Dynamics of *Pneumocystis carinii* Air Shedding During Experimental Pneumocystosis

Firas Choukri,1 El Moukhtar Aliouat,2 Jean Menotti,1 Anne Totet,3 Nausicaa Gantois,2 Yves J. F. Garin,1 Vance Bergeron,4 Eduardo Dei-cas,2 and Francis Derouin1

1Department of Parasitology-Mycology, Paris-Diderot University and Saint Louis Hospital (Assistance Publique-Hôpitaux de Paris), 2Department of Biology and Diversity of Emerging Eukaryotic Pathogens, Center for Infection and Immunity of Lille, Université Lille–Nord de France, 3Department of Parasitology-Mycology, University of Picardy and University Hospital, Amiens, and 4Physics Laboratory, Ecole Normale Supérieure de Lyon, France

To better understand the diffusion of *Pneumocystis* in the environment, airborne shedding of *Pneumocystis carinii* in the surrounding air of experimentally infected rats was quantified by means of a real-time polymerase chain reaction assay, in parallel with the kinetics of *P. carinii* loads in their lungs. *P. carinii* DNA was detected in the air 1 week after infection and increased until 4–5 weeks after infection before stabilizing. A significant correlation was shown between lung burdens and the corresponding airborne levels, suggesting the possibility of estimating the fungal lung involvement through quantification of *Pneumocystis* in the exhaled air.

The genus *Pneumocystis* designates opportunistic extracellular fungal species infecting a wide range of mammals, including humans. In immunocompromised patients, the airborne-transmitted *Pneumocystis jirovecii* (human-derived *Pneumocystis*) is responsible for a severe interstitial alveolar lung disease, *Pneumocystis* pneumonia (PcP), which is fatal without specific treatment. During infection, microorganisms multiply in the alveolar lumen according to a hypothetical life cycle that includes trophic forms, intermediate stages (called sporocytes), and mature cysts [1]. Most of our knowledge of the pathogenesis and transmission of *Pneumocystis* is gained from experimental models. Rat and mouse models of PcP have been used to demonstrate the airborne transmission of *Pneumocystis* from an infected host to a susceptible host [2, 3]. Furthermore, it has been shown that *Pneumocystis* organisms were able to multiply transiently in the lungs of immunocompetent hosts and to transmit the infection to either susceptible or immunocompetent hosts by the airborne route [3–6]. In human studies, analysis of PcP cluster cases, combined with genotyping of fungal isolates, has supported the theory of human-to-human transmission of *P. jirovecii* [7]. Indeed, the presence of *Pneumocystis* has been revealed in the immediate environmental air of infected animals [8] and humans [9], and *P. jirovecii* DNA was recently quantified in air samples from hospital rooms of infected patients, using real-time polymerase chain reaction (PCR) [10]. However, the air excretion of *Pneumocystis* during the course of PcP has yet to be characterized. Because this information is important to evaluate the transmission risk of *Pneumocystis*, we use an experimental model of PcP that closely mimics human PcP to sequentially quantify *Pneumocystis* in the air surrounding intratracheally infected rats.

**MATERIALS AND METHODS**

**Animal Model**

We used a rat model of *Pneumocystis carinii* (rat-derived *Pneumocystis*) pneumonia, as previously described [11]. Briefly, 10-week-old athymic Lou nu/nu rats, bred in sterile conditions at the Pasteur Institute of Lille (France) and free of latent *P. carinii* infection (as regularly checked in the rat colony by means of PCR on lungs of randomly selected rats), were used and housed in positive-pressure high-efficiency particulate air (HEPA)–filtered isolators. All animals (including uninfected controls) were treated with 1 mg/L of dexamethasone (MSD Chibret) in their drinking water, starting 2 weeks before *Pneumocystis* inoculation and maintained throughout the experiment. The inoculum consisted of 10⁷ cryopreserved parasites that had been collected and purified from the lungs of heavily infected rats [12]. Rats were inoculated intratracheally using a nonsurgical method after isoflurane anesthesia. All animal experimentation guidelines were followed.

**Air Sampling**

For collecting the environmental air surrounding infected rats, we developed a specific device in which a liquid medium biocollector Coriolis µ (Bertin Technologies) was directly connected to a HEPA-filtered air-sampling chamber (50 × 50 × 20 cm). To avoid environmental and cross-contamination,
experiments were performed in a positive-pressure laboratory and the sampling device was placed in a laminar airflow cabinet. Each rat was sampled individually (Figure 1).

Before and between each air sample, the chamber was carefully cleaned with a chemical disinfectant (Aniospray, Laboratoires Anios) and a control air sample was collected. Thereafter, 1 rat was placed in the chamber for a 90-min period before air sample collection. Control and experimental air samples were collected during 3 min at a flow rate of 300 L/min, allowing for a complete air purge of the chamber. Each air sample was collected on 15 mL of sterile phosphate-buffered saline (PBS) +0.002% polysorbate 80 and subsequently centrifuged at 2500 g for 10 min. The supernatant was removed to provide a 1-mL pellet that was stored at 4°C for later quantitative PCR assay.

Experimental Protocols
Initially, the kinetics of P. carinii excretion in air was examined in 3 infected nude rats, whose surrounding air was sampled just before inoculation (day 0) and then at days 12, 22, 29, 36, 41, and 49 after infection, with the knowledge that rats usually do not die before day 60 in this model.

A second experiment was designed to examine the correlation between the fungal load in the surrounding air and in the lungs of infected rats. Twenty-one nude rats were infected at day 0, and 6 noninfected rats were used as a control. At days 7, 14, 21, 28, 35, 42, and 49 after infection, 3 infected rats were randomly selected from each group. Between the fungal load in the surrounding air and in the lungs of infected rats. Twenty-one nude rats were infected at day 0, and 6 noninfected rats were used as a control. At days 7, 14, 21, 28, 35, 42, and 49 after infection, 3 infected rats were randomly selected from each group.

To express qPCR data in numbers of P. carinii organisms, a calibration curve was prepared from a suspension of P. carinii accurately counted on RAL-stained smears by a well-trained microscopist. Each qPCR run comprised 7 serial 10-fold dilutions of this suspension, ranging from 8.4 to 8.4 x 106 P. carinii organisms/mL. The following regression curve was calculated by means of linear regression analysis: \( y = x + S \log y_0 \), where \( y \) is the observed threshold cycle, \( x \) is the initial number of P. carinii organisms/mL, \( y_0 \) is the intercept for \( \log y_0 = 0 \), and \( S \) is the slope of the regression line. The slope of the regression line was \(-3.589 \pm 0.016\), and the regression coefficient \( r^2 \) was 0.9999 (\( P < .001 \)). From this curve, qPCR results from air and lung samples could be converted to the number of P. carinii organisms/mL of sample with an estimate of the qPCR detection limit at 8.4 P. carinii organisms/mL. Adjustment to the original sample size allowed final expression of the results in terms of number of P. carinii organisms per air sample or per lung.

Statistical Analysis
Statistical tests were performed using Graphpad Prism, version 5.01 for Windows (Graphpad Software, www.graphpad.com); \( P \) values of <.05 were considered to reveal a significant difference.

RESULTS
All qPCR results on control air samples and lungs from non-infected rats at days 0, 21, and 49 were negative. In experiment 1...
(Figure 2A), *P. carinii* organisms were detected and quantified in the surrounding air of the 3 rats from day 12, with concentrations ranging between 8 and 32 *P. carinii* organisms per air sample. Subsequently, the kinetics of excretion was similar for the 3 rats, with mild interindividual variations. Air burdens progressively increased to reach a maximum value at day 36, with fungal loads of between 890 and 3200 *P. carinii* organisms per air sample, followed by a stabilization or trend to decrease until day 49 (Figure 2A).

During the second experiment, a comparable kinetics of air excretion was observed. *P. carinii* load was detected in air from day 7 for the 3 rats, and it then increased until day 28 before stabilizing (Figure 2B).

In parallel, the kinetics of *P. carinii* load in the lungs was monitored. At day 7, fungal loads ranged between $3.2 \times 10^5$ and $1.3 \times 10^6$ *P. carinii* organisms per lung (Figure 2B). The fungal burden increased until day 49 [range, $(1.6–7.5) \times 10^6$ *P. carinii* organisms per lung], with a tendency to stabilize from day 35. As for the air samples, a low rate of interindividual variation was observed throughout the experiment.

Fungal loads in the lungs were also quantified microscopically. Overall, values estimated with use of microscopy were slightly higher than those estimated with use of qPCR (paired *t* test; mean difference, $0.67 \pm 0.17$ [log]; *P* < .001). However, both techniques yielded similar patterns of *P. carinii* infection kinetics, with a good correlation between them ($r^2 = 0.86; P < .001$) (Figure 2B). Finally, we examined the relationships between *P. carinii* air shedding and *P. carinii* infection level, as estimated by means of qPCR. A significant correlation was found between air and lung fungal burden ($r^2 = 0.48; P < .001$), with an approximate ratio of $1/10^6$ between air and lung burdens throughout infection.

**DISCUSSION**

To our knowledge, the kinetics of microbial shedding in exhaled air has rarely been quantified during the course of airborne-transmitted infections, even experimentally, mainly because air-sampling devices are more adapted to environmental monitoring than to individual sampling.

In this study, we first showed that using a liquid medium biocollector Coriolis branched directly to an HEPA-filtered experimental chamber provides a very well adapted and reproducible system for collecting the surrounding air exhaled by individual rats. By combining this method with a specific qPCR assay, *P. carinii* from the sampled air could be quantified. The results obtained using this method were reproducible in 2 separate experiments with a low rate of interindividual variability.

Second, sequential sampling after infection allowed us to describe for the first time the kinetics of the air excretion of *P. carinii* during the course of pneumocystosis. The main finding was that *P. carinii* could be detected as early as 7 days after infection. Indeed, this result stems from an experimental intratracheal infection with a high-concentration inoculum, but it
remains indicative of a short latency period between infection and fungal shedding. Such information has not previously been documented for Pneumocystis and could be important to consider in the risk estimate of transmission among immunocompromised hosts. During the course of infection, fungal load increased in the air over time to reach a peak value 4–5 weeks after infection and then showed a tendency to stabilize or to slightly decrease. This tendency was not related to a decrease in the fungal load in the lungs, because we showed that P. carinii burden increased continuously in the lungs over time, but could be explained by the decrease in breathing capacity at the final stage of infection. Additional study is warranted to estimate the viability and infectivity of airborne Pneumocystis and the rat infectiousness during the course of infection.

Third, fungal loads were examined in the lungs of rats that were euthanized at each air-sampling date. Results were in agreement with those of previous studies using the same Pneumocystis nude rat model, by showing a progressive increase in fungal burdens until the 7th week after infection [11] and an excellent reproducibility of the course of experimental infection between rats. Concordant P. carinii rates were observed between microscopy and qPCR, a result that validates the qPCR assay to quantify P. carinii and enables the conversion of Ct values into number of P. carinii microorganisms. By using qPCR on sequential air and lung samples, a significant correlation was shown between lung burdens and the corresponding burdens in air, with a lung:air ratio of approximately 10^6:1 throughout the course of the disease.

Despite the specificities of human pneumocystosis in AIDS and non-AIDS patients regarding incubation period and disease progression [13], an extrapolation of our results to human-acquired pneumocystosis is reasonable to consider because of the pronounced similarities between pulmonary lesions of PcP in immunocompromised patients and the nude rat model [14, 15]. We previously showed that P. jirovecii was detectable and quantifiable in the close environment of PcP patients, with a correlation trend between fungal burden in the patient’s pulmonary samples and the corresponding air samples taken in proximity to the patient [10]. The present observations in a relevant animal model support this correlation in human PcP and may open up the possibility of estimating the fungal lung involvement using a noninvasive method through quantification of Pneumocystis in exhaled air.

Funding
This work was supported by grants from the Agence Francaise de Sécurité sanitaire de l’Environnement et du Travail (nos. EST/2006/1/41 and 06-CRD-29 to F.C.).

Acknowledgments
We thank Claudine Sarfati, MD; Annie Sulahian, PhD (Saint Louis Hospital, Paris, France); and Magali Chabe, PharmD, PhD (Pasteur Institute of Lille, France) for helpful discussions.

References