomic clone B12-2 (Fig. 3A) as a hybridization probe was compared with that of a second *P. carinii*-specific DNA clone. The ssrRNA gene from *P. carinii* contains an intron (24) which, like clone B12-2, hybridizes specifically to *P. carinii* genomic DNA (18a). By analogy to ssrRNA genes from other organisms which are multiply represented in the genome (19) and from the observation that most if not all ssrRNA genes in *P. carinii* contain this intron (18a), we predicted that an intron probe would be both specific and sensitive. The results shown in Fig. 3B indicate that clone B12-2 is approximately 10-fold more sensitive as a hybridization probe. Since the two probes used in this experiment had equivalent specific activities, it follows that clone B12-2 is represented more often in the *P. carinii* genome than the ssrRNA gene. Accordingly, B12-2 is a more desirable hybridization probe for this assay.
Rats immunosuppressed for 9 weeks were sacrificed at weekly intervals starting at 3 weeks. A portion of lung tissue from each animal in each group (at least four and generally eight animals per group) was processed for visual *P. carinii* cyst and nucleus counts. Organism load in the balance of the tissue was evaluated by hybridization with clone B12-2. The accumulation of *P. carinii* over the course of the immunosuppressive treatment period as measured by the two methods is depicted in Fig. 4. There was a gradual increase in the number of cysts and nuclei (intracystic bodies and trophozoites) as determined by microscopic quantitation with the respective stains. Cyst and nuclei appeared to increase proportionally, suggesting a link between these two developmental stages. The utility of the DNA probe as an assay tool is also demonstrated by this figure. Organism load determined by the DNA hybridization assay closely paralleled the data obtained by microscopic evaluation. This fact is vividly demonstrated by plotting the correlation between *P. carinii* organism load determined by the two assay procedures (Fig. 5).

**Use of clone B12-2 to monitor clearance of *P. carinii* with known antipneumocystis agents.** The efficacies of two therapeudic regimens used to treat *P. carinii* pneumonia clinically (14, 30) were tested in the rat model and monitored both visually and by DNA hybridization with clone B12-2. Animals immunosuppressed for 6 weeks to establish a *P. carinii* infection were treated intravenously with pentamidine or with a combination of TMP and SMZ administered orally. Lung tissue was assayed in both experimental and control groups of animals after 4, 7, 14, and 21 days. Cyst counts determined by staining with toluidine blue are plotted in Fig. 6A as a function of the treatment period. Assay of organism load by DNA hybridization is shown in Fig. 6B. Both assays indicated that the two forms of chemotherapy effectively cleared the *P. carinii* burden from rats. A 99% reduction in organism load was realized by 21 days of treatment. The kinetics of *P. carinii* clearance was virtually the same for the two forms of therapy, and this was demonstrated by both assay approaches. By deduction, the results generated by the DNA-based assay, which measures both documented stages of the *P. carinii* life cycle, suggest that trophozoite elimination parallels cyst clearance for TMP-SMZ and pentamidine treatments.

**DISCUSSION**

The data presented here highlight the utility of using a DNA hybridization probe to monitor *P. carinii* burden in the experimental rat model system. This approach to quantitation is desirable because each stage in an organism’s life cycle is in theory quantifiable. Immunohistochemical assays for assessing parasite burden are complicated by the potential for stage-specific antigen expression. Microscopic quantitation of *P. carinii* cysts and trophozoites with selective strains is tedious and requires multiple staining procedures. Accurate determination of the trophozoite contribution is compromised by their small size. Consequently, they are easily confused with debris from the lung homogenate. Trophozoite forms also tend to aggregate together in large clumps. Data obtained with impression smears are subject to the criticism that only a portion of the lung is sampled. This might not accurately reflect the *P. carinii* burden in the entire tissue. In situ hybridization of *P. carinii* rRNA, which has been proposed as a method for diagnosis (13), would be subject to the same criticism if it were to be used to quanitate organism load. More importantly, in situ hybridization is very demanding technically and not applicable to large numbers of experimental samples.

We have shown that genomic clone B12-2 hybridizes specifically to *P. carinii* DNA. A caveat of this conclusion becomes obvious when one considers the manner in which the *P. carinii* infection is induced. Immunosuppression of rats is known to permit the expansion of populations of a wide variety of opportunistic organisms (23). In situ hybridization of lung sections prepared from immunosuppressed
rats with clone B12-2 (or a portion thereof) will unequivocally prove that the hybridization which we are measuring is specific. Lacking this result, we remain confident that hybridization with clone B12-2 is specific for the following reasons. No hybridization of clone B12-2 to genomic DNA prepared from organisms commonly found in hosts displaying pulmonary pathology was detected (Fig. 1). These include A. fumigatus, C. albicans, C. neoformans, C. kutscheri, P. multocida, P. aeruginosa, and S. pneumoniae. Secondly, there was a strict correlation between the results generated by DNA hybridization and visual counts (Fig. 4 and 5). Furthermore, the qualitative hybridization intensity realized with clone B12-2 as a probe parallels that seen with a P. carinii-specific (8, 24) ssrRNA gene probe (Fig. 3, for example). Finally, the efficacy of documented antipneumocystis therapeutic regimens was accurately predicted by hybridization with clone B12-2 (Fig. 6).

Clone B12-2 represents a repetitive DNA element in the P. carinii genome. The P. carinii-specific ssrRNA intron was less sensitive when used as a hybridization probe in a side-by-side experiment (Fig. 3). On the basis of this, we contend that clone B12-2 has a copy number at least as great as that of the ssrRNA gene family of P. carinii. Partial nucleotide sequence analysis of clone B12-2 (18a) indicates that it does not correspond to any P. carinii rRNA sequence described in the literature (8, 27, 32). In addition, restriction enzyme mapping of B12-2 differentiates it from another DNA repetitive element cloned from P. carinii (20).

Making the assumption that 100% recovery of genomic DNA was achieved from a P. carinii preparation that was quantitated microscopically, the results in Fig. 2 indicated that clone B12-2 can detect approximately 1,000 organisms in the current assay format. However, losses of nucleic acids during purification undoubtedly occur, which renders this assumption inaccurate. Consequently, it is safe to predict that the assay is sufficiently sensitive to detect fewer than 1,000 P. carinii organisms even in the presence of a tremendous excess of rat genomic DNA. Although the assay can specifically detect fewer than 1,000 P. carinii organisms, the limits of detection for organism load in an entire animal are in the order of \(5 \times 10^4\). Because of a limited surface area, the maximum amount of DNA that can be immobilized through the slot blot manifold onto the nylon membrane is 25 \(\mu\)g. This is only a fraction of a percentage of the DNA in a lung homogenate. Furthermore, the vast majority of this DNA is derived from the host, especially in a light infection. The raw data output from the assay is first compared with a standard curve that is generated by using DNA extracted from purified P. carinii organisms whose number has been determined by toluidine blue and Diff-Quik staining. This allows for the assignment of organism number to the portion of the lung homogenate which was assayed. From this, the total number of P. carinii organisms in the lungs of each experimental rat is projected.

The relationship between trophozoite and cyst forms of P. carinii is not well understood, although it is generally assumed that the two forms are interdependent. Indeed, the life cycle for this organism remains obscure. If P. carinii does in fact go through other stages in its life cycle, these are not yet recognized microscopically. Barring dramatic changes in DNA content or composition, it is safe to conclude that a DNA hybridization-based assay will capably detect each developmental stage in an organism’s life cycle. This, of course, also assumes that physical access to the genomic DNA can be achieved at each stage during the life cycle. Because a hybridization assay does not discriminate among developmental stages, it possesses a tremendous advantage over traditional approaches to quantitation. This is especially true for organisms like P. carinii whose biology is not thoroughly characterized. By the employment of a DNA hybridization assay in screens for novel antipneumocystis drugs, stage-specific therapeutic intervention will be detected. By way of example, compounds which selectively interfere with the trophozoite form of P. carinii might go undetected if cyst load as determined by toluidine blue staining was the sole assay to measure their efficacy. In summary, the clone B12-2 hybridization assay for the determination of P. carinii organism load offers several advantages and therefore should serve to complement conventional staining and immunohistochemical methods.

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REFERENCES


