Pneumocystis Colonization Is Highly Prevalent in the Autopsied Lungs of the General Population

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(See the brief report by Vargas et al and the editorial commentary by Calderón, on pages e19–21 and 354–6, respectively.)

Background. Increasing reports of *Pneumocystis* DNA in noninvasive respiratory specimens from immunocompetent asymptomatic adults and the characteristic lung tropism of *Pneumocystis* suggest that asymptomatic pulmonary infections with *Pneumocystis* occur after primary infection. However, studies searching for *Pneumocystis* in the autopsied lungs of healthy immunocompetent adults have not met with success.

Methods. Lungs of people who died of violent causes (accidents, homicide, and suicide) and of nonviolent causes (diseases causing a rapid demise in the street) in Santiago, Chile—for whom an autopsy was legally required—were examined for *Pneumocystis* by nested polymerase chain reaction (PCR) DNA amplification of the mitochondrial large subunit ribosomal RNA–specific *P. jirovecii* gene and immunofluorescent microscopic analysis. Lung tissue concentration methods and analysis of ~3% of the weight of the right upper lobe (RUL) were needed to reach the sensitivity threshold of the assays. Individuals determined to be *P. jirovecii* negative after analysis of 3% of the RUL weight in the violent death group were confirmed to be negative by analyzing additional tissue, totaling 6%–7% of the RUL weight.

Results. *P. jirovecii* was identified by nested PCR in 50 (64.9%) of 77 individuals (34 [61.8%] of 55 in the violent death group and 15 [78.9%] of 19 in the nonviolent death group; $P > .05$) and additionally by microscopic analysis in all individuals who tested positive for *P. jirovecii* DNA in the violent death group. Analysis of tissue beyond 3.0% of the RUL weight for the individuals who tested negative yielded consistently negative results.

Conclusions. A mild *P. jirovecii* pulmonary infection is prevalent in more than half of the general adult population. Our results strengthen the concept that immunocompetent adults develop frequent self-limited re-infections throughout life and participate in the circulation of *P. jirovecii* as an infective reservoir for susceptible individuals.

The development of highly sensitive molecular diagnostic techniques for DNA amplification, such as nested polymerase chain reaction (PCR), has allowed detection of minute burdens of *Pneumocystis* organisms in immunocompetent hosts that would otherwise remain undetected by techniques used for the diagnosis of *Pneumocystis* pneumonia (PCP) in immunocompromised patients, such as microscopic analysis and DNA amplification by single-round PCR. This highly sensitive nested PCR DNA amplification technique is used in epidemiological studies to detect *Pneumocystis* DNA in noninvasive respiratory specimens (such as nasal swabs, oropharyngeal washes, nasopharyngeal aspirates, sputum, and others) from different populations of immunosuppressed and immunocompetent individuals without overt PCP, a condition variously referred to as *colonization*, *carrier state*, and *subclinical infection* [1]. However, despite the inherent lung tropism of *Pneumocystis* [2] and the correlation between the number of *Pneumocystis* organisms recovered from noninvasive versus lung specimens shown in immunosuppressed animal models of PCP that develop a characteristically high load of *Pneumocystis* organisms in the lungs [3], results of *Pneumocystis jirovecii* nested PCR DNA amplification in noninvasive respiratory specimens from immunocompetent individuals have not been demonstrated to predict lung involvement. Therefore, lung

autopsy studies are needed to characterize the true incidence of pulmonary colonization and to permit a better understanding of the epidemiology, natural history, and eventual pathogenic power of *P. jirovecii* infection in the general population.

Reported incidences of *P. jirovecii* DNA in healthy immunocompetent adults vary from 0% in studies of induced or expectorated sputum and nasal swab specimens [4–7] to 20% in a study using oropharyngeal wash specimens from hospital administrative workers [8]. Nevez et al [9] have reported a 19.5% incidence in invasive bronchoalveolar lavage specimens from human immunodeficiency virus (HIV)–negative individuals. Similarly, *P. jirovecii* DNA has been documented in 55% of sputum specimens and in 19.1% of lung biopsy specimens in different studies of patients with chronic obstructive pulmonary disease (COPD) [10, 11]. In addition, immunocompetent adult health care workers develop an antibody response suggestive of asymptomatic reinfection when exposed to patients with PCP [12]. Furthermore, immunocompetent adult rodents develop a mild self-limited infection, as documented by microscopic analysis and nested PCR, in the lungs after exposure to an infective source [13–15]. These data suggest that reinfections with *Pneumocystis* occur after the primary infection in healthy individuals and therefore that *P. jirovecii* may be common in the lungs of immunocompetent adults. However, efforts to identify *Pneumocystis* in lung tissue specimens from the general adult population have not met with success [16, 17].

In contrast to the incidence reported among immunocompetent adults, that reported among healthy infants is remarkably high, as has been documented using both noninvasive [18–21] and lung tissue [22–26] specimens (varying from 15.9% to 32% in the former and from 9.4% to 44.6% in the latter). Beard et al [27] reported an incidence of 100% by examining 4 lung tissue specimens per infant.

Unlike with organisms that can be grown in culture, the type, quality, quantity, and number of specimens examined—plus the processing technique—are critical for the detection of minimal *Pneumocystis* loads. These aspects are even more important for immunocompetent hosts, in whom *Pneumocystis* replicates to low numbers and tends to cluster. Variations in processing techniques and in the type and amount of respiratory specimens analyzed may explain the differences in the incidences of colonization reported, and this possibility motivated us to retest the hypothesis that *Pneumocystis* colonizes the lungs of healthy adults by scaling up the amount of lung tissue examined for adults in proportion to the amount of lung tissue examined for infants in nested PCR and immunofluorescence analyses, under the simple secondary hypothesis that analysis of more lung tissue with concentration techniques is needed to reach the sensitivity threshold of the assays used and prove the concept.

Autopsies are infrequently performed for people who die naturally at home. Therefore, we sought individuals who died of violent causes (accidents, homicide, and suicide) or of nonviolent causes (diseases causing a rapid demise in the street), whose autopsies are legally required in Chile. Individuals who had been admitted to the hospital before death were excluded, to avoid the potential bias of hospital environment in colonization rates.

METHODS

Ethics. The Ethics Commission of the North Metropolitan Area of Health in Santiago approved this study. Chilean law requires autopsies for accidental deaths.

Autopsy specimens and data collection. Between May 2005 and November 2008, lung specimens were prospectively obtained at autopsy from individuals who died without being admitted to the hospital and whose autopsy was legally required at the Servicio Medico Legal (Chilean coroner's office) in Santiago. Subjects were selected on the basis of not having been admitted to the hospital, unexpected or violent death occurring in the street, absence of known immunocompromising conditions, and absence of obvious pulmonary disease on macroscopic examination. Autopsy diagnosis was established on the basis of clinical history, results of postmortem laboratory tests, and gross findings. Medical information (including age, date of death, autopsy findings, and autopsy diagnoses) were collected from the coroner's report before *Pneumocystis* analyses.

Processing of specimens. The right lung was removed using sterile equipment, placed in a sterile plastic bag, and stored at −80°C until processing for analysis. Only 1 lung was processed on a single day. The lung was slowly thawed by moving it to a −20°C refrigerator overnight and then to a −4°C refrigerator in the morning for ∼2 h. Once partially thawed, the lung was transported to a biosafety cabinet, removed from the bag, placed on a large sterile plate, soaked and cleaned with sterile phosphate-buffered saline (PBS) (pH 7.2), and moved to a new sterile plate, on which the lobes were separated. The right upper lobe (RUL) was selected in all cases. Weight was recorded, and the pleura was carefully removed using new, sterile equipment. Approximately 0.5-g specimens as needed to amount to 3% of the RUL weight were obtained from deep lung tissue through different 2-cm-deep incisions in the decorticated surface. Each specimen was obtained using separate sterile equipment and was processed individually.

***Pneumocystis* DNA extraction and amplification.** Specimens were cut into small pieces and homogenized by magnetic stirrer agitation in 20 mL of sterile PBS (pH 7.2) in ice pack–covered screw-capped flasks. The homogenate was then filtered using sterile gauze; the stirring flasks were washed with sterile PBS to collect any remnants of the specimens; centrifugation

Table 1. Autopsy Results and *Pneumocystis* Status, as Determined by Nested Polymerase Chain Reaction DNA Amplification and, in Addition, by Immunofluorescent Analysis for Those Who Died of a Violent Cause

Circumstance of death	Total no.	Age, median years (range)	Positive for *Pneumocystis*,[a] no. (%)
Violent death	55	…	34 (61.8)
Traffic accident	22	37.9 (12.7–66.6)	13 (59.0)
Suicide	20	42.7 (6.0–88.0)	13 (65.0)
Homicide	10	41.5 (23.6–50.1)	7 (70.0)
Other[b]	3	22.1 (10.0–68.3)	1 (NA)
Nonviolent death (medical diagnosis)[c]	19	60.1 (5.1–82.5)	15 (78.9)
Undetermined	3	55.0 (44.1–55.2)	1 (NA)
Total	77	…	50 (64.9)

NOTE. NA, not applicable (percentages were not estimated because of too few observations in each category).

[a] $P > .05$ for the comparison of prevalence between violent and nonviolent causes of death (2-tailed Fisher exact test).

[b] Electric shock ($n = 2$) and drowning ($n = 1$).

[c] Myocardial infarction ($n = 13$), cardiac tamponade ($n = 1$), stroke ($n = 1$), subarachnoid hemorrhage ($n = 1$), digestive hemorrhage ($n = 1$), peritonitis ($n = 1$), and intestinal obstruction ($n = 1$).

was performed at 4°C for 10 min at 2900 *g*; the supernatant was discarded; and the pellet was reconstituted in 700 μL of sterile PBS (pH 7.2) and stored at −20°C until processing. DNA was extracted and purified from 200 μL of this solution, using the QIAamp DNA Mini kit (Qiagen) as described elsewhere [28]. Liver tissue was processed in the same fashion as lung tissue during DNA extraction and purification, to monitor for cross-contamination. *Pneumocystis* DNA was identified by a 2-step nested PCR procedure using oligonucleotide primers pAZ102-E and pAZ102-H (which were designed for the gene encoding the mitochondrial large subunit ribosomal rRNA [rRNA] of *Pneumocystis* and which amplify all *Pneumocystis* species) and internal primers pAZ102-X and pAZ102-Y (which are specific for *P. jirovecii*), as described elsewhere [21]. PCR experiments were performed in a separate room in sterile hoods, with blinding to clinical details. *Pneumocystis*-negative and human β-globin internal controls were included with each specimen, to monitor for cross-contamination and DNA inhibitors. Standard cleaning and sterilization procedures were followed for reusable materials, including overnight immersion in a 20% dilution of superquaternary ammonia and moist heat sterilization (or dry, when applicable) before and after washing in neutral Extran solution (Merck). Standard cleaning and sterilization procedures using DNA breaking fluids (DNA Away; VWR Scientific Products) were applied to the biosafety cabinet and the hood units between processing specimens from each subject.

A subject was considered to be *Pneumocystis* positive when the expected *P. jirovecii* DNA–specific 267-bp band was visualized in 1 or more of his lung specimens. A subject was considered to be *Pneumocystis* negative if no *P. jirovecii* DNA was documented.

Microscopic analysis. Forms of *Pneumocystis* were studied by microscopic analysis using immunofluorescent stain (MeriFluor kit; Meridian Biosciences) in concentrated aliquots of homogenized specimens from individuals who died of violent causes. Microscopy observers (C.A.P., R.B., and S.L.V.) were unaware of the nested PCR results. A subject was considered to be *Pneumocystis* positive when 2 or more typical cysts or >10 small and noncharacteristic *Pneumocystis* forms were visualized. Results were agreed on by all observers (C.A.P., R.B., and S.L.V.).

Statistical analysis. The Stata statistical package, version 10.1 (StataCorp), was used to determine whether *Pneumocystis* carriage was associated with circumstance of death, by 2-tailed Fisher exact test (Table 1). Differences for which $P < .05$ were considered significant.

RESULTS

Subject characteristics and autopsy specimens. Complete right lungs were obtained from 77 individuals (59 males and 18 females). Their median age at death was 44.1 years (range, 5.1–88.0 years; mean, 43.3 years), and 10 individuals were <20 years old (range, 5.1–17.7 years; mean, 12 years). Causes of death were as follows: traffic accidents, 22; suicide, 20; homicides, 10; electric shock, 2; drowning, 1; medical causes, 19; and undetermined, 3. Autopsy diagnoses for medical causes were as follows: myocardial infarction, 13; cardiac tamponade, 1; stroke, 1; subarachnoid hemorrhage, 1; digestive hemorrhage, 1; peritonitis, 1; and intestinal obstruction, 1. The median es-

timated time from death to autopsy was 17 h (range, 7–54 h; mean, 18.34 h). Pneumonia was ruled out by microscopic analysis in all subjects. Information on history of use of drugs with reported anti-*Pneumocystis* activity or of exposure to immunosuppressed individuals was not available.

Each tissue specimen was analyzed individually. The median total RUL weight was 111 g (range, 29–280 g; mean, 116.5 g), and the median total weight of specimens analyzed was 0.480 g (range, 0.413–0.556 g; mean, 0.477 g). Tissue for microscopic analysis and DNA extraction was obtained from the same homogenized, concentrated specimens.

DNA amplification. Results of amplification reactions of controls for contamination of DNA extraction and purification using liver tissue were negative on all occasions. Single-step PCR showed negative results for *Pneumocystis* DNA in all specimens. *P. jirovecii* DNA was detected by nested PCR in 50 (64.9%) of the 77 subjects overall, in 38 (64.4%) of the 59 male subjects, and in 12 (66.6%) of the 18 female subjects. One patient yielded DNA bands that were very difficult to see in 2 of 7 specimens, and analysis of an additional 3% of RUL weight demonstrated clearly positive bands in 4 of 6 new tissue specimens. *P. jirovecii* DNA–negative individuals in the violent death group were retested using specimens with an additional 3% of RUL weight, and the results were consistently negative.

P. jirovecii DNA was detected more frequently in individuals with a medical condition as the autopsy diagnosis (78.9%) than in those dying in traffic accidents (59.0%), of suicide (65.0%), or of homicide (70.0%).

The median number of specimens analyzed per subject was 7 (range, 2–15 specimens; mean, 7.15 specimens). The median proportion of DNA-positive specimens per subject was 43% (range, 12%–100%; mean, 48.9%). All specimens were positive for 6 individuals; their causes of death were traffic accidents ($n = 3$), suicide ($n = 2$), and myocardial infarction ($n = 1$).

Among individuals who died of violent causes, *P. jirovecii* DNA was found more frequently in those <20 and >60 years old than in the other age groups (Figure 1).

Microscopic analyses. The 55 patients who died of violent causes were analyzed by immunofluorescent microscopy in addition to nested PCR. Each specimen was analyzed separately, and the microscopic reading took 30–90 min per patient. *Pneumocystis* forms, which were few in number, were visualized by immunofluorescence in all 34 individuals who tested positive and in none of those who tested negative for *P. jirovecii* DNA by nested PCR (Table 1 and Figures 1 and 2). However, nested PCR, in agreement with its greater sensitivity, identified a greater number of positive specimens per patient than did immunofluorescence—that is, the median number of positive specimens was 3 per patient (range, 1–7; mean, 3.15) by nested PCR and 1.5 per patient (range, 1–3; mean, 1.67) by immunofluorescence.
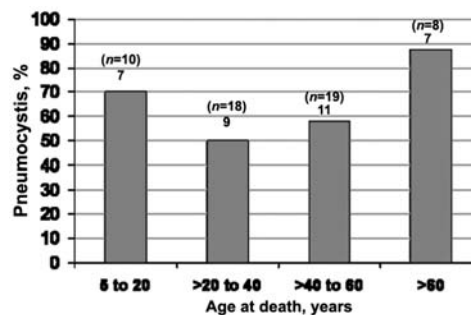

Figure 1. Detection of *Pneumocystis* in concentrated homogenates of autopsy lung specimens from 55 individuals who died of violent causes in Santiago, Chile, by age at death. *Pneumocystis jirovecii* DNA was tested for by nested polymerase chain reaction DNA amplification and immunofluorescence analysis in multiple lung specimens; diagnosis was consistent by both methods.

DISCUSSION

This study of legally required autopsies in Santiago, Chile, documents the presence of *Pneumocystis* organisms in the lungs of 34 (61.8%) of 55 physically healthy adults who died in the community of violent causes (such as traffic accidents, suicide, and homicide), as determined by both nested PCR DNA amplification and immunofluorescent microscopic analysis, and in the lungs of 15 (78.9%) of 19 persons who died of nonviolent causes, as determined by nested PCR DNA amplification only. These numbers are sufficient to confirm that *Pneumocystis* is highly prevalent in the lungs of immunocompetent individuals beyond primary infection, in agreement with the concept that healthy adults participate in the circulation of *Pneumocystis* in human populations as a reservoir of *Pneumocystis* infection (Table 1). Results obtained by both techniques (nested PCR DNA amplification and immunofluorescent microscopic analysis) were consistent and further strengthen serological data that show an increase in antibody titers in health care workers exposed to patients with PCP, suggesting that adults may acquire mild *Pneumocystis* infections after a primary infection if exposed to an infective source [12], as well as more recent data from Medrano et al [8] that documented *P. jirovecii* DNA in 12 (20%) of 50 oropharyngeal wash specimens from immunocompetent administrative hospital workers, showing that *P. jirovecii* carriage can frequently be detected in noninvasive respiratory specimens from the general adult population.

The higher frequency of *P. jirovecii* found in the group of adults who died of nonviolent causes in the present study suggests that medical conditions may favor carriage, as has been documented in patients with lung diseases (such as COPD) [11]. *Pneumocystis* was also more frequent in individuals who died of homicide and suicide than it was in those who died in a traffic accident, suggesting that social or living conditions can also favor *Pneumocystis* colonization. Unfortunately, medical
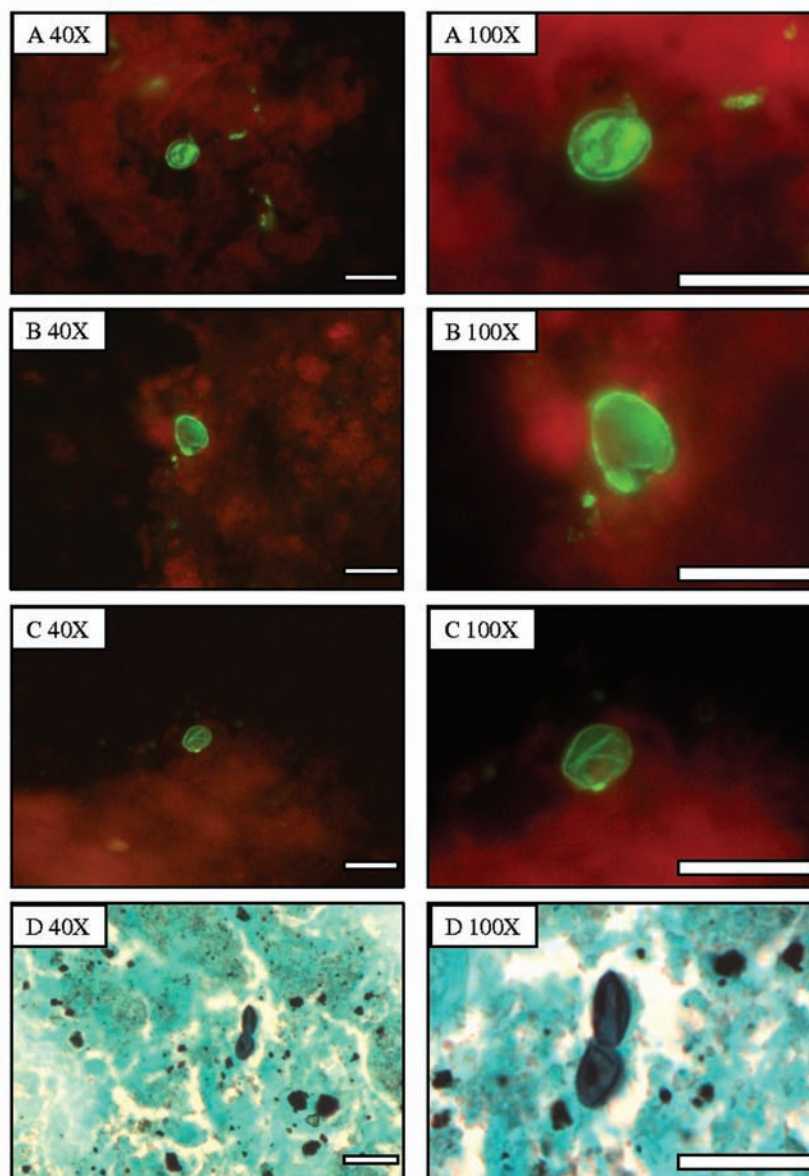
Figure 2. *Pneumocystis* in concentrated homogenates of autopsy lung specimens from 4 apparently healthy individuals who died in unrelated traffic accidents, as visualized by immunofluorescent staining (MeriFluor kit; Meridian Biosciences) (*A–C*) and by staining with Gomori-Grocott methenamine silver (for increased appreciation of the details of the cysts) (*D*). In all individuals, *Pneumocystis* forms were difficult to find, and few were found. Images correspond to a 12-year-old boy (*A*), a 42-year-old man (*B*), a 16-year-old girl (*C*), and a 52-year-old man (*D*). Bars show 10 μm in all panels.

history (including information on previous contact with immunodeficient patients) was not available.

Previous studies searching for *Pneumocystis* in the lungs of immunocompetent adults have been conducted among a small number of individuals by means of less sensitive methods (such as single-round PCR or immunohistochemical analysis) with negative results [16, 17], in agreement with our preliminary analyses using nested PCR DNA amplification without concentration techniques in multiple lung specimens from adults who died of accidental causes (data not shown). In contrast, previous studies of autopsied lungs from apparently healthy

infants dying in the community demonstrated the presence of *Pneumocystis* by careful microscopic examination of histologic sections [23, 25, 29] and by nested PCR DNA amplification of an amount of tissue close to 3% of their RUL weight [26]. Therefore, we reasoned that it might be fruitful to examine a proportion of RUL weight in adults similar to that examined in infants. The analysis of this larger amount of tissue proved to be essential to reach the sensitivity threshold of the nested PCR DNA amplification assay and of the immunofluorescent microscopy required to detect *Pneumocystis* in lung specimens from adults. An interesting finding of the present study is that

increasing the amount of lung tissue examined to >3.0% of lung weight (ie, above ∼7 specimens per patient) did not change the negative results. Therefore, the absence of *Pneumocystis* in the lungs of 21 (38.2%) of the 55 individuals who died of violent causes provides a strong indication that healthy persons are able to clear *Pneumocystis* from their lungs. This is in agreement with the findings of studies of noninvasive specimens showing that the majority of immunocompetent health care workers colonized with this organism clear the infection [31, 32] and more recently with the findings of the study of Medrano et al [8], who documented the obtainment of *P. jirovecii*–negative oropharyngeal wash specimens 6 months apart in 7 (77.7%) of 9 previously positive individuals.

The proportion of *Pneumocystis*-negative individuals in the present study also suggests that positive results for *Pneumocystis* in this population likely reflect pulmonary reinfections that occur after the primary infection and moreover that reinfections are frequent in immunocompetent individuals. Data to support the concept of reinfection are available from immunocompromised patients. For example, different *Pneumocystis* genotypes have been documented in HIV-infected patients who experience 2 episodes of pneumonia occurring >6 months apart [33]. In addition, an extensive epidemiological study showed that genotypes of *P. jirovecii* in AIDS-related PCP are more closely related to place of residence than to place of birth [34].

P. jirovecii identified in this study were not genotyped. Of interest, studies of immunocompromised patients dying of PCP have demonstrated variation in *P. jirovecii* genotypes among different lung lobes, indicating that their origin might not necessarily be clonal [28, 30]. Thus, it is possible that different clones of *P. jirovecii* in lobes other than the RUL of subjects in this study could have remained undetected [28, 30].

The scarce quantity of *Pneumocystis* organisms found in the lungs of individuals in this study possibly reflects the efficacy of their immune responses in clearing the organism. However, this minimal *Pneumocystis* load may also be interpreted as latent *P. jirovecii* organisms under the traditional—and less prevailing—view of latency reactivation. Evidence documenting different *P. jirovecii* genotypes in different episodes of PCP, reports of clusters of PCP in wards of immunocompromised patients (suggesting transmission), and additional evidence from animal models showing air transmission, clearance of *Pneumocystis* after PCP or primary infection, and lack of PCP reactivation in CD4 T cell–deficient mice that have cleared the infection all argue against this concept, making latency an unlikely explanation [35–38]. In contrast, in the present immunofluorescence analyses *P. jirovecii* of different sizes were stained that were compatible with cyst and trofozoite forms, suggesting active replication. Furthermore, viable, actively replicating *Pneumocystis* organisms were recently documented in immunocompetent animal models [14].

The age distribution among *Pneumocystis*-positive individuals who experienced a violent death—with higher percentages before the age of 20 and after the age of 60 years—may be explained by successive reinfections being more frequent before the age of 20 (for unknown reasons) and after the age of 60 (when cellular immunity may decrease) [39].

Lung histologic sections stained with hematoxilin-eosin showed normal parenchyma. Therefore, the *Pneumocystis* found in this study is compatible with the increasingly recognized concept of colonization, carriage, or asymptomatic infection [1]. The clinical significance of this mild infection in the immunocompetent host is being actively investigated [1]. Christensen et al [40] recently documented that infection with *Pneumocystis* in immunocompetent mice induces worsening of pulmonary function and histologic changes compatible with emphysema if exposed to tobacco smoke. Furthermore, the association between *P. jirovecii* colonization and the severity of COPD raises the possibility that *Pneumocystis* is involved in the pathogenesis of this condition [11]. Higher peripheral lymphocyte counts with increased CD4 T lymphocyte responses have been reported in subjects with chronic bronchitis, and a systemic inflammatory response with higher serum levels of tumor necrosis factor α, interleukin 6, and interleukin 8 has been documented in patients with COPD and *Pneumocystis* colonization [10, 41].

Whether *Pneumocystis* could have been detected in noninvasive specimens from this population cannot be concluded from this study, because noninvasive specimens were not obtained. The amount of *P. jirovecii* that can be recovered from noninvasive specimens from immunocompetent people—especially adults—may vary from sample to sample. These sampling methods need to be validated in immunocompetent individuals.

This work provides direct evidence that mild pulmonary infections with *Pneumocystis* organisms are highly prevalent among healthy adults, supporting the concept that healthy individuals can develop *Pneumocystis* reinfections throughout life after the primary infection and thus participate in the transmission cycle of *P. jirovecii*. The *Pneumocystis* load in adult human lungs is very low and lies below the sensitivity threshold of the nested PCR DNA amplification procedure. Therefore, analysis of large amounts of tissue with concentration techniques (as described above) is required for diagnosis. Additional studies to investigate whether these mild *Pneumocystis* reinfections in healthy adults are of clinical significance are warranted.

Acknowledgments

References

1. Morris A, Wei K, Afshar K, Huang L. Epidemiology and clinical significance of *Pneumocystis* colonization. J Infect Dis **2008**; 197:10–17.
2. Huang L, Morris A, Limper AH, Beck JM. An official ATS workshop summary: recent advances and future directions in *Pneumocystis* pneumonia (PCP). Proc Am Thorac Soc **2006**; 3:655–664.
3. Linke MJ, Rebholz S, Collins M, Tanaka R, Cushion MT. Noninvasive method for monitoring *Pneumocystis carinii* pneumonia. Emerg Infect Dis **2003**; 9:1613–1616.
4. Leigh TR, Kangro HO, Gazzard BG, Jeffries DJ, Collins JV. DNA amplification by the polymerase chain reaction to detect sub-clinical *Pneumocystis carinii* colonization in HIV-positive and HIV-negative male homosexuals with and without respiratory symptoms. Respir Med **1993**; 87:525–529.
5. Nevez G, Magois E, Duwat H, Gouilleux V, Jounieaux V, Totet A. Apparent absence of *Pneumocystis jirovecii* in healthy subjects. Clin Infect Dis **2006**; 42:e99–e101.
6. Vargas SL, Ponce CA, Gigliotti F, et al. Transmission of *Pneumocystis carinii* DNA from a patient with *P. carinii* pneumonia to immunocompetent contact health care workers. J Clin Microbiol **2000**; 38: 1536–1538.
7. Vargas SL, Ponce CA, Sanchez CA, Ulloa AV, Bustamante R, Juarez G. Pregnancy and asymptomatic carriage of *Pneumocystis jiroveci*. Emerg Infect Dis **2003**; 9:605–606.
8. Medrano FJ, Montes-Cano M, Conde M, et al. *Pneumocystis jirovecii* in general population. Emerg Infect Dis **2005**; 11:245–250.
9. Nevez G, Jounieaux V, Linas MD, et al. High frequency of *Pneumocystis carinii* sp.f. hominis colonization in HIV-negative patients. J Eukaryot Microbiol **1997**; 44:36S.
10. Calderon EJ, Rivero L, Respaldiza N, et al. Systemic inflammation in patients with chronic obstructive pulmonary disease who are colonized with *Pneumocystis jiroveci*. Clin Infect Dis **2007**; 45:e17–e19.
11. Morris A, Sciurba FC, Lebedeva IP, et al. Association of chronic obstructive pulmonary disease severity and *Pneumocystis* colonization. Am J Respir Crit Care Med **2004**; 170:408–413.
12. Giron JA, Martinez S, Walzer PD. Should inpatients with *Pneumocystis carinii* be isolated? Lancet **1982**; 2:46.
13. An CL, Gigliotti F, Harmsen AG. Exposure of immunocompetent adult mice to *Pneumocystis carinii* f. sp. *muris* by cohousing: growth of *P. carinii* f. sp. *muris* and host immune response. Infect Immun **2003**; 71:2065–2070.
14. Chabe M, Dei-Cas E, Creusy C, et al. Immunocompetent hosts as a reservoir of *Pneumocystis* organisms: histological and rt-PCR data demonstrate active replication. Eur J Clin Microbiol Infect Dis **2004**; 23: 89–97.
15. Dumoulin A, Mazars E, Seguy N, et al. Transmission of *Pneumocystis carinii* disease from immunocompetent contacts of infected hosts to susceptible hosts. Eur J Clin Microbiol Infect Dis **2000**; 19:671–678.
16. Millard PR, Heryet AR. Observations favouring *Pneumocystis carinii* pneumonia as a primary infection: a monoclonal antibody study on paraffin sections. J Pathol **1988**; 154:365–370.
17. Peters SE, Wakefield AE, Sinclair K, Millard PR, Hopkin JM. A search for *Pneumocystis carinii* in post-mortem lungs by DNA amplification. J Pathol **1992**; 166:195–198.
18. Larsen HH, von Linstow ML, Lundgren B, Hogh B, Westh H, Lundgren JD. Primary *Pneumocystis* infection in infants hospitalized with acute respiratory tract infection. Emerg Infect Dis **2007**; 13:66–72.
19. Nevez G, Totet A, Pautard JC, Raccurt C. *Pneumocystis carinii* detection using nested-PCR in nasopharyngeal aspirates of immunocompetent infants with bronchiolitis. J Eukaryot Microbiol **2001**; 48(suppl 1):122S–123S.
20. Totet A, Meliani L, Lacube P, et al. Immunocompetent infants as a human reservoir for *Pneumocystis jirovecii*: rapid screening by non-invasive sampling and real-time PCR at the mitochondrial large subunit rRNA gene. J Eukaryot Microbiol **2003**; 50(suppl):668–669.
21. Vargas SL, Hughes WT, Santolaya ME, et al. Search for primary infection by *Pneumocystis carinii* in a cohort of normal, healthy infants. Clin Infect Dis **2001**; 32:855–861.
22. Kasolo F, Lishimpi K, Chintu C, et al. Identification of *Pneumocystis carinii* DNA by polymerase chain reaction in necropsy lung samples from children dying of respiratory tract illnesses. J Pediatr **2002**; 140: 367–369.
23. Morgan DJ, Vargas SL, Reyes-Mugica M, Walterspiel JN, Carver W, Gigliotti F. Identification of *Pneumocystis carinii* in the lungs of infants dying of sudden infant death syndrome. Pediatr Infect Dis J **2001**; 20: 306–309.
24. Vargas SL, Ponce CA, Galvez P, et al. *Pneumocystis* is not a direct cause of sudden infant death syndrome. Pediatr Infect Dis J **2007**; 26:81–83.
25. Vargas SL, Ponce CA, Hughes WT, et al. Association of primary *Pneumocystis carinii* infection and sudden infant death syndrome. Clin Infect Dis **1999**; 29:1489–1493.
26. Vargas SL, Ponce CA, Luchsinger V, et al. Detection of *Pneumocystis carinii* f. sp. *hominis* and viruses in presumably immunocompetent infants who died in the hospital or in the community. J Infect Dis **2005**; 191: 122–126.
27. Beard CB, Fox MR, Lawrence GG, et al. Genetic differences in *Pneumocystis* isolates recovered from immunocompetent infants and from adults with AIDS: epidemiological implications. J Infect Dis **2005**; 192: 1815–1818.
28. Ambrose HE, Ponce CA, Wakefield AE, Miller RF, Vargas SL. Distribution of *Pneumocystis carinii* f. sp. *hominis* types in the lung of a child dying of *Pneumocystis* pneumonia. Clin Infect Dis **2001**; 33:e100–e102.
29. Sheldon WH. Subclinical *Pneumocystis* pneumonitis. AMA J Dis Child **1959**; 97:287–297.
30. Helweg-Larsen J, Lundgren B, Lundgren JD. Heterogeneity and compartmentalization of *Pneumocystis carinii* f. sp. hominis genotypes in autopsy lungs. J Clin Microbiol **2001**; 39:3789–3792.
31. Durand-Joly I, Soula F, Chabe M, et al. Long-term colonization with *Pneumocystis jirovecii* in hospital staffs: a challenge to prevent nosocomial pneumocystosis. J Eukaryot Microbiol **2003**; 50(suppl):614–615.
32. Miller RF, Ambrose HE, Wakefield AE. *Pneumocystis carinii* f. sp. *hominis* DNA in immunocompetent health care workers in contact with patients with *P. carinii* pneumonia. J Clin Microbiol **2001**; 39:3877–3882.
33. Keely SP, Stringer JR, Baughman RP, Linke MJ, Walzer PD, Smulian AG. Genetic variation among *Pneumocystis carinii hominis* isolates in recurrent pneumocystosis. J Infect Dis **1995**; 172:595–598.
34. Beard CB, Carter JL, Keely SP, et al. Genetic variation in *Pneumocystis carinii* isolates from different geographic regions: implications for transmission. Emerg Infect Dis **2000**; 6:265–272.
35. Chen W, Gigliotti F, Harmsen AG. Latency is not an inevitable outcome of infection with *Pneumocystis carinii*. Infect Immun **1993**; 61:5406–5409.
36. Hughes WT. Natural mode of acquisition for de novo infection with *Pneumocystis carinii*. J Infect Dis **1982**; 145:842–848.
37. Lundgren B, Elvin K, Rothman LP, Ljungstrom I, Lidman C, Lundgren JD. Transmission of *Pneumocystis carinii* from patients to hospital staff. Thorax **1997**; 52:422–424.
38. Morris A, Lundgren JD, Masur H, et al. Current epidemiology of *Pneumocystis* pneumonia. Emerg Infect Dis **2004**; 10:1713–1720.
39. Gruver AL, Hudson LL, Sempowski GD. Immunosenescence of ageing. J Pathol **2007**; 211:144–156.
40. Christensen PJ, Preston AM, Ling T, et al. *Pneumocystis murina* infection and cigarette smoke exposure interact to cause increased organism burden, development of airspace enlargement, and pulmonary inflammation in mice. Infect Immun **2008**; 76:3481–3490.
41. Varela JM, Respaldiza N, Sanchez B, et al. Lymphocyte response in subjects with chronic pulmonary disease colonized by *Pneumocystis jirovecii*. J Eukaryot Microbiol **2003**; 50(suppl):672–673.