Quantitative Real-Time Polymerase Chain-Reaction Assay Allows Characterization of Pneumocystis Infection in Immunocompetent Mice

Vibeke H. Vestereng,* Lisa R. Bishop, Beatriz Hernandez, Geetha Kutty, Hans H. Larsen,* and Joseph A. Kovacs

Critical Care Medicine Department, Warren G. Magnuson Clinical Center, National Institutes of Health, Bethesda, Maryland

Pneumocystis causes pneumonia in immunodeficient hosts but also likely causes infection in healthy hosts. To characterize infection in healthy mice, we developed and validated a real-time polymerase chain reaction assay for quantitation of Pneumocystis carinii f. sp. muris. In healthy mice exposed to Pneumocystis-infected animals, organisms were first detected at 2–3 weeks, peaked at 5–6 weeks, and were cleared by 7–9 weeks. The peak organism load in healthy animals was 2–3 logs lower than that in immunodeficient animals. This approach should facilitate studies of anti-Pneumocystis immune mechanisms in healthy hosts and provide insights into the development of Pneumocystis pneumonia in immunodeficient hosts.

Although Pneumocystis jiroveci causes pneumonia (PCP) in immunosuppressed individuals, serological data suggest that most healthy individuals have been exposed to Pneumocystis at an early age but do not develop clinically significant pneumonia [1, 2]. The characterization of the course of Pneumocystis infection and of the mechanisms of clearance in healthy hosts will lead to a better understanding of the pathogenesis of this disease in immunosuppressed hosts.

Animal models, especially rodent models, have been of critical importance in the study of Pneumocystis pneumonia. A more rapid and reliable method for quantitation of Pneumocystis organisms would be of great benefit in studying the course of this infection. The intensity of Pneumocystis infection has been difficult to quantitate, because the organisms exist in multiple forms and tend to cluster. Traditionally, organisms have been quantitated by the microscopic examination of homogenized lung tissue that has been stained for cysts or trophozoites, a procedure that is tedious and time consuming and dependent on observer interpretation [3, 4]. Alternatively, lung-tissue sections can be stained with either histochemical stains [4, 5] or Pneumocystis-specific monoclonal antibodies [6]; but these are only semiquantitative techniques. Traditional polymerase chain reaction (PCR) has also been used in this setting, but it is also semiquantitative at best and cannot be used to differentiate quantitatively among small numbers of organisms [7, 8].

Our group previously had developed a real-time PCR method to quantitate P. jiroveci in human clinical samples [9] and to quantitate Pneumocystis carinii in in vitro cultures [10]. To better study the natural history of Pneumocystis infection in healthy mice and to allow comparisons with genetically altered mice that are highly susceptible to PCP, we have developed a quantitative PCR (Q-PCR) assay that is specific for P. carinii f. sp. muris, targeting the single-copy dihydrofolate reductase gene (DHFR), and we have used this assay to characterize natural infection in immunocompetent mice.

**Materials and methods.** Healthy, 6–8-week-old BALB/c, BALB/c scid, or C57 black (C57BL) mice were purchased from the National Cancer Institute. CD40L-KO (strain B6, 129S-Tnfsf5tm1lmx) and Rag-1-KO (strain C57BL/6J-Rag-1) mice were obtained from Jackson Laboratory. CD40L-KO, Rag-1-KO, and BALB/c scid mice were subsequently bred at the National Institutes of Health (NIH). Immunocompetent mice were housed together in microisolator cages with P. carinii f. sp. muris–infected mice, to mimic the route and intensity of natural infection. Seeder mice were maintained in the cages during the entire study period whenever possible and for at least 1 week in all cages. Three experimental cages each housed 9 healthy BALB/c mice and 1 Pneumocystis-infected Rag-1-KO mouse, and 2 control cages each housed 9 healthy BALB/c mice only. One mouse from each of the 5 cages was killed periodically every 2–4 days for ∼30 days (experiments A and B) and every 3–15 days for 78 days beginning at day 21 (experiment C). Blood was obtained by cardiac puncture, and the lungs were removed. One lobe was fixed in HistoCHOICE (Amresco) with 20% ethanol, for paraffin embedding. The remaining lobes were weighed and stored at −70°C for DNA extraction. In a fourth experiment (experiment D), 4 cages each housed 3 C57BL mice, 3 CD40L-KO mice, and either 1 P. carinii f. sp. muris–infected...
BALB/c scid mouse (exposed) or no scid mouse (controls). Mice were exposed for 32, 39, or 75 days, at which time the occupants of 1 cage were killed and blood and lungs were extracted as described above. The animal-experimentation guidelines of the NIH were followed in these studies.

For DNA extraction, lung tissue was thawed and placed in NucliSens Lysis Buffer (Organon Teknika) at a concentration of 100 mg/mL and was homogenized using a polytron with a disposable generator (Omni International). DNA was extracted from 1 mg of homogenized lung tissue, by use of the NucliSens Isolation Kit (Organon Teknika), according to manufacturer’s instructions, and was eluted in a final volume of 50 μL.

For the Q-PCR, a LightCycler-FastStart DNA Master Hybridization Probes reaction mixture (Roche) that contained Taq polymerase and dNTPs was used, with the addition of 1 U of Taq polymerase and dNTPs was used, with the addition of 1 U of uracil-DNA glycosylase (UNG) to prevent carryover contamination, 5.0 mmol MgCl2/L, 0.5 μmol each primer/L (Life Technologies), 0.2 μmol each probe/L (Idaho Technology), and 4 μL of either extracted DNA or cloned template DNA, in a total reaction volume of 20 μL. The primers and probes were vhv5a (5′-TTCCGGGCCTCTTAAAGGTG-3′) and vhv3a (5′-CTAGGATGTTCCATAGCCGC-3′) and fluorescence-resonance energy-transfer probes MDHFR1 (5′-AGACAAGGAAATACATTGTCGACAGAT-fluorescein-3′) and MDHFR2 (5′-LC Red 640-TGGATGATGCTTTAGAATCTTCT-phosphate-3′).

The PCR reaction was performed as a step-down procedure. To inactivate UNG, the reactions were brought to 95°C for 10 min before cycling was performed. The first 5 cycles included denaturation at 95°C, annealing at 65°C for 10 s, and extension at 72°C for 10 s. For the next 5 cycles, the annealing temperature was 60°C, and then, for 35 cycles, it was 55°C.

A standard curve was generated by amplifying 8 × 10³, 8 × 10², and 8 × 10¹ copies of a cloned P. carinii f. sp. muris DHFR template per PCR reaction, together with glycogen (35 μg/mL) as a DNA carrier. LightCycler software was used to determine the P. carinii f. sp. muris copy number per PCR reaction, which was multiplied by 12.5 to determine the copy number per milligram of lung tissue. Samples were run in duplicate, and control reactions with no template were included in each run.

An ELISA to detect anti–P. carinii f. sp. muris antibodies was performed by a method described elsewhere [11]. Antigen prepared by sonication of partially purified Pneumocystis organisms was used to coat Immunomodule Maxisorp plates (Nunc). Serum samples were assayed at a 1:100 dilution, and the reactions were developed using horseradish peroxidase–conjugated goat anti–mouse IgG and o-phenylenediamine dihydrochloride substrate (Sigma).

For immunohistochemistry, lung-tissue sections were incubated with an anti-Pneumocystis monoclonal antibody, 4D7, followed by horseradish peroxidase–conjugated secondary antibody and substrate, as described elsewhere [6]. Clusters of Pneumocystis in 1 complete lung-tissue section for each animal were quantitated.

**Results.** To study Pneumocystis infection in healthy as well as in immunodeficient animals, we wanted to develop a Q-PCR assay with a broad dynamic range that used the DHFR gene. DHFR is a single-copy gene; therefore, quantitating the number of its copies allows a direct quantitation of the number of Pneumocystis nuclei. We used a step-down PCR amplification procedure, which increases specificity and sensitivity. After optimization, this Q-PCR assay had a quantitative range of >5 logs when a plasmid template was used. Plotting the theoretical copy number versus the experimental copy number gave a correlation coefficient, R, of 0.99 (n = 17), by linear regression.

We then used the assay to quantitate the number of organisms in infected lung tissues. Initially, DNA was extracted by a traditional extraction method using proteinase K and phenol/chloroform, but reproducibility was inadequate. Reproducibility was markedly improved when the NucliSens DNA extraction kit was used. The quantitation (log copies) of replicate extractions varied by <2%, with the exception of 1 sample, which had very low levels that varied by ~18%. Day-to-day reproducibility was good, with 2.4% variation when DNA was extracted from the same lung tissue on 2 different days.

To study the natural history of Pneumocystis infection by use of a mouse model, immunocompetent mice were housed together with an infected seeder mouse (3 cages with 9 healthy BALB/c mice plus 1 seeder) or without a seeder mouse (2 control cages). Initially, we observed animals for ~30 days after they had been housed together and found a similar course of infection in 2 separate experiments (figure 1A, experiments A and B). Because the Pneumocystis-organism load was still increasing at day 30, we conducted an additional experiment, to determine the peak of infection and the rate of clearance (figure 1C). Infection was similar to that in immunodeficient animals, we wanted to develop a Q-PCR assay with a broad dynamic range that used the DHFR gene. DHFR is a single-copy gene; therefore, quantitating the number of its copies allows a direct quantitation of the number of Pneumocystis nuclei. We used a step-down PCR amplification procedure, which increases specificity and sensitivity. After optimization, this Q-PCR assay had a quantitative range of >5 logs when a plasmid template was used. Plotting the theoretical copy number versus the experimental copy number gave a correlation coefficient, R, of 0.99 (n = 17), by linear regression.

We then used the assay to quantitate the number of organisms in infected lung tissues. Initially, DNA was extracted by a traditional extraction method using proteinase K and phenol/chloroform, but reproducibility was inadequate. Reproducibility was markedly improved when the NucliSens DNA extraction kit was used. The quantitation (log copies) of replicate extractions varied by <2%, with the exception of 1 sample, which had very low levels that varied by ~18%. Day-to-day reproducibility was good, with 2.4% variation when DNA was extracted from the same lung tissue on 2 different days.

To study the natural history of Pneumocystis infection by use of a mouse model, immunocompetent mice were housed together with an infected seeder mouse (3 cages with 9 healthy BALB/c mice plus 1 seeder) or without a seeder mouse (2 control cages). Initially, we observed animals for ~30 days after they had been housed together and found a similar course of infection in 2 separate experiments (figure 1A, experiments A and B). Because the Pneumocystis-organism load was still increasing at day 30, we conducted an additional experiment, to determine the peak of infection and the rate of clearance (figure 1A, experiment C). P. carinii f. sp. muris DNA could be detected in individual animals as early as days 10–12 after mice had been housed together. Subsequently, the number of organisms steadily increased, peaking at days ~36–39 (geometric mean, 365–379 copies/mg in experiment C; n = 6). Infection was cleared by days 53–63. Experiment D was performed to verify that the course of Pneumocystis infection in healthy C57BL mice was similar to that in BALB/c mice and to examine the course of Pneumocystis infection in CD40L-KO mice. Both healthy C57BL and CD40L-KO mice exposed to a P. carinii f. sp. muris–infected seeder mouse became infected, with similar numbers of Pneumocystis organisms, by day 32 (P > .2; Student’s t test). However, the organisms were cleared in C57BL mice by day 75, whereas the number of organisms increased in the CD40L-KO mice until the end of the experiment, at day 75 (291,583 copies/mg, range, 254,875–345,750 copies/mg [P < .001 vs. C57BL]). The highest organism load observed in immunocompetent mice was 3848 copies/mg, which is substantially lower than that in
Figure 1. A. No. of copies of *Pneumocystis* organisms in exposed and unexposed animals, as determined by quantitative polymerase chain reaction. Each panel represents the results of a single experiment; each time point represents the mean for 3 (exposed) or 2–3 (unexposed) animals. Individual healthy exposed mice became infected as early as days 10–13. Organism load peaked at day \( \sim 36 \) (experiment C) and cleared in all animals by days 63–75 (experiments C and D). *Pneumocystis* copy nos. in CD40L-KO mice continued to increase through day 75 (experiment D). The SE is indicated by the error bars. B. Anti–*Pneumocystis carinii* f. sp. *muris* IgG antibodies, as detected by ELISA, for animals in experiments C and D. Antibody levels began to increase at day \( \sim 39 \) and remained elevated through the end of the experiment, at days 75–78. Neither unexposed mice nor exposed CD40L-KO mice produced anti–*P. carinii* f. sp. *muris* IgG antibodies. Each time point represents either the average optical-density value for 3 exposed or 2 unexposed mice (1 mouse from each cage at each time point), in the case of experiment C, or the average optical-density value for 3 exposed or unexposed mice from 1 cage, in the case of experiment D. Background values (no primary antibody) have been subtracted. The SE is indicated by the error bars.

Lung-tissue sections from both infected and control mice from experiment C, days 32–43, were stained with a monoclonal antibody, 4D7, that is specific for *Pneumocystis* (figure 2). Clusters of *Pneumocystis* in 1 complete lung-tissue section for each animal were quantitated. The counting of clusters of organisms by histological testing showed a good correlation with the copy numbers detected by Q-PCR \( (R = 0.8) \).

Anti–*P. carinii* f. sp. *muris* IgG antibodies could be detected in immunocompetent *Pneumocystis*-exposed mice beginning at day 39, and these remained detectable until the end of the study (figure 1B). As expected, neither the CD40L-KO mice (which are not capable of Ig-class switching) nor the unexposed, healthy mice produced anti–*P. carinii* f. sp. *muris*–specific IgG antibodies.

**Discussion.** We have developed and validated a Q-PCR assay that uses DHFR, a single-copy gene, to reproducibly quantitate *P. carinii* f. sp. *muris* organisms in animals with high or low levels of infection, without the need to count organisms in either tissue sections or impression smears. This Q-PCR assay enabled us to monitor natural *Pneumocystis* infection in im-
Figure 2. Immunohistochemical staining of lung-tissue sections from immunocompetent *Pneumocystis carinii* f. sp. *muris*-infected BALB/c mice that had been housed together with *Pneumocystis*-infected mice. *A,* Very light infection (day 32; 17 copies/mg of lung tissue, according to the results of quantitative polymerase chain reaction [Q-PCR]); *B,* Light infection (day 36; 3168 copies/mg of lung tissue, by Q-PCR). Each lung-tissue section is shown at 100× (left) and 1000× (right) magnification. Clusters of *P. carinii* f. sp. *muris* organisms stained brown (arrows). In panels A, 2 clusters were seen in the entire lung field, whereas, in panels B, 18 clusters were seen. For comparison, panel C shows the staining of a section of lung (magnification, 100×) from a *P. carinii* f. sp. *muris*-infected Rag-1 mouse (457,000 copies/mg of lung tissue).
munocompetent mice, detecting as few as 12.5 copies/mg lung tissue. We detected *Pneumocystis* in exposed mice as early as 10–13 days after exposure. Organism loads in immunocompetent animals peaked after ~5–6 weeks of exposure, and organisms were cleared after ~7–9 weeks of exposure. Our results are similar to those reported in a recent study that used microscopic quantitation to examine natural infection in immunocompetent mice [7], as well as to those in studies of the course of *Pneumocystis* infection in weaning rabbits [12, 13].

Although the initial course of infection was similar in immunodeficient (CD40L-KO) mice, the organism burden in those animals continued to increase during the 75 days of observation. Given that there is no (or very minimal) restriction of growth of *Pneumocystis* by the immune system in these mice, the similar organism number through day 32, with the subsequent divergence by day 39, suggests that the immune response in immunocompetent animals begins to effectively control infection only after ~5 weeks. The quantitation of the number of organisms in CD40L-KO mice over time allows an estimation of 5–8 days as the organism (nucleus) doubling time, in the absence of any effective immune response. This suggests that *Pneumocystis* is a relatively slowly replicating organism—a factor that may contribute to the difficulties in culturing the organism in vitro [14]. This assay should greatly facilitate studies that require the quantitation of *Pneumocystis* organisms. Because it uses a single-copy gene, quantitation by Q-PCR should accurately reflect the number of organisms (nuclei). A recently described Q-PCR assay based on the single-copy Kex1 gene should generate results similar to those produced by our assay, although, to date, the sample processing and reproducibility for that recently described assay have not been validated [15].

In the present study, levels of IgG antibodies did not clearly rise before a decrease in organism load, which suggests that these antibodies may not be critical to the control of *Pneumocystis* infection in immunocompetent animals. However, IgM antibodies, which may have developed before IgG antibodies, were not evaluated.

This mouse model should be useful in characterizing the immunological responses involved in the clearance of *Pneumocystis* from the lungs of immunocompetent animals. A better understanding of the immunological responses to *Pneumocystis* in healthy hosts should provide insight into potential immunological defects that allow *Pneumocystis* infection to progress in immunosuppressed hosts.

### Acknowledgments

We thank Rene Costello and Howard Mostowski for their assistance.

### References