MOLECULAR DIAGNOSIS

Detection of *Pneumocystis carinii* among children with chronic respiratory disorders in the absence of HIV infection and immunodeficiency

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A nested polymerase chain reaction (PCR) assay was investigated for detection of *Pneumocystis carinii* in 96 respiratory tract specimens from 82 children, of whom 28 were immunocompetent but with chronic lung disorders (CLD), eight had AIDS and *P. carinii* pneumonia (PCP), 16 had AIDS but no respiratory symptoms, and 30 were healthy immunocompetent children. Gomori methenamine silver stain (GMS) and indirect immunofluorescence assay (IFA) were performed in parallel. Of 36 specimens from children with CLD, 12 were *P. carinii* PCR-positive compared to 10 positive by GMS-IFA. Of eight specimens from children with AIDS and PCP, seven were *P. carinii*-positive by PCR and six by GMS-IFA, and of 22 specimens from HIV-positive children without respiratory symptoms, two were positive by PCR and none by GMS-IFA. *P. carinii* DNA was also detected by PCR in blood samples from four children with *P. carinii*-positive nasopharyngeal aspirates. Specimens from healthy children were negative for *P. carinii* by both PCR and GMS-IFA. Of the seven children with CLD, who were *P. carinii*-positive, two had clinical and microbiological improvement with co-trimoxazole treatment, two improved initially but relapsed, and one had *P. carinii* cysts persistently in follow-up specimens despite co-trimoxazole treatment. These results suggest an association between *P. carinii* and exacerbations of CLD in childhood, in the absence of HIV infection or other immunodeficiency syndromes.

Introduction

In immunocompromised patients, and particularly in those with AIDS, *Pneumocystis carinii* pneumonia (PCP) is one of the most frequent opportunist infections. It may result from a pneumocystis infection *de novo* or from reactivation of latent childhood infection.

Reports of PCP in non-immunocompromised patients are rare and appeared before testing for HIV and the detailed assessment of immune function were available [1]. DNA amplification studies have failed to detect *P. carinii* in sputum or autopsy lung tissue from immunocompetent patients [2, 3], or from bronchoalveolar lavage fluid except for patients with small cell lung cancer [4]. However, others have reported the detection of *P. carinii* in specimens from healthy adults without immunological impairment or evidence of neoplasms or HIV infection [5–7]. *P. carinii* has also been detected

by conventional stains in non-immunosuppressed patients with chronic obstructive lung disease [8].

This study evaluated the presence of *P. carinii* in respiratory specimens from immunocompetent children with chronic lung diseases (CLD). *P. carinii* detection was investigated by conventional stains and by a nested polymerase chain reaction (PCR) assay with rat-derived *P. carinii* oligonucleotides known to show significant homology with *P. carinii* found in man. In a previous study, these oligonucleotides proved to be efficient primers for amplification of *P. carinii* DNA from both rats and human patients with PCP and did not amplify DNA from a range of other pathogens including fungi [9].

Materials and methods

Clinical specimens and patients

A total of 96 respiratory specimens including nasopharyngeal aspirates (NPA) and induced sputum samples was assayed for *P. carinii*. Clinical specimens

and the study population consisted of: *group 1*, 36 specimens from 28 HIV-seronegative children (1–14 years) according to the CDC criteria, with normal B- and T-lymphocyte counts, T-cell subsets and total immunoglobulins, who presented with various respiratory disorders; *group 2*, eight specimens from eight HIV-positive children with PCP diagnosed on clinical and radiological findings of whom six were *P. carinii*-positive by staining (one patient had earlier *Toxoplasma gondii* encephalitis and was receiving anti-*P. carinii* specific treatment at the time of sampling); *group 3*, 22 specimens from 16 HIV-positive patients without respiratory symptoms or chest X-ray abnormalities, of whom 10 presented with a high degree of immunosuppression (CD4+ lymphocyte counts < 200/mm³) and were receiving specific prophylaxis against *P. carinii* at the time of specimen collection; *group 4*, 30 specimens from 30 healthy, afebrile infants without active pulmonary infection who were not receiving immunosuppressive drugs and were age-matched and sex-matched for group 1.

Patients with CLD (group 1) were recruited between Nov. 1992 and Jan. 1995 from the Paediatric Institution of Rome after approval of the local Ethics Committee. An NPA was collected at admission for all patients. Some patients were evaluated prospectively to establish clinical or microbiological response, or both, to treatment and required further specimens ($n = 1-6$). Induced sputum samples were also assayed from five patients. Informed consent to perform anti-HIV testing was obtained. Nine HIV-infected children (group 3) had further single specimens which were investigated retrospectively for *P. carinii* after discharge from hospital, usually coincident with disappearance of clinical symptoms.

Respiratory specimens were assayed for *P. carinii* by PCR, indirect immunofluorescence assay (IFA) and tinctorial Gomori methenamine staining (GMS). Samples were also screened for bacteria, fungi, mycoplasmas, *Mycobacterium tuberculosis* and respiratory syncytial virus. The presence of other viruses, such as cytomegalovirus, coronaviruses and rhinoviruses, was not investigated. Single or multiple blood samples were also obtained from each patient and the controls and these were tested by PCR.

Specimen processing

NPA specimens were collected on admission as described previously [10]. NPA and induced sputa were liquefied with sputolysin (6.5 mM dithiothreitol; Unipath, Basingstoke) and then vortex mixed with glass beads for 3 min. An equal volume of phosphate-buffered saline (PBS, pH 7.2) was added and samples were centrifuged at 3000 rpm for 15 min. A portion of the pellet was resuspended in 1 ml of PBS and stored until required for PCR assay. A further portion was either smeared on IFA slides or stained by a rapid GMS [11].

Immunofluorescence antibody test (IFA)

Specimens were fixed in cold acetone for 10 min and then treated with trypsin (2.5 g/L) to remove proteins covering antigenic sites of the pathogen. Anti-*P. carinii* monoclonal antibodies (MAbs) (Monofluo kit *Pneumocystis*, Diagnostic Pasteur, Marnes La Coquette, France) prepared against human *P. carinii* cysts and specific for cyst-wall antigen were employed [12]. An IFA specimen was considered positive if two or three good cysts were seen in at least three high power fields. Both the IFA and GMS preparations were examined blindly by different people.

Polymerase chain reaction procedure (PCR)

Approximately 1 ml of sample was centrifuged at 15 000 rpm for 3 min; pellets were resuspended in 400 μ l of proteinase K buffer containing 500 μ g of proteinase K (Boehringer Mannheim Biochemicals). After overnight incubation at 37°C, DNA was extracted by phenol-chloroform-isoamyl alcohol followed by ethanol precipitation. Two primer sets based on the chromosomal rRNA sequence of rat-derived *P. carinii* were used in a nested PCR as described previously [9]. The amplification mix contained 10 mM Tris-HCl (pH 8.3, supplemented with 50 mM KCl), 200 mM of each deoxynucleotide triphosphate, 0.25 mM of each primer, AmpliTaq DNA Polymerase 25 U/ml, and 2.5 mM MgCl₂ in a final reaction volume of 50 μ l (all reagents from Perkin-Elmer, Norwalk, USA). Samples were subjected to initial amplification with external primers (35 cycles of 1 min, 94°C; 1 min, 50°C; 2 min, 72°C) followed by re-amplification with inner primers (25 cycles). The DNA products of 378 bp for the external primers and 312 bp for the inner primers were detected by gel electrophoresis and staining with ethidium bromide. The PCR product (10- μ l samples) was further analysed by Southern hybridisation with a radiolabelled oligonucleotide probe. Samples were declared positive if a band of appropriate size was detected by autoradiography.

For blood PCR, peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation (Lymphoprep, Nycomed) within 2 h of collection. DNA was extracted by the method of Kawasaki [13]. PBMCs were incubated overnight with proteinase K and DNA was extracted as above.

Precautions appropriate for the prevention of cross-contamination of PCR assays were strictly observed [14]. Each stage was performed in separate rooms, pipettes and bottles were regularly autoclaved and gloves were changed frequently.

Controls. For each assay, positive and negative controls were included. Purified *P. carinii* cysts extracted from infected human lung tissue (autopsy material) and BAL specimens from AIDS patients with documented PCP were used as positive controls. Lung tissue homo-

genates prepared from uninfected human lung with no detectable *P. carinii* or other respiratory pathogens, and BAL specimens from immunocompetent patients, negative for *P. carinii* or fungi at time of their collection, served as negative controls. The PCR method was also tested with a number of other organisms (*Toxoplasma gondii*, *Cryptococcus neoformans*, *Candida albicans*, *C. tropicalis*, *Aspergillus fumigatus*) to assess specificity. A buffer negative control was also included to test for cross-contamination.

Results

Clinical and laboratory data are summarised in Tables 1 and 2.

Group 1. Among the 28 immunocompetent children with CLD, asthma (n = 4) and chronic bronchitis (8) were the most frequent diagnoses. Other underlying conditions included immotile cilia syndrome (1) and middle lobe syndrome (2), atopy and respiratory allergy with recurrent episodes of bronchopneumonia (2) and bronchiolitis with otitis media (2). Principal symptoms leading to admission were productive cough for at least 3 months of a year according to published

CLD definition [15] with fever of ≤7 days. In one patient, the duration of fever had lasted several weeks and had not responded to antibiotic treatment. Chest radiological findings included bilateral infiltrates in three patients, emphysema in five, diffuse interstitial infiltrate in one and bronchovascular markings in five. In patients for whom arterial blood gas values were determined, values were normal.

Of 36 NPA samples from these patients, 12 (33%) were positive for *P. carinii* by PCR (seven patients), compared to 10 (27.7%) positive by GMS and IFA (six patients). All positive PCR specimens produced a strong band of amplified DNA easily identified after ethidium bromide staining. All GMS and IFA positive specimens were positive by both staining techniques. Induced sputum specimens were available from five patients with *P. carinii*-positive NPA; these were all positive by both PCR and GMS/IFA. The characteristics, treatment and outcome of the seven CLD patients positive for *P. carinii* are shown in Table 2. Other potential respiratory pathogens were isolated from four of these patients, including *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Pseudomonas* spp. and *Moraxella catarrhalis*. Five children were treated with co-trimoxazole; two improved, two improved initially but then relapsed, and one had persistent *P.*

Table 1. Results of *P. carinii* investigation on respiratory specimens from different patient groups

Patient group	Number of NPA samples	Number of samples positive for <i>P. carinii</i> by	
		PCR	GMS-IFA
Group 1 (n = 28)*	36	12 (7)*	10 (6)
Group 2 (n = 8)	8	7 (7)	6 (6)
Group 3 (n = 16)	22	2 (1)	0
Group 4 (n = 30)	30	0	0

NPA, nasopharyngeal aspirate; GMS-IFA, gomori methenamine stain – indirect immunofluorescence assay.
*() number of patients.

Table 2. Clinical and microbiological findings among children positive for *P. carinii* with chronic respiratory diseases

Case no.	Underlying disease	<i>P. carinii</i> detected by				Antimicrobial therapy	Outcome
		NPA		PBMC PCR	Other pathogens isolated		
		GMS-IFA	PCR				
1	Middle lobe syndrome	Yes	Yes	Yes	None	TMP-SMX	Microbiological improvement
2	Middle lobe syndrome	Yes	Yes	No	<i>S. aureus</i> , <i>Str. pneumoniae</i> , <i>M. catarrhalis</i>	TMP-SMX	Improvement, then relapse
3	Middle lobe syndrome	Yes	Yes	No	<i>Str. pneumoniae</i> , <i>M. catarrhalis</i>	TMP-SMX	Improvement, then relapse
4	Chronic bronchitis; post-measles pneumonia	No	Yes	No	<i>Pseudomonas</i> spp. <i>Str. pneumoniae</i>	Other	Improvement, then relapse
5	Chronic bronchitis	Yes	Yes	No	ND	TMP-SMX	Clinical and microbiological improvement
6	Chronic bronchitis	Yes	Yes	No	ND	Other	Improvement, then relapse
7	FUO; chronic bronchitis	Yes	Yes	No	<i>S. aureus</i>	TMP-SMX	Persistence of <i>P. carinii</i> cysts

NPA, nasopharyngeal aspirate; GMS, gomori methenamine stain; IFA, indirect immunofluorescent assay; FUO, fever of unknown origin; other, antimicrobial therapy without known efficacy against *P. carinii*; ND, not determined; PBMC, peripheral blood monocytes; TMP-SMX, co-trimoxazole.

carinii cysts in follow-up specimens. Two children were treated with other antibiotics with no activity against *P. carinii*, both improved initially but relapsed.

Group 2. Of the eight HIV-positive patients with probable PCP, two presented with productive or dry cough without dyspnoea, four had fever and dyspnoea, and two had dyspnoea only. Radiological signs varied from perihilar markings to bilateral interstitial shadowing. Five patients were hypoxaemic. In six patients, PCP had been confirmed previously by GMS. Of the eight specimens collected from these patients, seven (87.5%) were PCR-positive, including the six known GMS-positive specimens which were also confirmed as positive by IFA. The single PCR-positive, GMS-IFA-negative specimen was from the child receiving anti-PCP prophylaxis.

Group 3. The other 16 HIV-infected children had advanced AIDS but no pulmonary symptoms. In this group *P. carinii* DNA was also detected in two (9%) specimens obtained from one patient.

Group 4. All 30 control children were negative for *P. carinii* by PCR, GMS and IFA.

P. carinii DNA was detected in four blood specimens: one from a CLD case with NPA samples positive for *P. carinii* by both microscopy and PCR (case 1, Table 2), two from HIV-infected patients with documented PCP and with NPA positive by both staining and PCR, and one from an HIV-infected patient with no respiratory symptoms but with an NPA positive by PCR only.

Discussion

In HIV-positive children and adults, PCP is the most common AIDS-defining diagnosis, occurring in 30–50% of all cases. In children, PCP has also been described in patients with predisposing conditions such as neoplasms, organ transplants, hypogammaglobulinaemia and immunosuppressive therapy [16]. Subclinical *P. carinii* infection has been recognised at autopsy in healthy immunocompetent children [17]. Furthermore, specific antibodies have been shown to develop by the age of 4 years, indicating ubiquitous early exposure to the organism, [18, 19].

The present study investigated the role of *P. carinii* in immunocompetent children with CLD. *P. carinii* was detected by PCR in 12 of 36 NPA specimens from children admitted with exacerbations of CLD; 10 of these were confirmed by IFA and GMS. In contrast, *P. carinii* was not detected in NPA samples from 30 healthy children. However, the role of *P. carinii* in the respiratory disease of children with CLD was unclear. Cross-contamination between cases was unlikely, as bronchoscopes or other special equipment were not

required to obtain NPA specimens. Several authors have suggested that *P. carinii* may be a transmissible pathogen in both animals and man [20, 21]. However, none of the children positive for PCP was known to have been exposed to patients with PCP. The isolation of co-pathogens in these patients is not surprising. Culture results of NPAs and other respiratory samples are often difficult to interpret because of the carriage of potential respiratory pathogens in the upper airways. Furthermore, clinical and radiological features were non-specific in many study patients some of whom were already receiving antimicrobial therapy, further complicating the clinical and microbiological picture. Although the role of *P. carinii* in exacerbations of CLD needs further clarification, the inflammatory reaction and bronchial obstruction seen in CLD might permit proliferation of *P. carinii* in the lung.

Two CLD children with *P. carinii* improved with co-trimoxazole therapy, suggesting that *P. carinii* may have been responsible for the worsening of their pulmonary disease. Four patients improved on co-trimoxazole but then relapsed clinically and one was found to have *P. carinii* cysts in follow-up specimens. These failures may have been related to relapse or re-infection. Recurrent episodes of PCP are thought to be caused by re-infection rather than by reactivation [22, 23].

Overall, PCR detected five more positive samples than GMS and IFA. This may be related to the ability of PCR to detect DNA from both trophozoite and cyst forms; IFA and GMS are specific for cysts only, and may be negative in specimens with a low cyst load. Cross-contamination with *P. carinii* DNA from positive samples was unlikely as negative controls were included in all PCR runs.

Sputum is difficult to collect from infants and young children and NPAs are often relied upon. Our detection of *P. carinii* by both PCR and GMS-IFA in NPA samples confirms the data from other groups showing that NPA is a useful analytical sample for the diagnosis of PCP among HIV-infected and leukaemic infants [24, 25].

Among the eight HIV-positive children with established PCP, one had a negative PCR result. The failure of PCR to amplify DNA in this specimen was probably due to long-term anti-PCP treatment, but loss of DNA during storage or routine manipulation could not be ruled out.

One of the PCR-positive GMS-IFA-negative specimen was from an HIV-positive child, who had taken prophylaxis for PCP; this patient had received both co-trimoxazole and aerosolised pentamidine. PCP prophylaxis may contribute to under-detection of *P. carinii* by conventional staining methods by reducing the number of organisms present in the respiratory

tract [26]. In this situation PCR positivity may represent subclinical colonisation with *P. carinii*. PCR examination of serial specimens over time may offer prognostic information about the development of symptomatic PCP as well as the efficacy of current treatment regimens.

The lack of false positive PCR results among healthy children suggests that the specificity of the PCR assay for *P. carinii* was high.

Four children (1, CLD; 3, AIDS) with positive NPAs for *P. carinii* also had detectable *P. carinii*-specific amplification products in PBMCs, confirming previous data from rats and human patients with PCP [11, 21, 27, 28]. Recently, *P. carinii* sequences were detected by PCR in serum from patients with PCP [29]. The practical value of *P. carinii* PCR assays of whole blood, serum or PBMCs as a diagnostic tool for PCP remains to be determined.

Further work is required to determine whether long-term carriage of *P. carinii* alone or with accompanying co-pathogens contributes to exacerbations of CLD and whether appropriate prophylactic or therapeutic regimens should be considered in these patients.

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