MEDICAL SCIENCE

Detection of *Pneumocystis carinii* with DNA amplification

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Oligonucleotide primers and probes were used in the polymerase chain reaction to amplify *Pneumocystis carinii* specific DNA sequences from alveolar lavage samples from 47 diagnostic bronchoscopies. No *P carinii* DNA was found in lavage from 10 immunocompetent patients; only low levels were found in 3 of 13 samples from immunosuppressed individuals without *P carinii* pneumonia (PCP), and the highest levels, readily demonstrated by simple ethidium bromide staining, were found in all of 16 samples from immunosuppressed patients with PCP confirmed by means of standard silver staining and in 4 from patients with clinical PCP but negative silver staining. DNA amplification provides a highly sensitive and specific technique for the identification of *P carinii* that should be valuable in epidemiological studies on this parasitic infection and in diagnosis.

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Introduction

Pneumocystis carinii is the major cause of fatal pneumonia in patients with profound T cell deficiency due to immunosuppressive therapy for organ transplantation or cancer or to AIDS. Culture of *P carinii* in vitro is difficult because of the parasite's fastidious nature, and diagnostic identification of the organism, in bronchoscopic lavage or induced sputum, depends on direct microscopy after silver or giemsa stains,[1] which are inherently non-specific. The development of monoclonal antibodies[2] has improved specificity, but the sensitivity is uncertain,[3] and there remains the need for a method combining high sensitivity and high specificity, applicable in diagnosis and in studies to clarify the natural history and epidemiology of *P carinii* infection.

Organisms are most specifically characterised by their DNA sequences, and the development of the polymerase chain reaction (PCR), which allows efficient amplification of specific DNA sequences, affords the opportunity of applying such highly sensitive but specific tools in microbial diagnosis.[4-6] We have cloned DNA from *P carinii*, affirmed by characteristic in-situ hybridisation patterns, and determined the DNA sequence of part of the gene coding for the large subunit of mitochondrial ribosomal RNA of *P carinii* derived from the rat. Chosen oligonucleotide sequences proved to be efficient primers for amplification of *P carinii* DNA from both rat and human lung infected with the parasite but not from a series of other isolates that included potential pulmonary pathogens: *Aspergillus nidulans, Cryptococcus neoformans, Candida (albicans* and non-*albicans* strains), *Mycobacterium tuberculosis*, and *Saccharomyces cerevisiae*.[7] Comparison of sequences of the amplified product from the rat and human host showed limited but consistent differences and allowed us to construct an internal oligonucleotide suitable for confirmatory Southern hybridisation (oligoblotting) studies on DNA amplified from human material.

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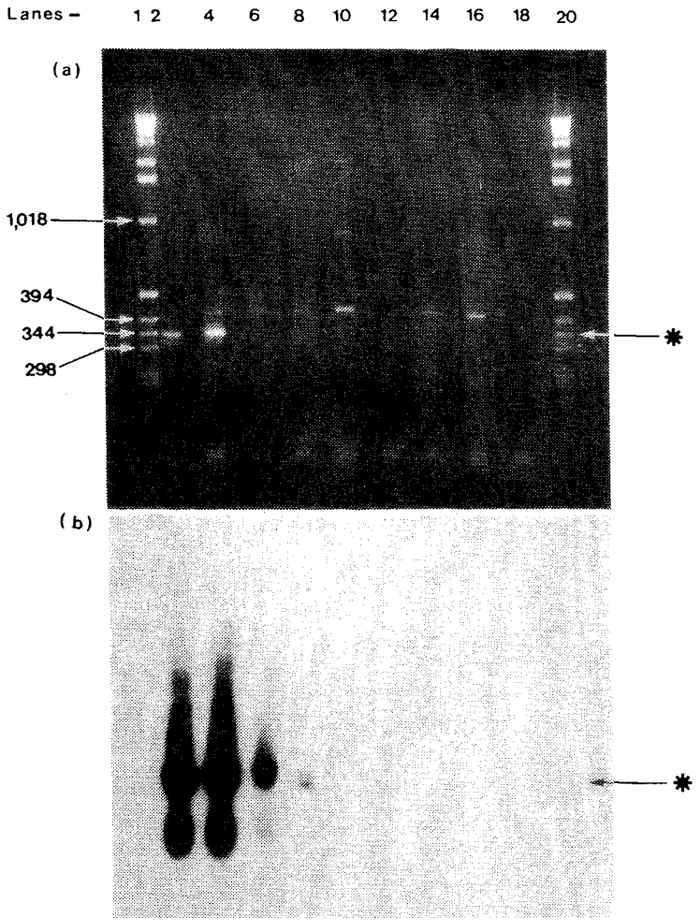

DNA amplification from a series of bronchoscopic samples.

Products of DNA amplification using DNA template prepared from 2 ml of sample· (a) ethidium bromide stained gel, (b) autoradiograph of oligoblot Lanes 2, 4: immunosuppressed patients with silver stain confirmed *P carinii* pneumonia. Lanes 6, 8, 10, 14. immunosuppressed patients without PCP Lane 12: washings from cleaned and sterilised bronchoscope Lane 16 immunocompetent patient. Lane 18 no DNA control. Lanes 1, 20 1 kb molecular weight markers Asterisk denotes position of *P carinii* specific amplification product

We report the application of these techniques to the detection of *P carinii* in bronchoscopic lavage samples.

Methods

Clinical specimens

Bronchoalveolar lavage samples were obtained from 47 patients undergoing bronchoscopy at the Churchill Hospital, Oxford, and the Middlesex Hospital, London.

37 patients were immunosuppressed, either by HIV infection (33) or by treatment for lymphoma (2), vasculitis (1), or leukaemia (1). All patients had symptoms of acute respiratory illness with one or more of the following features: abnormal chest signs, arterial hypoxaemia, or abnormal chest radiograph. The 10 remaining patients were immunocompetent and were undergoing bronchoscopy for the investigation of various chronic respiratory disorders. Routine microbiological and cytological analysis, including methenamine silver staining, was done on each lavage, and a sample was reserved for the DNA amplification study.

DNA amplification and detection

DNA was extracted from the lavage samples by means of proteinase K digestion and phenol/chloroform extraction. The oligonucleotide primers pAZ102-E: -5'-GATGGCTGTTTCCAAGCCCA-3'- and pAZ102-H: -5'-GTGTACGTTGCAAAGTACTC-3'- were used in an amplification reaction mixture (50 mmol KCl, 10 mmol 'Tris', pH 8·0, 0·01% [w/v] gelatin, 3 mmol $MgCl_2$, 400 µmol dNTPs [Boehringer Mannheim], 0·4-1·0 µmol oligonucleotide primer, and 3 units of 'Amplitaq' [Perkin Elmer Cetus]) with denaturation at 94°C for 90 s, annealing at 50°C for 90 s, and extension at 72°C for 2 min for 40 cycles. Negative controls with no added template were included.

The amplification products were subjected to electrophoresis in 1·5% agarose gels, and the presence of pneumocystis specific band (346 base pairs) was tested by: (i) visualisation with ultraviolet light after ethidium bromide staining; and (ii) oligoblotting after transfer to 'Hybond N' (Amersham) filters and hybridisation with the ^{32}P end-labelled internal primer, pAZ102-L2: -5'-ATAAGGTAGATAGTCGAAAG-3'-, specific to human *P carinii* (see figure). High-stringency washes were at 48°C, and the filters were exposed overnight to radiographic film at −80°C with intensifying screens. Each lavage sample was coded, and amplification and detection were repeated in two separate experiments blind to clinical data.

RESULTS OF DNA AMPLIFICATION OF P CARINII DNA IN BRONCHOALVEOLAR LAVAGE SAMPLES; COMPARISON WITH METHENAMINE SILVER STAINING AND CLINICAL RESPONSE TO TREATMENT

Patient group (no of patients)	Silver stain	Response to treatment	Positive DNA amplification for *P carinii*		Negative DNA amplification for *P carinii*
			Ethidium bromide stain + oligoblot	Oligoblot alone	
Immunosuppressed (16)	+	+	16	0	0
Immunosuppressed (6)	−	+	4	0	2
Immunosuppressed (15)	−	−	0	3†	12††
Immunocompetent (10)	−	−	0	0	10

†Alternative diagnosis *Toxoplasma gondii*, disseminated *Mycobacterium avium intracellulare*, pulmonary lymphoma
††Alternative diagnosis endobronchial Kaposi's sarcoma (2), disseminated *Mycobacterium avium intracellulare* and cytomegalovirus pneumonitis (1), *Salmonella typhimurium* bacteraemia (1), *Pseudomonas putida* chest infection (1), *Streptococcus pneumonia* and *Haemophilus influenzae* chest infection (1), bronchiectasis (1), no diagnosis (3)

Results

On the basis of clinical progress, response to treatment with nebulised pentamidine (20 cases) or co-trimoxazole (7 cases), and results of standard investigations (including methenamine silver staining), the 47 patients were divided into four groups (see table): (i) 16 immunosuppressed patients with a positive diagnosis of pneumocystis pneumonia by silver staining of lavage and response to treatment; (ii) 6 immunosuppressed patients with clinical response to treatment but negative silver staining on lavage; (iii) 15 immunosuppressed patients with neither response to treatment nor positive silver staining on lavage (an alternative diagnosis to account for the respiratory disease was available in 12); and (iv) 10 immunocompetent patients from the routine bronchoscopy list. The results of DNA amplification assayed by the visualisation of a 346 base-pair DNA band after (a) ethidium bromide staining and (b) autoradiography after oligoblotting were compared with these clinical categorisations (table).

No *P carinii* DNA was detectable in the samples from the immunocompetent group. All 16 immunosuppressed individuals with *P carinii* identified by means of methenamine silver staining on alveolar lavage had amplified *P carinii* DNA visible by both ethidium bromide

staining and oligoblotting (see figure). Of the 6 individuals judged to have had pneumocystis pneumonia by clinical symptoms and response to treatment but not confirmed by identification of parasites in methenamine silver stained lavage samples, 4 were positive on DNA amplification after both ethidium bromide staining and oligoblotting, and 2 were negative after both methods.

Lesser degrees of *P carinii* infection were detected with oligoblotting but not ethidium bromide staining of the DNA amplification product in lavage samples from 3 of 15 of the immunosuppressed subjects without pneumocystis pneumonia. The intensity of the signal was much less strong than that obtained from patients with acute *P carinii* infection but significantly stronger than a barely visible signal obtained in 4 other samples from this patient group and in 3 of 12 preliminary washings of the bronchoscope after routine cleaning and sterilisation (figure).

Discussion

The construction of suitable oligonucleotides and their application in DNA amplification and subsequent oligoblotting allows highly sensitive and specific identification of *P carinii*. Specificity results from the use of DNA sequences unique to the parasite and sensitivity from two amplification steps—the polymerase chain reaction and oligoblotting.[4-6]

No amplified *P carinii* DNA was detectable in the lavage samples from the 10 immunocompetent subjects, either with ethidium bromide staining or with the more sensitive oligoblotting. This result accords with the failure, in a recent study, to identify *P carinii* with monoclonal antibody in a necropsy survey of lungs from non-immunosuppressed patients.[8] These observations seriously question the assumption that *P carinii* pneumonia (PCP) in immunosuppressed patients necessarily results from reactivation of dormant foci of organisms within the lung, the legacy of asymptomatic infection in childhood.[9] The epidemiology of *P carinii* infection in man has yet to be satisfactorily clarified.

Of the 16 subjects with a clinical diagnosis of PCP and positive silver staining for the parasite on lavage, all produced a strong band of amplified DNA easily identified after ethidium bromide staining. 6 subjects had a final clinical diagnosis of PCP but negative silver staining on lavage. 2 of these had no detectable *P carinii* DNA, but 4 had strong bands detected with ethidium staining. The sensitivity of DNA amplification suggests that the pulmonary illness in the 2 patients with negative *P carinii* DNA must be attributable to some other cause despite the apparent response to pentamidine or co-trimoxazole. Entities such as non-specific and lymphocytic pneumonitis—clinically indistinguishable from PCP except that they may remit spontaneously—are well-recognised but ill-understood aspects of AIDS.[10] The results in these 6 individuals suggest that DNA amplification can identify both false-positive and false-negative diagnoses derived from clinical data and silver staining. This improved diagnostic power may be particularly valuable in trials to determine the efficacy of novel therapies for PCP. We are urgently trying to examine more patients in this diagnostic category.

3 of 15 samples from immunosuppressed subjects without a clinical diagnosis of PCP had observable bands on oligoblotting alone which were easily distinguishable from the very faint signal obtained from the bronchoscope washings. We estimate that oligoblotting offers an increased sensitivity of 10^2 fold over ethidium band staining,[7] showing that the number of parasites must be much smaller in these samples than in the those from patients with PCP. These findings are not unexpected, since each individual was heavily immunosuppressed and data from the rat model of PCP indicate that the number of parasites increases relatively slowly over a 6-8 week spell before pneumonia ensues.[11] The sensitivity of oligoblotting after DNA amplification may thus offer special advantages for planning prophylactic therapy. Stringent precautions to eliminate contamination of samples are essential both in the laboratory and at collection—further studies must acknowledge the possibility that cleaned bronchoscopes may not necessarily be free of *P carinii* DNA. We are currently testing the efficacy of the method applied to samples obtained non-invasively, including induced sputum.[12]

DNA amplification provides a highly sensitive and specific technique for identifying *P carinii*. Its application in further studies should help to elucidate the unresolved epidemiology of this parasitic infection. The increasing availability of automation and calibration[13,14] may also allow the method to become a readily applied and powerful diagnostic tool.

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