Detection of *Pneumocystis carinii* f. sp. *hominis* and Viruses in Presumably Immunocompetent Infants Who Died in the Hospital or in the Community

Sergio L. Vargas,[1] Carolina A. Ponce,[1] Vivian Luchsinger,[2] Cecilia Silva,[1] Myriam Gallo,[3] René López,[3] José Belletti,[3] Luis Velozo,[4] Ricardo Avila,[4] Maria A. Palomino,[4] Samuel Benveniste,[5] and Luis F. Avendaño[2]

Programas de [1]Microbiología and [2]Virología, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, [3]Servicio Médico Legal, [4]Departmento de Patología, Hospital Roberto del Rio, and [5]Departmento de Patología, Hospital Exequiel Gonzalez Cortés, Santiago, Chile

Fresh-frozen lung and tracheal-aspirate specimens obtained from 112 infants who died in Santiago, Chile, during 1998–2000 were analyzed for the presence of *Pneumocystis* DNA, by use of nested DNA amplification of the large subunit mitochondrial rRNA, and for the presence of viruses, by use of culture and immunofluorescence. *Pneumocystis* DNA was detected in specimens from 45 (51.7%) of 87 infants who died in the community and from 5 (20%) of 25 infants who died in the hospital ($P = .006$). Primary infection with *Pneumocystis* was highly frequent among infants who die unexpectedly in the community. Infection with viruses was more common in infants who died in the hospital.

The few existing autopsy reports of histologically mild *Pneumocystis carinii* f. sp. *hominis* infection in presumably immunocompetent infants support the serologic evidence that primary infection with *Pneumocystis* is acquired early in life and underscore the need to characterize this pediatric infection [1–4]. *P. carinii* f. sp. *hominis,* also known as *Pneumocystis jiroveci* (a human-derived *Pneumocystis* species), as determined by use of genotyping [5], has been described after careful microscopy examination in the lungs of 35%, 14%, and 13.9% of infants who died of sudden infant death syndrome (SIDS) in Santiago, Chile; Oxford, United Kingdom; and Rochester, New York, and New Haven, Connecticut, respectively [1, 2]. Unlike the usually massive infection seen in immunocompromised hosts, which is easily detected by microscopy or single-round polymerase chain reaction (PCR), the use of more-sensitive techniques, such as nested PCR, might be necessary to diagnose primary infection in immunocompetent hosts.

Methods. Lung-tissue and tracheal-aspirate specimens from 112 infants without known immunocompromising conditions, whose autopsies were performed between May 1998 and October 2000, were prospectively collected, within 6–48 h after death, from the Servicio Medico Legal (Chilean coroners' office) and from 2 children's hospitals in Santiago. Eighty-seven specimens were obtained from infants whose death was unexpected, according to the infant's medical history, and had occurred in the community; 25 specimens were obtained from infants who died in the hospital. Autopsy diagnosis was established on the basis of clinical history, laboratory test results, and gross and microscopic findings. A diagnosis of SIDS was given when there was no recognized premortem disease, no significant micro- or macroscopic pathological findings, and negative toxicological test results. Before the results of the *Pneumocystis* or viral analyses were available, data on age, date of death, antibiotic use, and autopsy findings were collected from medical history records, for deaths that occurred in the hospital, or from the coroner's report, for deaths that occurred in the community. The Ethics Commission of the University of Chile School of Medicine approved the study. Signed consent for autopsy was obtained from the parents of infants who died in the hospital. Chilean law requires autopsies for all infants who die in the community.

Lung-tissue specimens were stored at −80°C until processing. One specimen from the upper lobe of the right lung of each infant was examined. DNA was extracted from a 0.2–0.5-g portion of tissue, as described elsewhere [6]. *Pneumocystis* DNA was identified by use of a 2-step nested PCR procedure using oligonucleotide primers pAZ102-E and pAZ102-H, which are designed for the gene encoding the large subunit mitochondrial rRNA of *Pneumocystis* and amplify all *Pneumocystis* species, and internal primers pAZ102-X and pAZ102-Y, which are specific for *P. carinii* f. sp. *hominis,* as described elsewhere [7]. Negative controls were included with each specimen, to monitor for cross-contamination. PCRs were performed in duplicate and without knowledge of clinical details.

Table 1. Circumstance of death and autopsy diagnosis for 112 infants who died in Santiago, Chile, during 1998–2000 and for whom *Pneumocystis carinii* f. sp. *hominis* and common respiratory viruses were respectively sought by nested polymerase chain reaction DNA amplification, viral culture, or immunofluorescence assay.

Circumstance of death/autopsy results	Total	No. (%) of infants with		
		P. carinii f. sp. *hominis*[a]	Virus[b]	Coinfection
Unexpected death at home	87	45 (51.7)	9 (10.3)	7 (8.0)
Unremarkable autopsy (SIDS)	75	38 (50.6)	8 (10.6)	6 (8.0)
With an autopsy finding	12	7 (58.3)	1 (8.3)	1 (8.3)
Pneumonia[c]	7	5	1	1
Brain malformation	1	0	0	0
Congenital heart defect	2	1	0	0
Sepsis[c]	2	1	0	0
Hospital death	25	5 (20.0)	15 (60.0)	3 (12.0)
Pneumonia[d]	25	5	15	3

NOTE. In cells where no percentage is given, data are no. of infants. SIDS, sudden infant death syndrome.

[a] $P = .006$, infants who died at home vs. those who died in the hospital; $P = .01$, diagnosis of SIDS vs. pneumonia among infants who died in the hospital; and P was not significant for SIDS vs. an unexpected autopsy finding among infants who infants died in the community (2-tailed Fisher's exact test for all comparisons).

[b] $P < .001$, infants who died in the hospital vs. those who died at home; and $P < .001$, pneumonia vs. SIDS (2-tailed Fisher's exact test).

[c] Detected as an unexpected finding by microscopic examination using hematoxylin-eosin stain.

[d] Associated diagnoses in 11 infants included surgically operated tracheoesophageal fistula ($n = 1$), bronchopulmonary dysplasia ($n = 3$), bacterial meningitis ($n = 1$), trisomy 21 with associated ventricular septal defect ($n = 2$), ventricular septal defect ($n = 1$), unspecified chronic lung damage ($n = 1$), unspecified genetic defect ($n = 1$), and cerebral atrophy ($n = 1$).

Cysts of *Pneumocystis* were studied by use of microscopy using Grocott-Gomori silver methenamine stain in 1 histological lung section for each of the 87 infants who died in the community; observers were unaware of PCR results. A specimen of tracheal secretion and 2 specimens of lung tissue, obtained during autopsy using sterile equipment, were processed for viral isolation and immunofluorescence assay (IFA) for respiratory syncytial virus (RSV), adenovirus, influenza, parainfluenza, herpes simplex virus, and cytomegalovirus (CMV). Specimens were inoculated (0.2 mL each) into HEp-2 cells, MDCK cells, Vero cells, and human lung fibroblast diploid cells (CMV shell vial) and were incubated at 37°C. Cultures were observed for 10 days, and confirmatory IFAs were performed for cultures with and without development of cytopathic effect, as described elsewhere [8]. Two independent observers reported results separately. Viral identification in any specimen was considered to be diagnostic.

STATA software (version 8.0; StataCorp) was used to test whether *Pneumocystis* and viruses were associated with circumstance of death and autopsy diagnoses, by use of a 2-tailed Fisher's exact test. $P < .05$ was considered to be significant.

Results. The age range of 39 female and 48 male infants who died unexpectedly at home was 20–366 days (mean, 87 days; median, 72 days). The age range of 9 female and 16 male infants who died in the hospital was 17–330 days (mean, 147 days; median, 137 days).

Identification of *Pneumocystis* and viruses according to circumstance of death and autopsy diagnosis are listed in table 1. Twelve of the infants who died in the community had an unexpected autopsy finding. In none of the 7 infants with pneumonia diagnosed by histological examination was *Pneumocystis* considered to be the etiological agent (table 1). Pneumonia was the main clinical and autopsy diagnosis in 14 (56%) of the 25 infants who died in the hospital and was an associated diagnosis in the remaining 11 infants (44%) (table 1). None of the infants who died in the hospital received trimethoprim-sulfamethoxazole, dapsone, atovaquone, pentamindine, azithromycin, or other drugs with reported anti-*Pneumocystis* activity.

DNA of *P. carinii* f. sp. *hominis* was detected in 50 (44.6%) of the 112 infants (28 male and 22 female). The incidence was higher for infants 2–4 months old and was significantly more frequent for infants <6 months old than those ⩾6 months old ($P = .03$). No particular seasonal pattern of distribution was detected (figure 1).

A significantly higher incidence of *Pneumocystis* infection was found in infants who died in the community (45/87 [51.7%]) than in infants who died in the hospital (5/25 [20%]) ($P = .006$) (table 1). For the 5 infants who died in the hospital and who were positive for *Pneumocystis* DNA, the length of the hospital stay was 0.3, 1, 12, 21, and 50 days, respectively; for the 20 infants who died in the hospital and who were negative for *Pneumocystis* DNA, the length of the hospital stay was 2–

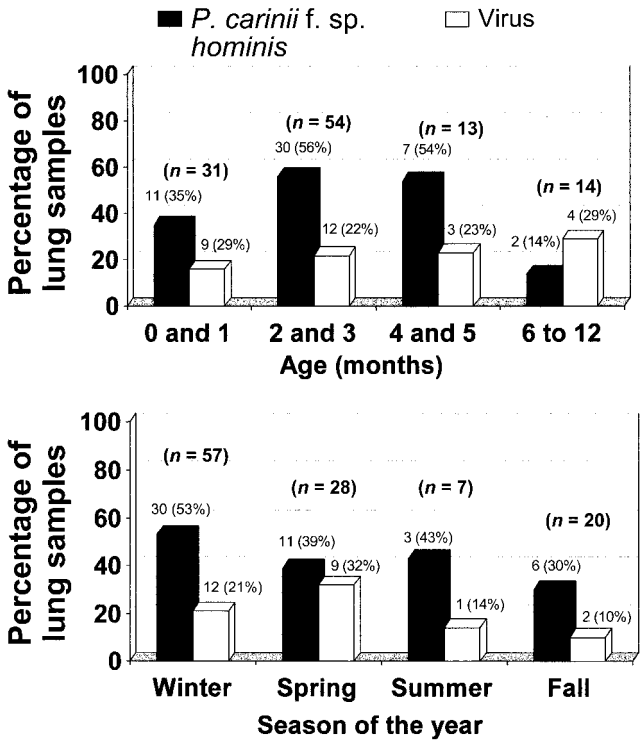

Figure 1. Detection of *Pneumocystis carinii* f. sp. *hominis* DNA and respiratory viruses in autopsy lung specimens from 112 Chilean infants, according to age *(top)* and season when death occurred *(bottom)*. Nos. over the bars represent the no. of infants with *Pneumocystis* or viruses and the total no. of infants examined for each age range or season. Infants who died in the community ($n = 87$) and in the hospital ($n = 25$) are included.

180 days (mean, 27 days; median, 10 days). Each shared hospital room holds 6 infant beds.

Among the infants who died in the community, cyst forms grouped in clusters were seen, by use of microscopic examination, in specimens from 14 (31%) of the 45 infants who tested positive for *Pneumocystis* DNA but were not seen in specimens from the 42 infants who tested negative for *Pneumocystis* DNA ($P = .002$). Single-round PCR results were positive in specimens from 2 infants who had more frequent cysts visualized by use of microscopy. Of the 5 infants who died in the community with unexpected pneumonia diagnosed by histological examination and who tested positive by nested PCR for *P. carinii* f. sp. *hominis* DNA, none tested positive by single-round PCR. Cysts were visualized by microscopy in specimens from 2 of them (table 1). Specimens from infants who died in the hospital were not examined with Grocott-Gomori stain.

Virus detection was similar at different ages and was more frequent in winter and spring (figure 1). Viruses were identified in 24 infants: 15 (60.0%) of the 25 infants who died in the hospital and 9 (10.3%) of the 87 infants who died in the community ($P < .001$) (table 1). Viruses isolated from infants who died in the community included RSV ($n = 3$), adenovirus ($n = 2$), echovirus ($n = 2$), parainfluenza ($n = 1$), and CMV ($n = 3$). Viruses isolated from infants who died in the hospital were RSV ($n = 8$), adenovirus ($n = 7$), parainfluenza ($n = 1$), and CMV ($n = 1$). Mixed viral infections were identified in 2 infants who died in the hospital (both had adenovirus plus RSV) and in 2 infants who died in the community (1 had adenovirus plus echovirus and 1 had parainfluenza virus plus CMV). *Pneumocystis* was found to be associated with a virus in 7 infants who died in the community (RSV [$n = 3$], CMV [$n = 2$], adenovirus [$n = 1$], and echovirus [$n = 1$]) and in 3 infants who died in the hospital (adenovirus [$n = 1$], adenovirus plus RSV [$n = 1$], and CMV [$n = 1$]) (table 1).

Discussion. The present study has documented the presence of *Pneumocystis* DNA in the lungs of approximately half (51.7%) of presumably immunocompetent infants who died unexpectedly in the community in Santiago, Chile (table 1), thus further suggesting that *Pneumocystis* is highly endemic among infants and is not restricted to immunocompromised patients. This result also supports the hypothesis that infants may play an important role in the circulation of *Pneumocystis* organisms as an infective reservoir for susceptible patients, as documented in animal models [9]. The age distribution of *Pneumocystis* infection seen in the present study, with a higher incidence at age 2–4 months and decreasing incidences thereafter, suggests that the primary infection occurs early in life [4, 7] and is eliminated from the lungs of infants without establishing commensalism or long-term latency, as shown in animal models [11, 12] (figure 1).

The present results are consistent with those from our previous report in documenting that *Pneumocystis* infection was significantly more frequent among infants who died unexpectedly in the community than among infants whose parents requested medical attention and who died in the hospital (table 1) [1]. This suggests that infection with *Pneumocystis* does not produce overt disease among immunocompetent infants who die in the community and supports the longstanding view that primary infection is largely asymptomatic (table 1).

An association between *Pneumocystis* infection and the autopsy diagnosis of SIDS cannot be concluded from the present study, because similar incidences of *Pneumocystis* infection were detected in the 12 infants who had unexpected pathological findings at autopsy who had died suddenly in the community and in the 75 infants with diagnosis of SIDS. However, the role of these findings in precipitating death was uncertain, and a diagnostic finding of pneumonia in 7 of these infants was based solely on inflammation detected by histological examination (table 1). A recent histological study found no differences in pulmonary inflammation in infants who died of either accidental death or of SIDS [13]. Accordingly, the presence of histological inflammation in infants in the present study does not

necessarily rule out SIDS. In addition, infants who died at the hospital are not an ideal control group for SIDS, because their deaths were not sudden. However, we must recall that they did not receive drugs with known anti-*Pneumocystis* activity and that their exposure to *Pneumocystis* in the hospital may have been similar to that of infants who died in the community, because they were hospitalized in rooms shared with other children.

The striking differences in clinical presentation and intensity of the primary *Pneumocystis* infection between these infants and immunocompromised hosts provide additional evidence that clinical disease and pathogenesis mechanisms in *Pneumocystis* infection are mostly host mediated [14, 15]. Effective inflammatory responses are required to control *P. carinii* pneumonia (PCP), and the degree of respiratory impairment correlates with the degree of lung inflammation [15]. Pathogenesis of the primary infection remains poorly understood, and inflammatory responses with diffuse histological damage were not detected in these infants by use of hematoxylin-eosin stain. However, the interaction between immunocompetent infants and *Pneumocystis* during primary infection generally leads to elimination of *Pneumocystis*. Further study using sensitive technology might provide an explanation to this paradox [10].

Infection with respiratory viruses, which frequently causes overt respiratory illness, was significantly more frequent among infants who died in the hospital (table 1). The incidence of virus detection in infants with SIDS in the present study (9/87 [10.3%]) is similar to or smaller than that in previous reports [16]; these variations might reflect viral epidemics [16].

The present study may also suggest a seasonal distribution for viruses that was not seen for *Pneumocystis* (figure 1). PCP has been reported to occur more frequently in winter months. However, the incidence of PCP might be influenced by environmental conditions and by the number of susceptible immunocompromised patients. Rabbit models of spontaneous primary infection reveal that *Pneumocystis* DNA may be detected over several weeks and thus make a seasonal pattern of transmissible respiratory infection less apparent [17].

The present study has shown that autopsy diagnosis of primary *Pneumocystis* infection requires the use of highly sensitive tools. Although single-round PCR is adequate to diagnose PCP in immunocompromised patients [18], it is not sensitive enough to detect primary infection and is even less sensitive than careful microscopy using Grocott-Gomori stain.

The visualization of cysts grouped in few clusters in 31% (14/45) of *Pneumocystis* DNA–positive specimens documents the mild nature of primary infection and the greater sensitivity of nested PCR and suggests the presence of an active infection. This greater sensitivity is also reflected in the higher incidence of *Pneumocystis* infection in infants in the present study, compared with that obtained stochastically with stains in previous microscopy series [1, 2].

Nested PCR does not offer advantages over traditional microscopy and is less specific than single-round PCR in detecting clinical PCP in immunocompromised patients [18]. However, nested PCR permits the identification of asymptomatic infections or carrier states in less immunocompromised or immunocompetent individuals [7, 19].

Histological documentation of few *Pneumocystis* cysts in the present study suggests that a positive nested PCR result in a lung specimen from an immunocompetent individual indicates a mild pulmonary infection and not merely the presence of *Pneumocystis* DNA. Furthermore, trophozoites, which are 5–10 times more numerous than cysts, are not detected by Grocott-Gomori stain.

Therefore, when the characteristic mild histological pattern of the primary infection by *Pneumocystis* makes histological readings very laborious, nested PCR in fresh-frozen lung specimens should be preferred over Grocott-Gomori stain in formalin-fixed, paraffin-embedded tissue sections as a screening method for autopsy diagnosis. Additional epidemiological autopsy studies from other sites and studies of subclinical pathogenic mechanisms to document whether *Pneumocystis* infection plays a role in sudden-onset, unexpected deaths at home in infants are warranted.

Acknowledgments

We thank Walter T. Hughes for critical review of the manuscript, Francisco Cumsille for statistical support, Rebeca Bustamante and Cristián Donoso for excellent technical assistance, and Patricia Pizarro for assistance with data management. We also honor the memory of Ann E. Wakefield (deceased; formerly Professor of Infectious Diseases, University of Oxford, Oxford, United Kingdom) for highlighting discussions that stimulated this work.

References

1. Vargas SL, Ponce CA, Hughes WT, et. al. Association of primary *Pneumocystis carinii* infection and sudden infant death syndrome. Clin Infect Dis **1999**; 29:1489–93.
2. Morgan D, 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**; 20:306–9.
3. 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**; 140:367–69.
4. Peglow SL, Smulian AG, Linke MJ, et al. Serologic responses to *Pneumocystis carinii* antigens in health and disease. J Infect Dis **1990**; 161:296–306.
5. Chabé M, Vargas SL, Eyzaguirre I, et al. Molecular typing of *Pneumocystis jirovecii* found in formalin-fixed paraffin-embedded lung tissue sections from sudden infant death victims. Microbiology **2004**; 150:1167–72.
6. 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:e100–2.
7. 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**; 32:855–61.
8. Avendaño LF, Palomino MA, Larrañaga C. Surveillance for respiratory syncytial virus in infants hospitalized for acute lower respiratory infection in Chile (1989 to 2000). J Clin Microbiol **2003**; 41:4879–82.

9. Dumoulin A, Mazars E, Gargallo D, et al. Immunocompetent hosts transiently parasitized by *Pneumocystis carinii* are a potential infectious source to susceptible hosts. Eur J Clin Microbiol Infect Dis **2000**; 19:671–8.
10. Casadevall A, Pirofski LA. The damage-response framework of microbial pathogenesis. Nat Rev Microbiol **2003**; 1:17–24.
11. Chen W, Gigliotti F, Harmsen AG. Latency is not an inevitable outcome of infection with *Pneumocystis carinii*. Infect Immun **1993**; 61:5406–9.
12. Vargas SL, Hughes WT, Wakefield AE, Oz H. Limited persistence and subsequent elimination of *Pneumocystis carinii* from the lungs after *P. carinii* pneumonia. J Infect Dis **1995**; 172:506–10.
13. Krous HF, Nadeau JM, Silva PD, Blackbourne BD. A comparison of respiratory symptoms and inflammation in sudden infant death syndrome and in accidental or inflicted infant death. Am J Forensic Med Pathol **2003**; 24:1–8.
14. Limper AH, Offord KP, Smith TF, Martin WJ II. *Pneumocystis carinii* pneumonia: differences in lung parasite number and inflammation in patients with and without AIDS. Am Rev Respir Dis **1989**; 140:1204–9.
15. Wright TW, Gigliotti F, Finkelstein JN, McBride JT, An CL, Harmsen AG. Immune-mediated inflammation directly impairs pulmonary function, contributing to the pathogenesis of *Pneumocystis carinii* pneumonia. J Clin Invest **1999**; 104:1307–17.
16. Samuels M. Viruses and sudden infant death. Paediatr Respir Rev **2003**; 4:178–33.
17. Tamburrini E, Ortona E, Visconti E, et al. *Pneumocystis carinii* infection in young non-immunosuppressed rabbits: kinetics of infection and of the primary specific immune response. Med Microbiol Immunol **1999**; 188:1–7.
18. Sing A, Trebesius K, Roggenkamp A, et al. Evaluation of diagnostic value and epidemiological implications of PCR for *Pneumocystis carinii* in different immunosuppressed and immunocompetent patient groups. J Clin Microbiol **2000**; 38:1461–7.
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**; (Suppl):122S–3S.