MAJOR ARTICLE

Search for Primary Infection by *Pneumocystis carinii* in a Cohort of Normal, Healthy Infants

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To determine whether *Pneumocystis carinii* is associated with clinical illness in the competent host, 107 normal, healthy infants were enrolled in a 2-year prospective cohort study in Chile. *P. carinii* was identified by specific stains and nested–deoxyribonucleic acid (DNA) amplification of the large subunit mitochondrial ribosomal ribonucleic acid gene of *P. carinii* f. sp. *hominis*, and seroconversion was assessed by enzyme-linked immunosorbent assay of serum samples drawn every 2 months. *P. carinii* DNA was identified in nasopharyngeal aspirates obtained during episodes of mild respiratory infection in 24 (32%) of 74 infants from whom specimens were available for testing. Three (12.5%) of those 24 infants versus 0 of 50 infants who tested negative for *P. carinii* had apnea episodes. Seroconversion developed in 67 (85%) of 79 infants who remained in the study by 20 months of age and occurred in the absence of any symptoms of disease in 14 (20.8%). The study indicates that *P. carinii* DNA can be frequently detected in healthy infants, and it raises the hypothesis that they may be an infectious reservoir of *P. carinii* in the community. Further investigation is needed to identify whether *P. carinii* causes overt respiratory disease in infants.

Primary infection by *Pneumocystis carinii* can cause fatal pneumonia in infants immunocompromised by AIDS, cancer chemotherapy, or organ transplantation and in those who have congenital immunodeficiency disorders [1]. Occurrence of the infection in undernourished infants has also been reported [2, 3]. However, beyond these reports on infants with compromised immune systems, serological surveys indicate that the great majority of healthy, immunocompetent infants have primary *P. carinii* infection before 3 years of age [4–7]. These surveys suggest that primary *P. carinii* infection is among the most common infections in pediatrics and lead us to hypothesize that pneumonitis diagnosed in the immunocompromised host is only a small proportion of the *P. carinii* infections affecting infants and children.

The current belief is that the primary infection in the competent host is asymptomatic [8]. However, previous studies were designed to examine seroprevalence and not to determine the relationship between *P. carinii* and acute disease in healthy children [4–7]. Whether or not a clinically identifiable pattern occurs is not

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The study was approved by the Ethics Commission at the University of Chile School of Medicine, and signed informed consent was obtained from the parents or legal guardians of all children who participated.

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known. Disease due to *P. carinii* in healthy infants may have been overlooked because of the view that *P. carinii* is restricted to the immunocompromised host and because the definitive diagnosis of *P. carinii* requires an invasive procedure not normally used for healthy pediatric subjects.

We hypothesized that primary infection by *P. carinii* causes an overt respiratory illness in the healthy infant and that the clinical manifestations may be different or less severe than those known to occur in the compromised host. Previous reports support our hypothesis, including the demonstration of *P. carinii* in immunocompetent infants who underwent bronchoalveolar lavage (BAL) during a lower respiratory tract infection because their mothers had AIDS; the finding of *P. carinii* antigenemia (with few histologically documented cases) in 18% of premature infants with pneumonitis; and the finding of *P. carinii* in 14% of infants <3 months of age with bronchopneumonia who were admitted to the hospital [9–11]. Furthermore, we have histologically documented *P. carinii* in the lungs of 15%–35% of infants dying unexpectedly at home for whom autopsy indicated sudden infant death syndrome [12].

Therefore, we designed a cohort study to determine whether it was possible to identify a pattern of clinically overt *P. carinii* primary infection in healthy infants and to assess the circumstances of seroconversion.

METHODS

Study subjects, evaluations, and design. One hundred seven newborns attending Rosita Renard outpatient clinic in Santiago, Chile, were selected on the basis of normal nutritional and physical-examination status, absence of morbid conditions, and absence of immunocompromised hosts living in their households. They were enrolled before 1 month of age and followed until 2 years of age in a well-baby clinic opened for the purpose of this study at the Luis Calvo Mackenna Hospital in Santiago. Follow-up consisted of a monthly physical examination and the recording of a detailed medical history registering every infection. Parents were also instructed to bring the infant to the clinic should an infection develop or to report infections by telephone.

A 2-mL blood sample was obtained every other month, and the serum was stored at −70°C until processed by ELISA to detect seroconversion. Nasopharyngeal aspirates were obtained when an upper or lower respiratory tract infection was diagnosed, especially if cough was an accompanying symptom. Respiratory infection was diagnosed as an upper respiratory infection if a cold, nasopharyngitis, or pharyngitis was present and as a lower respiratory infection if the infant developed bronchitis, pneumonia, or bronchiolitis. Otitis media, tonsillitis, and tonsillopharyngitis were distinguished as separate entities. Nasopharyngeal aspirates were obtained with a 6.5-French suction catheter with control port; a portable suction pump was used, and secretions were collected in 15-mL capped sterile tubes.

A volume of up to 0.2 mL of saline was added to the tube when secretions were too thick or scarce, and one-half of the final volume was used for detection of *P. carinii* by Grocott-Gomori methenamine silver staining. DNA was extracted as below and stored at −70°C until processed by DNA amplification. Chest radiographs were obtained when medically indicated. The study was approved by the Ethics Commission of the University of Chile School of Medicine, and an informed consent form was signed by both parents for each infant.

DNA amplification. A volume of ~200 μL of aspirate material was examined for *P. carinii* by DNA amplification as described elsewhere [13, 14]. In brief, the samples were digested with proteinase K (20 mg/mL) at 60°C in the presence of 10 m*M* EDTA and 0.5% SDS. Total DNA was purified and concentrated with use of the Pharma-Gen "clean up" system (Pharma-Gen) and recovered in a volume of 50 μL. Five μL of this DNA preparation was used for DNA amplification with *P. carinii* oligonucleotide primers pAZ102-E (5′-GATGGCTGTTTCCAAGCCCA-3′) and pAZ102-H (5′-GTGTACGTTGCAAAGTACTC-3′). Amplification was carried out at 94°C for 1.0 min, 55°C for 1.0 min, and 72°C for 2.0 min for 40 cycles. In order to achieve a greater level of sensitivity and specificity, we used primers pAZ102-X (5′-GTGAAATACAAATCGGACTAGG-3′) and pAZ102-Y (5′-TCACTTAATATTAATTGGGGAGC-3′), which are internal to the first set of primers and specific for human *P. carinii*, in a second round of PCR [13, 14], taking 1 μL from the first-round PCR product.

Thirty-five cycles of amplification were carried out under the same conditions used for the external primers. The final PCR products were separated on 2.0% agarose gels and visualized with ethidium bromide. *P. carinii* DNA amplified in this fashion had a predicted length of 267 bp. Negative controls with no added template DNA were included after each sample to monitor for cross-contamination. A sample of human-derived *P. carinii* DNA was used as a positive control in each experiment. All manipulations during DNA extraction and amplification were performed in laminar flow cabinets, with use of disposable pipettes, tubes, and reagent aliquots to avoid contamination.

Antibody determinations. Serum samples obtained sequentially at 2-month intervals from every infant were studied. Antibody titer determinations were made by comparison of ELISA readings obtained with a crude sonicate of *P. carinii*–infected mouse lungs and a crude sonicate of noninfected mouse lungs (demonstrated by PCR to be free of *P. carinii*) as the solid-phase antigen [15, 16]. In brief, *P. carinii*–infected and –noninfected mouse lungs were homogenized and sonicated, and the supernatant was then clarified by centrifugation and microfiltration (0.45 μm pore size), diluted to a protein

Table 1. Identification of *Pneumocystis carinii* in sequential nasopharyngeal aspirate samples from 5 infants who had >1 episode of *P. carinii*–positive respiratory infection.

Patient no. (age at first DNA-positive sample)	Results of DNA testing for *P. carinii* in nasopharyngeal aspirate sample obtained at indicated month[a]																
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1 (3 mo, 24 d)	+								−			−					+
2 (3 mo, 20 d)	+		+		−				−								
3 (6 mo, 24 d)	+				+		+										
4 (4 mo, 16 d)	+			+													
5 (3 mo)	+	+			+	−											

[a] Following obtainment of first DNA-positive sample.

concentration of ~10 μg/mL in carbonate buffer, and used to coat wells in Linbro flat-bottomed microtiter plates (Flow Laboratories). Plates were incubated, tightly covered, in a moist chamber at 37°C for 90 min to allow the antigen to adhere. Coated wells were blocked with 5% commercial nonfat dry milk in PBS for 1 h at 37°C or overnight at 4°C. Serum samples were diluted 1:50 in PBS containing 0.05% Tween 20 (PBS-T; Sigma), and 50 μL/well of each serum sample was incubated overnight at 4°C. Plates were then washed 3 times with PBS-T, and bound antibody was detected with use of goat antibody to human IgG and IgM conjugated to alkaline phosphatase (Jackson Immunoresearch Laboratories), diluted 1:5000 in PBS-T. After a 4-h period of incubation at 37°C, the plates were washed 4 times with PBS-T and developed by the addition of *p*-nitrophenyl phosphate (Sigma) at a concentration of 1 mg/mL in diethanolamine buffer. This ELISA was previously standardized by use of a variety of monoclonal and polyclonal antibodies known to be specific for *P. carinii*. Irrelevant monoclonal antibodies give optical densities (ODs) of <0.05 in this ELISA format.

A sample was labeled as positive if the OD in the *P. carinii* ELISA (OD Pc) was >0.15, with an OD of <0.15 in the ELISA using noninfected mouse lung (OD non-Pc); possibly positive if the OD Pc was 0.11–0.14 and the OD non-Pc was <0.05; uninterpretable if the OD in both was >0.15; or negative if the OD Pc was <0.10 [15, 16].

Statistical analysis. The STATA statistical package (StataCorp) was used to test whether *P. carinii*–positive and *P. carinii*–negative respiratory infection episodes were associated with specific clinical diagnoses or specific symptoms, by means of Fisher's exact test. Ages of subjects at the time at which DNA-positive or DNA-negative nasopharyngeal wash specimens were obtained were compared by use of Student's *t* test. A *P* value of <.05 was considered significant.

RESULTS

From March to June 1997, 107 healthy 1-month-old infants (mean weight at birth, 3393 g; range, 2370–4610 g; median, 3380 g) were recruited at the Rosita Renard Outpatient Health Clinic, National Health System, in Santiago, Chile. Of these infants, 16 were lost from the study before 6 months of age and 12 were lost between 6 and 18 months of age. The reasons for discontinuation were noncompliance with the drawing of blood every 2 months ($n = 16$) and transfer of care to a facility in another city ($n = 12$). Thus, 91 infants remained in the study for >6 months and 79 for >18 months.

A total of 804 episodes were registered during the study. Four hundred ninety-seven (61.8%) were upper respiratory tract infections, and 307 (38.1%) were lower respiratory tract infections.

Direct detection of* P. carinii *in respiratory samples. One hundred seventy-eight nasopharyngeal aspirate samples obtained during separate respiratory infection episodes were available for DNA analysis from 74 (69%) of the 107 infants participating in the study (range, 1–5 samples per infant). *P. carinii* DNA was detected in 31 samples from respiratory infection episodes for 24 (32%) of the 74 infants; in 3 (9.7%) of these samples, *P. carinii* cysts were also found by Grocott-Gomori methenamine–silver nitrate staining. Seventeen of the 24 infants who had a *P. carinii* DNA–positive sample had a second nasopharyngeal sample obtained. The second sample, obtained at intervals between 2 and 3 months after the first positive sample was obtained, was positive for *P. carinii* in 4 of these 17 infants. A *P. carinii* DNA–positive nasopharyngeal aspirate sample was obtained from 1 infant 16 months after the first positive result, although 2 samples obtained in between were negative (table 1). One infant for whom samples had been positive twice before still had *P. carinii* detectable 2 months after the second sample tested positive, although no respiratory symptoms were present at the time of sampling (table 1, patient 3).

Clinical diagnoses in the 178 patients with samples available for DNA amplification were not different for *P. carinii*–positive and *P. carinii*–negative respiratory infection episodes (table 2). *P. carinii* DNA–positive nasopharyngeal aspirate samples were obtained more frequently from younger infants (aged 1.9–19 months; mean, 7.1 months; median, 5 months; SD, 4.9) than were *P. carinii* DNA–negative samples (obtained from patients

Table 2. Clinical diagnosis in 178 episodes of respiratory infection for which *Pneumocystis carinii* was sought by DNA amplification.

Diagnostic category, specific diagnosis	DNA analysis results[a]	
	Positive (n = 31)	Negative (n = 147)
Upper respiratory infection	16 (51.6%)	81 (55.1%)
Cold	4/13	8/66
Nasopharyngitis	1/1	2/7
Laryngotracheitis	0/0	1/2
Laryngotracheobronchitis	0/0	1/1
Tonsillitis or acute otitis media	1/2	3/5
Lower respiratory infection	14 (45.2%)	64 (43.5%)
Bronchitis	0/8	11/34
Bronchiolitis or infectious asthma	1/6	10/23
Pneumonia	0/0	2/5
Not ill	1 (3.2%)	2 (1.4%)
Total	31 (100%)	147 (100%)

[a] No. of episodes with fever (rectal temperature >38°C)/total no. of episodes. Comparisons are not statistically significant. P = .562 by Fisher's exact test.

aged 1–23 months; mean, 10.4 months; median, 10.5 months; SD, 5.6) ($P < .005$).

Clinical findings. Signs and symptoms documented during *P. carinii*–positive respiratory infection episodes were generally indistinguishable from those recorded during *P. carinii*–negative episodes, except for apnea (table 3). Three of the 24 infants with *P. carinii* DNA–positive respiratory infections were brought to the clinic because of antecedent apneic episodes, and 1 of them required stimulation by the parents. Other respiratory pathogens were not sought. Physical examination findings were normal for these 3 infants except for mild coryza and minimal dry cough, and chest radiographs were not obtained. The infant who required stimulation (table 1, patient 3) was positive for *P. carinii* on 2 subsequent occasions, the last being at 6 months after the first positive sampling (when he presented with apnea).

Seroconversion. ELISA antibody titer determinations documented seroconversion in 47 (53.4%) of the 88 infants remaining in the study at 8 months of age and in 67 (84.8%) of 79 infants at 20 months of age (figure 1). Once seroconversion was documented, 93% of infants remained seropositive on all subsequent serum sampling, a finding supporting the reproducibility of this assay. Seven percent of the infants who seroconverted had 1 or 2 negative titers in between positive titers. Fifty-three (60%) of the 88 infants had at least 1 documented respiratory infection 15 days to 10 months (median, 20 days; mean, 51 days) prior to seroconversion. These were diagnosed as upper respiratory tract infections in 33 infants and as lower respiratory tract infections in 20 infants. The first positive ELISA titer was noted in the absence of any detectable prior respiratory illness in 14 (20.8%) of the 67 seropositive infants who were followed through the end of the study.

Seroconversion was detected in 21 (87.5%) of the 24 infants with 1 *P. carinii*–positive nasopharyngeal aspirate. In 10 of the 21 infants who seroconverted and also had at least 1 *P. carinii*–positive nasopharyngeal aspirate, the first detection of *P. carinii* DNA preceded seroconversion at between 1 and 9 months. *P. carinii* DNA was detected 2–9 months after seroconversion in another 10 of these 21 infants. One infant had his first positive serum sample obtained simultaneously with the *P. carinii* DNA–positive nasopharyngeal aspirate.

Three of the 24 infants with *P. carinii*–positive nasopharyngeal aspirate samples did not seroconvert. Two of them still had negative titers after a follow-up period of 16 and 18 months following nasopharyngeal aspirate–positivity and were healthy at the end of the study. The third infant had acute lymphoblastic leukemia diagnosed at 9 months of age, 3 months after his *P. carinii*–positive nasopharyngeal aspirate was obtained, and was withdrawn from the study.

DISCUSSION

This study provides evidence that primary infection by *P. carinii* can be detected by sensitive DNA amplification techniques in noninvasive respiratory samples from healthy infants. Direct detection of *P. carinii* DNA in nasopharyngeal aspirate samples suggests that healthy infants might play an important role in the human epidemiology of *P. carinii* infection, e.g., as a reservoir for transmission to susceptible individuals.

DNA amplification studies of BAL and lung tissue samples show that *P. carinii* DNA can be detected in most if not all patients with *P. carinii* pneumonia (PCP) [17–19]. Recent re-

Table 3. Clinical signs and symptoms in 178 episodes of respiratory infection for which *Pneumocystis carinii* was sought by DNA amplification.

Sign or symptom	No. (%) of episodes		P[a]
	P. carinii–positive (n = 31)	*P carinii*–negative (n = 147)	
Fever (rectal temperature >38° C)	7 (22)	38 (26)	.87
Coryza	27 (87)	129 (88)	.80
Cough	7 (22)	22 (15)	.43
Any bronchial auscultatory signs	18 (58)	73 (50)	.51
Tonsillitis or acute otitis media	5 (16)	11 (7)	.23
Apnea	3 (10)	0 (0)	.002

[a] By Fisher's exact test.

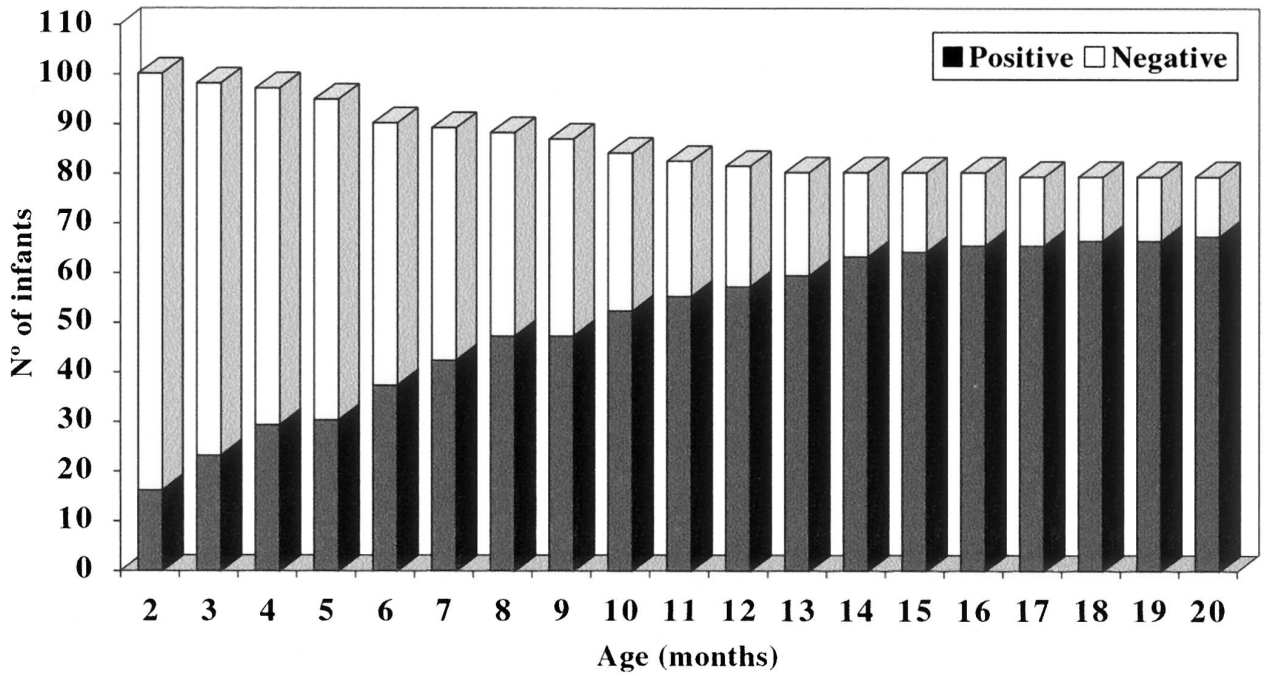

Figure 1. Proportions of infants with positive and negative ELISA titers of antibody to *Pneumocystis carinii* at different ages in a cohort of 107 initially enrolled healthy infants. Of 100 infants tested at 2 months of age, 16 (16%) were seropositive, probably because of maternally acquired antibodies. Forty-seven (53%) of 88 infants and 67 (85%) of 79 infants continuing in the follow-up had seroconverted by 8 and 20 months of age, respectively.

ports show that the detection of *P. carinii* DNA in oral secretions is highly indicative of active PCP in immunocompromised patients [13, 14, 20–22]. However, the sensitivity of this diagnostic procedure increases when applied to lower respiratory tract specimens, such as BAL fluid or lung tissue [18, 20–23]. Obtaining this type of specimen could not be justified for infants in our cohort. Therefore, we probably detected a smaller proportion of the *P. carinii* infections that occurred in this group of infants [13, 14, 19, 23]. This is also suggested by the high rates of seroconversion that were detected.

A point of concern in this study is whether *P. carinii* DNA might be detectable in nasal secretions without even subclinical infection and whether false-positive DNA amplification reactions are encountered. We were unable to examine children without respiratory illnesses in this study for *P. carinii* DNA. However, in a separate study, we examined 258 nasopharyngeal, pharyngeal, and salivary secretions from 86 immunocompromised and immunocompetent individuals without any evidence of respiratory infection. None of the samples had detectable *P. carinii* DNA, a finding suggesting spurious *P. carinii* DNA is at a low titer or absent in individuals without the infection [24], as reported by other authors [23, 25]. We have found transient *P. carinii* DNA carriage in immunocompetent contacts of a patient with documented PCP [26]. However, none of the infants in this cohort were known to have been in contact with a patient with PCP, and the presence of an immunocompromised person in the household was a criterion for exclusion from participation in the study.

The serological data indicating that the great majority of infants in our cohort seroconverted by 20 months of age are consistent with early exposure to *P. carinii* and are similar to those from earlier studies [4–7]. The serological assay performed in this study was done with mouse *P. carinii*. Because mouse *P. carinii* may have some antigenic differences from human *P. carinii* [27], a negative ELISA titer does not necessarily mean that antibodies to human *P. carinii* were absent, leaving the possibility that *P. carinii* infection could indeed be even more prevalent than detected in our pediatric population. However, our seroprevalence data are similar to previously published data from studies involving the use of either rat or human *P. carinii* as the test antigen [4–7].

The detection of *P. carinii* DNA during episodes of mild upper or lower respiratory tract infections during this study does not exclude the current view that primary infection by *P. carinii* can be asymptomatic. Except for 3 occasions, infants without respiratory symptoms were not tested for *P. carinii* (table 2). Moreover, the evidence that seroconversion occurred without the antecedent of an upper or lower respiratory tract infection in 14 (20.8%) of the 67 seropositive infants completing the study follow-up period supports that primary in-

fection can occur asymptomatically in at least that proportion of infants. The occurrence of asymptomatic infections in a proportion of infants is in agreement with the finding in our previous studies that primary *P. carinii* infection is associated with sudden infant death syndrome [12].

The analysis of the respiratory episodes in those cases identified as positive by DNA amplification did not reveal a distinctive clinical pattern for this infection, with the possible exception of apnea. The signs and symptoms encountered are common to those of other causes, and a causative relationship cannot be established on the basis of our findings. However, 3 patients had a history of apneic episodes at presentation. Physical examination revealed mild coryza accompanied by dry, discrete cough. In 1 of these 3 patients, *P. carinii* DNA was subsequently identified during a consecutive respiratory infection episode, and, in addition, it was detected in 1 nasopharyngeal washing sample taken during follow-up while the patient was asymptomatic, 6 months from the first *P. carinii* DNA–positive episode (table 1, patient 3). This patient required stimulation efforts, as described by the parents.

Isolated reports document apnea as a clinical sign in infants with PCP [28–31]. The occurrence of apnea might be explained by characteristic abnormalities in pulmonary surfactant that can be induced by *P. carinii* infection [32–41]. However, in these patients no signs of respiratory distress were present, a chest radiograph was not obtained, and no other respiratory pathogens were sought.

P. carinii DNA was identified on more than 1 occasion in 5 of 17 patients who had more than 1 respiratory sample taken during the period of the study. In all of them (table 1), except for the 6-month sample from patient 3, subsequent nasopharyngeal samples for *P. carinii* analysis were taken during respiratory infections. Consecutive nasopharyngeal aspirates were positive for *P. carinii* DNA up to 6 months from the time at which the first sample was positive, suggesting that elimination of *P. carinii* after the onset of the primary *P. carinii* infection might take a period of few months in some infants, as has been shown in animal models [42]. Genotyping was not available to assess whether the *P. carinii* types identified in sequential samples were related. However, studies of AIDS patients with recurrent episodes of PCP have suggested that *P. carinii* strains identified in samples <6 months apart are likely related [43]. The issue of limited carriage of *P. carinii* warrants further investigation, especially if a role for infants in the circulation of *P. carinii* organisms in the community can be demonstrated [44].

This study provides direct evidence of *P. carinii* primary infection in healthy, immunocompetent infants, showing that it can be asymptomatic in at least 20% of cases. Whether *P. carinii* primary infection alone causes overt respiratory disease in the healthy infant cannot be determined from this study, since respiratory infections are common in this age group and a comprehensive microbiological analysis to exclude other known causes of respiratory infection was not done. To determine whether *P. carinii* can directly cause overt respiratory illness will require further investigation. The frequent finding of *P. carinii* in respiratory infections in infants in this study supports the possibility that infants might play a role in the circulation and transmission of *P. carinii* organisms in the community [44]. Infants might constitute a currently unrecognized infectious reservoir for other susceptible infants or for immunocompromised patients at risk of severe pneumonia.

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