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Use of Semiquantitative PCR To Assess Onset and Treatment of *Pneumocystis carinii* Infection in Rat Model

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The use of semiquantitative PCR (SQPCR) to assess *Pneumocystis carinii* pneumonia (PCP) infection and its response to treatment was studied with rats. Groups of eight rats were immunosuppressed with steroids for 3 to 12 weeks. Untreated controls were maintained for the same periods. Three groups of rats were treated with pentamidine, three groups were treated with trimethoprim-sulfamethoxazole, and three groups of rats were tapered from steroids. At various times during suppression, rats from the different groups were sacrificed. At necropsy, lungs were lavaged to obtain bronchoalveolar fluids and then homogenized. Bronchoalveolar fluids and homogenates were assayed by cyst counting and SQPCR. An increase in the SQPCR signal was seen throughout immunosuppression, with a slow decrease upon the withdrawal of steroids and a faster decrease with drug treatment. SQPCR results with lung homogenates and bronchoalveolar fluids strongly correlated with each other and with cyst counts. These results warrant investigation of SQPCR for assessing treatment results of human *P. carinii* pneumonia infection.

Pneumocystis carinii pneumonia (PCP) is a frequent complication of immunosuppression, particularly that associated with human immunodeficiency virus infection (2, 3). Although drug prophylaxis has reduced the incidence of PCP in patients at high risk, it remains a common presentation of human immunodeficiency virus disease (17, 18, 26). Definitive diagnosis requires evidence of the organism in sputum, bronchoalveolar lavage (BAL) fluid, or open biopsy; demonstration generally requires the use of special stains, such as methenamine silver (1), or immunohistochemical assays (1, 15).

Although treatment of PCP with anti-*Pneumocystis* drugs has a high success rate, between 5 and 40% of patients fail to respond adequately to the initial course of therapy (18, 27). Early identification of treatment failure may improve clinical outcome, but detection is complicated by several factors. (i) The initiation of therapy is often associated with a decline in the partial pressure of oxygen, whether or not this treatment is ultimately successful. Thus, clinical response is not tightly associated with successful antimicrobial therapy (27). (ii) Radiologic improvement generally follows clinical improvement by a substantial period, limiting its utility (39). (iii) Patients may harbor other organisms, such as cytomegalovirus, which result in similar organ system effects and radiologic findings (14). (iv) Silver stains may fail to differentiate between viable and nonviable organisms (13, 19, 29, 40). (v) The visualization of developmental forms other than cysts by routine histopathologic methods is difficult (8, 29).

PCR assays have proven to be sensitive and reliable techniques for the diagnosis of *Pneumocystis* infection with sputum, BAL fluid, and lung tissue (4, 5, 7, 9, 15, 21, 22, 25, 30, 33–35, 41, 42). Quantitative PCR assays have proven to be useful in quantitating such diverse phenomena as oncogene amplification (36), gene expression (31), and organism load (28). Indeed, qualitative assessment of PCP infection by PCR seems to correlate with the expected organism load for animals on various therapeutic regimens (37). We hypothesized that a semiquantitative PCR (SQPCR) assay would correlate well with the results of cyst counting methods. If so, it would be useful for following the clinical course of infections and might serve as a reliable and rapid surrogate for other organism quantitation procedures (6, 20) or immunological techniques (12). In this paper, we show that SQPCR methods provide a method for assessing the effect of treatment in a rat *Pneumocystis* model.

MATERIALS AND METHODS

Experimental design. *P. carinii* infections were produced in Sasco Sprague-Dawley (SSD^+) (O'Fallon colony; Room OM1, Omaha, Nebr.) rats as previously described (10). Briefly, animals were maintained in barrier-isolated conditions and fed a normal diet. Some groups of eight rats were maintained without immunosuppression, while others were immunosuppressed with methylprednisolone (4.0 mg/week) (Depo Medrol; The Upjohn Co., Kalamazoo, Mich.). After 6 weeks of this treatment, all rats treated with steroids established clinically significant immunosuppression and *P. carinii* infections (45), as assessed by cyst and nucleus counts (45, 46) and impression smears stained with a modified Giemsa stain (10). After 6 weeks of initial immunosuppression, three groups of eight immunosuppressed rats were treated with the anti-*P. carinii* drug pentamidine isethionate (LymphoMed, Inc., Melrose Park, Ill.) at 10 mg/kg three times per week for a total of 5, 7, or 18 doses (44). Three additional groups of eight rats were treated with trimethoprim-sulfamethoxazole (TMP-SMX) (Burroughs Wellcome Co., Research Triangle Park, N.C.) at 250 mg/kg for 10, 21, or 42 days while they remained immunosuppressed by methylprednisolone (44). The treatments given to each group of eight rats are summarized in Table 1. Groups of rats were sacrificed at time zero and at regular intervals for the duration of this study. At the time of sacrifice, all rats underwent necropsy. Blood and several tissues, including lung, liver, spleen, bone marrow, testis, and kidney, were taken at necropsy and fixed in 95% ethanol. Before fixation, lungs were lavaged with four 5-ml aliquots of phosphate-buffered saline (pH 7.2). The recovered fluid, which ranged from 15 to 18 ml, was concentrated by centrifugation and then resuspended in 5 ml of supernatant. Some of this concentrated lavage fluid was retained for cyst counting; the remainder was centrifuged and separated into two fractions, one of which contained cell pellet while the other consisted of supernatant.

Tissue preparation. Ethanol-fixed lung tissue was picked up with a sterile needle, placed on a clean microscope slide, minced, and homogenized with a fresh razor blade. This tissue was transferred to a microcentrifuge tube, suspended in 100 μl of PCR buffer (10 mM Tris-HCl [pH 8], 50 mM KCl, 3 mM $MgCl_2$) that contained 20 μg of proteinase K (Sigma Chemical Co., St. Louis, Mo.), digested at 52°C for at least 1 h, and then heated to 95°C for 10 min to destroy proteinase K activity before storage (47).

BAL cell pellets were prepared by centrifuging BAL fluid in a microcentrifuge tube and then digesting pellets in proteinase K buffer as described above.

Cyst counts were carried out as described by Kim et al. (20).

PCR. Primer sequences for the gene that codes for the large subunit of rat *P.*

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TABLE 1. Treatment regimens, sampling times, and SQPCR results

Regimen	Wk after start of study[a]	Mean lung SQPCR (range)	Mean BAL SQPCR (range)
Steroid immunosuppression, 4 mg/wk	3	70 (50–117)	40 (0–103)
	6	ND[b]	50 (7–97)
	7.5	51 (51–51)	90 (25–142)
	9	115 (22–362)	139 (69–362)
	12	84 (46–120)	83 (36–177)
Tapered steroid immunosuppression			
4 mg/wk for 6 wk, 2 mg/wk for 1 wk, 1 mg/wk for 1 wk, 0.5 mg/wk for 1 wk	9	70 (0–280)	99 (12–286)
Same tapered regimen as described above, off steroids for 3 wk	12	42 (0–184)	24 (0–74)
Same tapered regimen as described above, off steroids for 5 wk	15	48 (0–384)	1 (0–8)
Same tapered regimen as described above, off steroids for 8 wk	18	5 (0–31)	2 (0–7)
Nonimmunosuppressed	3	10 (0–28)	1 (0–10)
	6	0 (0–0)	0 (0–0)
	7.5	0 (0–0)	0 (0–0)
	9	2 (0–16)	0 (0–0)
	15	0 (0–0)	0 (0–0)
TMP-SMX			
250 mg/kg/day for 10 days	7.5	21 (4–34)	113 (35–342)
250 mg/kg/day for 21 days	9	9 (0–33)	13 (1–38)
250 mg/kg/day for 42 days	12	1 (0–4)	1 (0–5)
Pentamidine			
10 mg/kg for 5 doses	7.5	51 (0–127)	82 (38–197)
10 mg/kg for 7 doses	9	28 (15–51)	35 (0–239)
10 mg/kg for 18 doses	12	16 (5–46)	24 (0–74)

[a] Time zero was defined as the first day of immunosuppression therapy.
[b] ND, not done.

carinii mitochondrial rRNA were taken from Wakefield et al. (43). Primers for rat β-globin were based on sequences published by Wong et al. (46). The *Pneumocystis* primers produce a 346-bp PCR product, and the globin primers produce a 400-bp product when given an appropriate template. A 0.5-μl sample of each lysate was used in a PCR mix that contained 10 mM Tris-HCl (pH 8), 50 mM KCl, 3 mM $MgCl_2$, 0.5 mM deoxynucleoside triphosphates, 0.5 μM each primer (Table 2), and 1 U of *Taq* polymerase. Each sample was overlaid with 25 μl of mineral oil, denatured for 2 min at 94°C, and then subjected to 30 cycles which consisted of a 60-s 94°C denaturation step, a 50-s 50°C annealing step, and a 50-s 72°C elongation step. Each set of PCR assays included lung tissue from one rat (1245) which had been immunosuppressed for 9 weeks and which served as a reference standard and a blank sample for a negative control. The use of a reference standard compensated for small day-to-day variations in PCR conditions as well as for variations in the amount of DNA actually available for amplification. The rat β-globin gene was chosen for convenience since it was expected to reflect the relative amount of rat tissue present in the amplification reaction, much as globin may serve as an internal reference gene for assessing oncogene amplification (36). Analyses of rat β-globin and *P. carinii* DNA were performed simultaneously in separate reaction tubes from PCR through Southern blotting.

Electrophoresis and blotting. A 10-μl aliquot of PCR product was electrophoresed for 1 to 2 h on a 2% agarose gel, blotted overnight on a sheet of nylon hybridization membrane (Oncor, Gaithersburg, Md.), air dried, and then blocked with Oncor membrane blocking solution. Hybridization was carried out at 45°C in 5 ml of Oncor Hybrisol III which contained 4 pmol of ^{32}P-labeled probe. After hybridization, the blot was washed three times at room temperature in 100 ml of washing solution that contained 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate. The blot was air dried and then exposed to Kodak XAR-5 film for 0.5 to 1 h to obtain autoradiograms for densitometry.

TABLE 2. Primers and probes for PCR

Gene	Primer or probe	Sequence
Globin	Primer 1	5′ GGTGCACCTAACTGATGTTG 3′
	Primer 2	5′ GCTTGTCACAGTGGAGTTCAC 3′
	Probe	5′ GATAATGTTGGCGCTGAGGCCC 3′
PCP	PAZ102-E	5′ GATGGCTGTTTCCAAGCCCA 3′
	PAZ102-H	5′ GTGTACGTTGCAAAGTACTC 3′
	PAZ102-L2	5′ ATAAGGTAGATAGTCGAAAG 3′

Densitometry. Densitometry was carried out on a Leitz TAS image analysis computer, and assay results were expressed as *Pneumocystis*/globin signal ratios. Then each ratio was adjusted to its relative value with respect to that of lung tissue from rat 1245, which was adjusted to be 100 for every reaction. For example, BAL fluid from rat 1286 yielded a PCP/globin ratio of 1.31 during an amplification reaction in which the control rat (1245) demonstrated a ratio of 19.4. Therefore, each ratio was multiplied by 5.15, giving an adjusted ratio of 6.75 for rat 1286 BAL fluid and an adjusted ratio of 100 for rat 1245. In this way, we compensated for differences in reaction conditions and the incorporation of radioactive dATP into probes.

Data analysis was carried out with the Systat computer program (Systat, Inc., Evanston, Ill.). Analysis of variance was followed by posttest comparison by the Tukey HSD method (38). Linear regression analyses were also carried out. Correlations among lung homogenate and BAL fluid cyst count results and among lung homogenate and BAL fluid SQPCR results were tested with the Wilcoxon signed rank test.

RESULTS

Initial immunosuppression. The effects of initial immunosuppression on lung homogenate SQPCR results are shown in Fig. 1. There is a substantial increase in mean SQPCR signals for pulmonary tissue in the interval between 0 and 3 weeks, which increases slightly over the following 9 weeks. However, individual rats differed substantially in their responses to immunosuppression. For example, while one rat immunosuppressed for 3 weeks had a SQPCR result of 15, another rat immunosuppressed for 9 weeks had a SQPCR result of 22. These variations may represent interindividual variations in response to immunosuppression, individual organism inocula, or limitations in the assay techniques used in this series of experiments. Nevertheless, statistically significant differences in SQPCR results were seen within 9 weeks (Table 3).

The effects of initial immunosuppression on BAL fluid SQPCR results are shown in Fig. 2. There is a substantial increase in mean SQPCR signals for BAL fluid in the interval

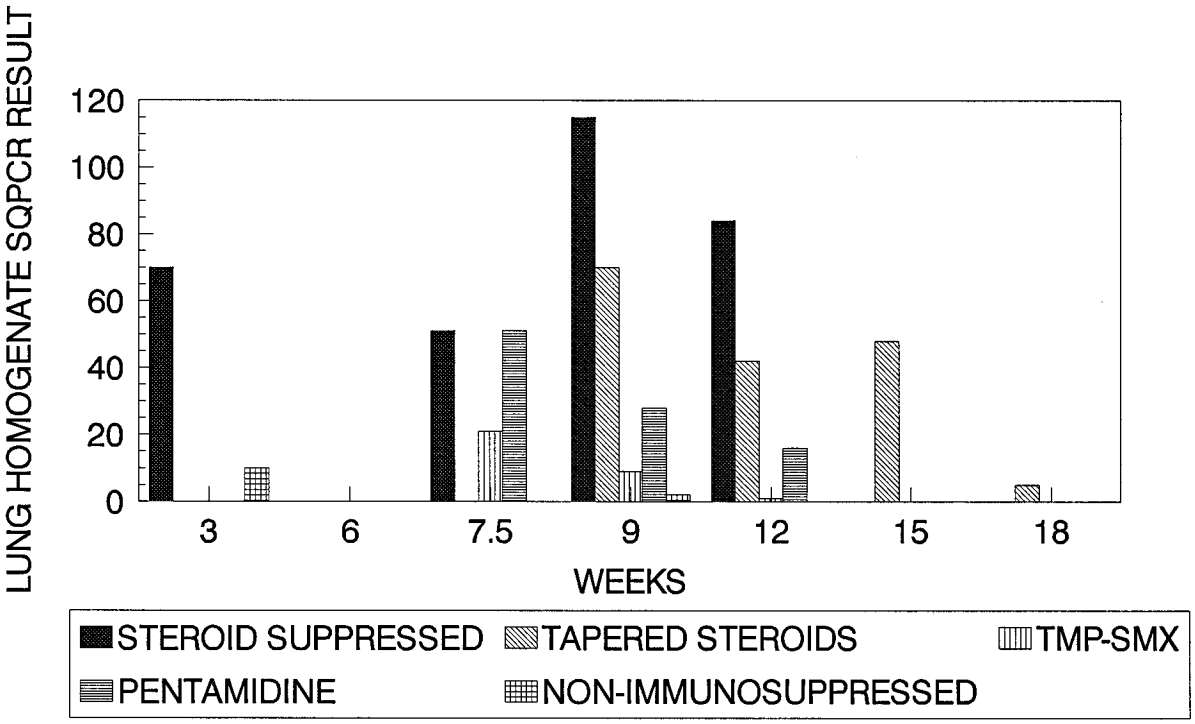

FIG. 1. Average lung homogenate SQPCR results for rats immunosuppressed with methylprednisolone for 3, 7.5, 9, and 12 weeks (steroid suppressed); rats immunosuppressed for 6 weeks, tapered off steroids for the following 3 weeks, and then left off steroids (tapered steroids); rats immunosuppressed for 6 weeks and then treated with TMP-SMX while remaining immunosuppressed; rats immunosuppressed for 6 weeks and then treated with pentamidine while remaining immunosuppressed; and rats which received no immunosuppression (nonimmunosuppressed).

between 0 and 3 weeks, which gradually increases over the ensuing 9 weeks. As in the analysis of lung homogenates, it was observed that individual rats within a given group differed in their responses. Statistically significant differences in SQPCR results were seen by week 7.5 and persisted through the 12-week sample.

The results obtained by SQPCR are qualitatively similar, although not identical, to those obtained by cyst counting (Fig. 3 and 4). At the 3-week time point, the organism burden in terms of the number of cyst forms was barely detectable in lung homogenate samples and undetectable in BAL fluid. In contrast, strong SQPCR signals were apparent in both compartments. By 7.5 weeks, approximately 10,000,000 cysts were counted in lung tissue, while about 200,000 were found in BAL fluid. The SQPCR signal from BAL fluid doubled, but the signal from lung tissue was about the same as that at the 3-week time point. By 9 weeks, it was apparent that the organism density was quite heavy, as evidenced by strong SQPCR signals as well as higher cyst counts in both compartments. By 12 weeks, SQPCR signal and cyst numbers were on the decline for all samples but lung homogenate samples, whose cyst counts were at their highest level during the experiment.

Tapering of steroids. Steroids were tapered to cause a gradual decline in *P. carinii* organism load as previously reported (45). The gradual decline in organism load was in contrast to the more dramatic effects observed with drug therapy and permitted further evaluation of the sensitivity of the techniques used in this study. The effects of withdrawing steroids

TABLE 3. *P* values for the differences in means between groups of rats, as computed by Tukey's HSD method for SQPCR results based on pulmonary tissue

Rat group	*P* value[a]								
	Control[b]	Ster (9 wk)	Ster (12 wk)	TMP 1	TMP 2	TMP 3	Pent 1	Pent 2	Pent 3
Control	1.000								
Ster (9 wk)	0.000	1.000							
Ster (12 wk)	0.020	0.999	1.000						
TMP 1	1.000	0.033	0.671	1.000					
TMP 2	1.000	0.004	0.316	1.000	1.000				
TMP 3	1.000	0.002	0.193	1.000	1.000	1.000			
Pent 1	0.447	0.465	0.998	0.999	0.952	0.853	1.000		
Pent 2	0.994	0.052	0.790	1.000	1.000	1.000	1.000	1.000	
Pent 3	1.000	0.011	0.486	0.903	1.000	1.000	1.000	1.000	1.000

[a] *P* values labeled 0.000 represent any *P* value of <0.0005. Statistically significant results ($P < 0.05$) are underlined.
[b] Rats that were never immunosuppressed.

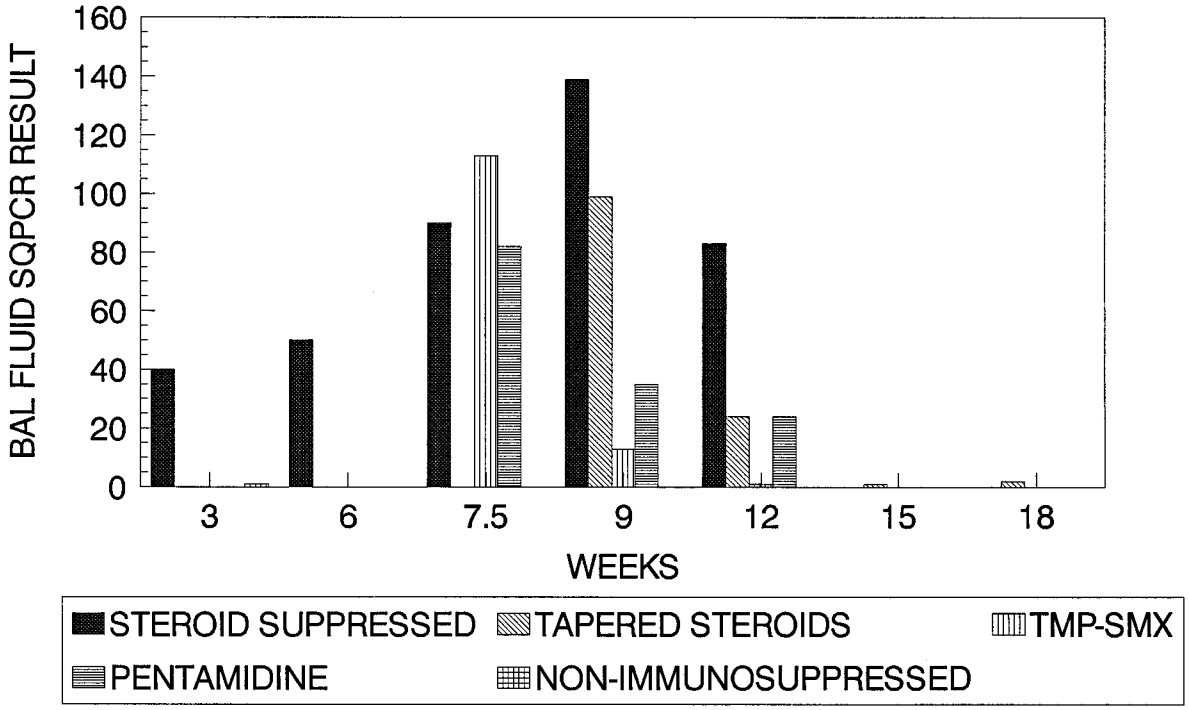

FIG. 2. Average BAL fluid SQPCR results for rats immunosuppressed with methylprednisolone for 3, 7.5, 9, and 12 weeks (steroid suppressed); rats immunosuppressed for 6 weeks, tapered off steroids for the following 3 weeks, and then left off steroids (tapered steroids); rats immunosuppressed for 6 weeks and then treated with TMP-SMX while remaining immunosuppressed; rats immunosuppressed for 6 weeks and then treated with pentamidine while remaining immunosuppressed; and rats which received no immunosuppression (nonimmunosuppressed).

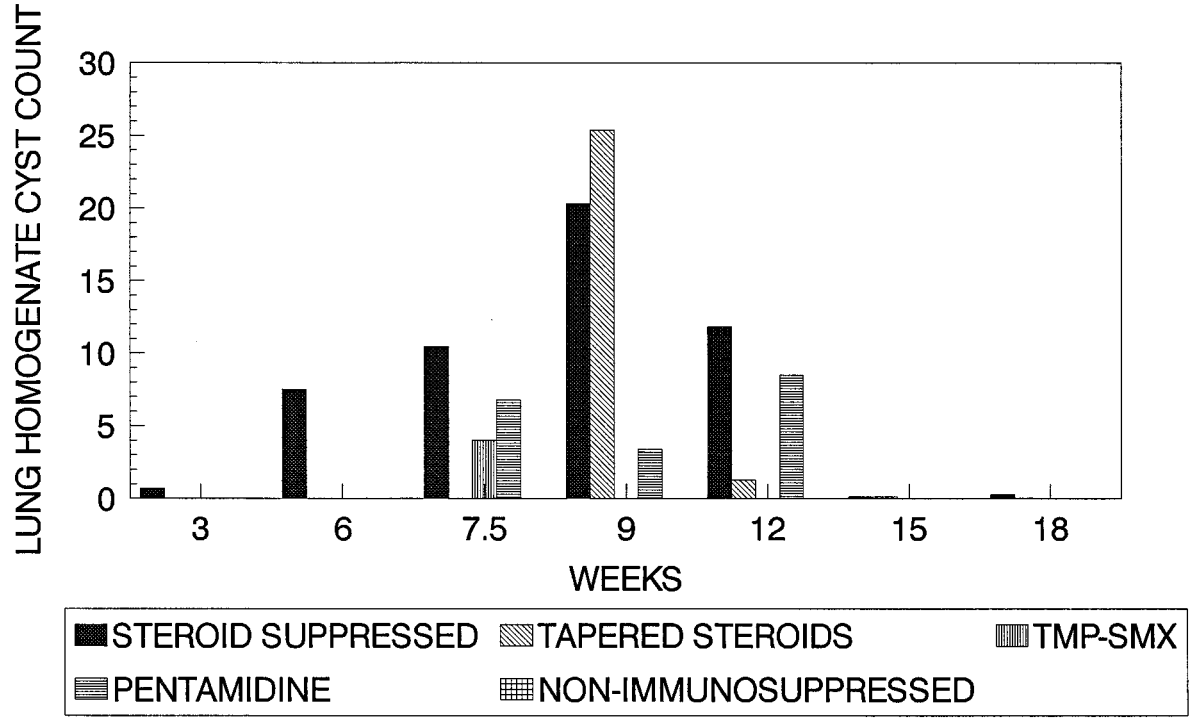

FIG. 3. Average lung homogenate cyst counts (10^6) for rats immunosuppressed with methylprednisolone for 3, 7.5, 9, and 12 weeks (steroid suppressed); rats immunosuppressed for 6 weeks, tapered off steroids for the following 3 weeks, and then left off steroids (tapered steroids); rats immunosuppressed for 6 weeks and then treated with TMP-SMX while remaining immunosuppressed; rats immunosuppressed for 6 weeks and then treated with pentamidine while remaining immunosuppressed; and rats which received no immunosuppression (nonimmunosuppressed).

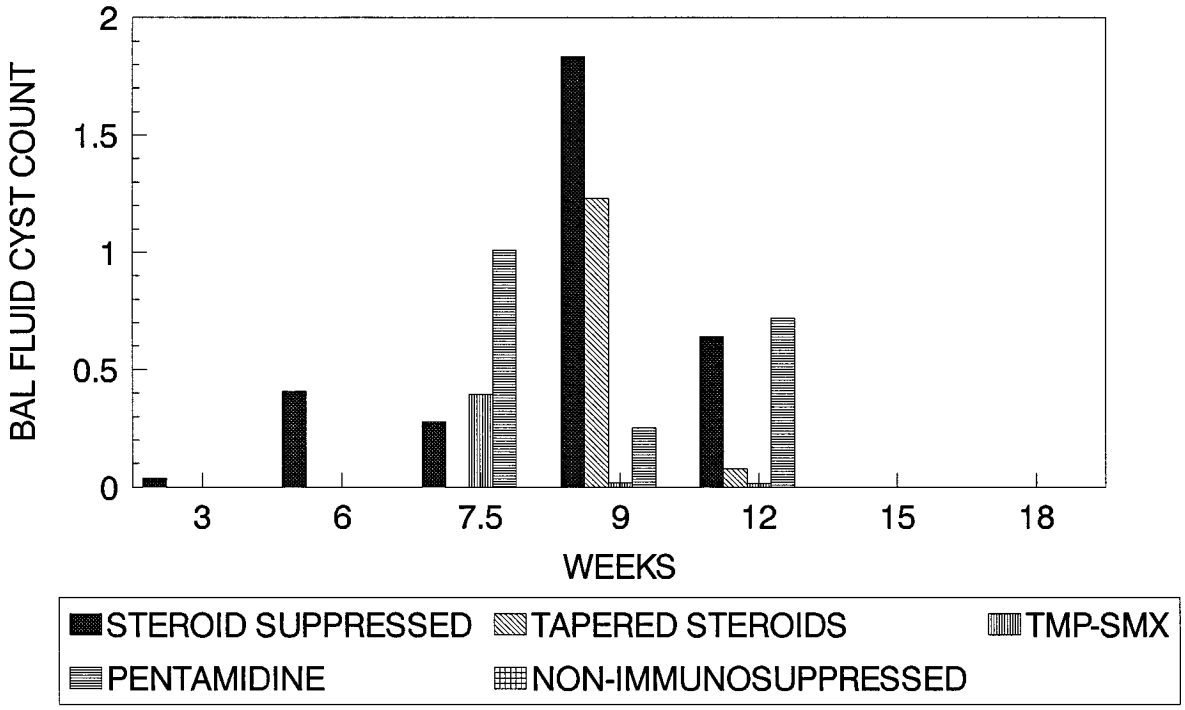

FIG. 4. Average BAL fluid cyst counts (10^6) for rats immunosuppressed with methylprednisolone for 3, 7.5, 9, and 12 weeks (steroid suppressed); rats immunosuppressed for 6 weeks, tapered off steroids for the following 3 weeks, and then left off steroids (tapered steroids); rats immunosuppressed for 6 weeks and then treated with TMP-SMX while remaining immunosuppressed; rats immunosuppressed for 6 weeks and then treated with pentamidine while remaining immunosuppressed; and rats which received no immunosuppression (nonimmunosuppressed).

on pulmonary SQPCR results are illustrated in Fig. 1. Rats withdrawn from steroids show decreased SQPCR results for PCP that approach preimmunosuppression levels by the end of the experiment. Similar results are seen for assays performed with BAL fluid (Fig. 2), except that the reduction of signal from this compartment is accelerated compared with the decrease observed in homogenate preparations.

Treatment with anti-*P. carinii* drugs. The effects of treatment with TMP-SMX on pulmonary tissue SQPCR results are seen in Fig. 1. Treatment with TMP-SMX causes a rapid decrease in the PCP SQPCR result; by the first time point (10 days of treatment), the results are not significantly different from those of control animals that have never been immunosuppressed. The effects of treatment on BAL fluid SQPCR results are not as dramatic after 10 days of TMP-SMX (Fig. 2) but then mirror the trend of the signal from lung homogenate after extended treatment.

The effects of treatment with pentamidine on pulmonary tissue and BAL fluid SQPCR results are also illustrated in Fig. 1 and 2. The results with BAL fluid closely parallel those with lung homogenate. Treatment with pentamidine appears to be less effective, as measured by SQPCR, than treatment with TMP-SMX; the differences are not statistically significant, however (Table 4).

Correlation between lung homogenate and BAL fluid SQPCR results. The results of correlation analyses between BAL fluid SQPCR results and lung homogenate SQPCR results are shown in Fig. 5. The relationship between values may

TABLE 4. *P* values for the differences in means between groups of rats, as computed by Tukey's HSD method for SQPCR results based on BAL fluid

Rat group	*P* value[a]								
	Control[b]	Ster (9 wk)	Ster (12 wk)	TMP 1	TMP 2	TMP 3	Pent 1	Pent 2	Pent 3
Control	1.000								
Ster (9 wk)	0.000	1.000							
Ster (12 wk)	0.003	0.747	1.000						
TMP 1	0.000	0.033	0.998	1.000					
TMP 2	1.000	0.000	0.271	0.003	1.000				
TMP 3	1.000	0.000	0.098	0.001	1.000	1.000			
Pent 1	0.000	0.618	1.000	0.995	0.175	0.052	1.000		
Pent 2	0.833	0.003	0.867	0.075	1.000	0.998	0.803	1.000	
Pent 3	0.022	0.286	1.000	0.903	0.614	0.299	1.000	0.991	1.000

[a] See Table 3, footnote *a*.
[b] See Table 3, footnote *b*.

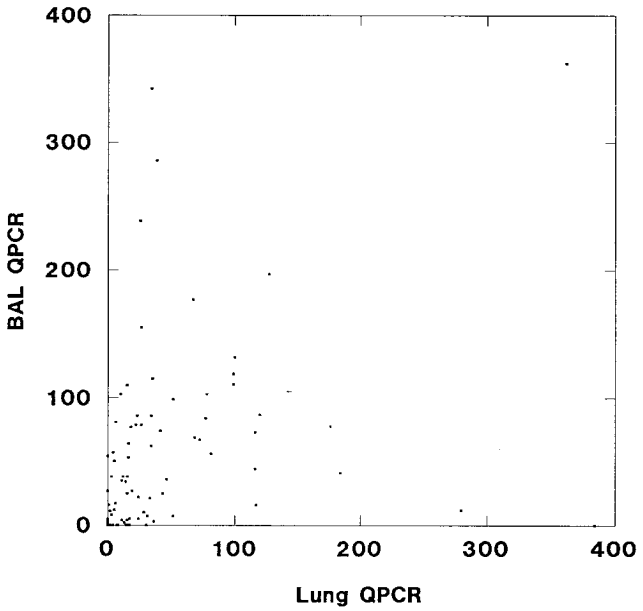

FIG. 5. Scatter plot demonstrating the relationship between lung homogenate SQPCR results and BAL fluid SQPCR results.

be summarized by the following equation: BAL fluid SQPCR = (0.462 · lung homogenate SQPCR) + 21.6251. The Pearson correlation coefficient for this relationship is 0.435, and the correlation is significant ($P < 0.001$). Nevertheless, as the scatter plot in Fig. 5 shows, there is substantial scatter. This may reflect a sampling effect. Although *Pneumocystis* organisms are not homogeneously distributed within a rat lung, only a portion of each rat's pulmonary tissue was extracted for PCR; thus, scatter might be expected to result from variations in the number of organisms present in any given sample of pulmonary tissue. One might expect this effect to be diminished when BAL fluid is assayed.

Correlation between SQPCR results and cyst counts. The correlation between cyst counting and SQPCR was tested with the Wilcoxon signed rank test; all cases were included in this analysis. The results are shown in Table 5. There is a strong correlation between cyst counting results and SQPCR results; the apparently weaker correlation between lung homogenate and BAL fluid SQPCR results is a result of the reduced statistical power associated with the use of a distribution-independent statistical test.

Detection of SQPCR signal in other organs. Very low-level SQPCR signals were detected in the livers of some rats, but not in whole blood, spleens, bone marrow, testes, and kidneys. Because the signals were weak and the results were sporadic, the assessment of hepatic tissue was not a reliable indicator of the presence of PCP infection in lungs.

TABLE 5. *P* values for the differences in results from cyst counting and SQPCR, as determined by the Wilcoxon signed ranks test

Method	*P* value[a]			
	Lung homogenate cyst count	Lung homogenate SQPCR	BAL fluid cyst count	BAL fluid SQPCR
Lung homogenate cyst count	1.000			
Lung homogenate SQPCR	0.000	1.000		
BAL fluid cyst count	0.000	0.000	1.000	
BAL fluid SQPCR	0.000	0.061	0.000	1.000

[a] See Table 3, footnote *a*.

DISCUSSION

The experimental results presented above suggest that SQPCR is an effective approach by which both to identify clinically significant *P. carinii* infections in rats and more importantly to follow treatment. Peters et al. (32) presented semiquantitative data that established that the intensity of a *P. carinii* PCR product signal correlates with the organism count. This work extends these results by demonstrating that such an SQPCR assay may be used to assess treatment efficacy in animals with PCP and by establishing the utility of an internal control (in this case, the β-globin gene) to assess DNA integrity as well as to provide an estimate of the amount of tissue sampled and subjected to PCR. SQPCR assay results strongly correlated with those obtained by cyst counting methods, whose clinical utility is already well established (6, 20). In addition, SQPCR results were qualitatively similar to cyst counting results for groups of animals withdrawn from steroids or treated with either pentamidine or TMP-SMX. As a result, we believe that the less laborious SQPCR method represents an attractive alternative to cyst counting for the assessment of potential therapeutic agents in the rat model.

In addition to molecular biologic assessment (16, 24, 32), enzyme-linked immunosorbent assays (ELISAs) (11, 12, 23) have been proposed as alternatives to cyst counting for organism quantification. The SQPCR approach described above is not yet as easily implemented as immunologic approaches but has the advantage of being readily used with a variety of sample types, including sputum and tissue, for which the implementation of ELISAs is difficult. With BAL specimens, SQPCR provides the advantage of normalizing for efficacy of specimen collection via the use of a reference gene. For this reason, we believe that SQPCR is potentially an attractive alternative to ELISAs for organism quantification. Nevertheless, our experiments have also demonstrated that the degree of rat-to-rat variation is high. Only by serially monitoring infection in individual animals by SQPCR can the true utility of this method for the assessment and management of *P. carinii* infections be clearly demonstrated.

Our data also indicate that in early infection, there is a threshold number of organisms (more than one million) that must be present in pulmonary tissue to produce detectable numbers of cysts in BAL fluid. However, this requirement is obviated by the application of SQPCR. Strong signals were present in both BAL fluid and lung homogenate after 3 weeks of immunosuppression and during the phase of early infection in which few cysts were detected in pulmonary tissue by staining and no organisms were seen in BAL fluid. Thus, this technique may have particular application to the early diagnosis of PCP or the evaluation of therapies in which small numbers of organisms may remain.

ACKNOWLEDGMENTS

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