Granulomatous *Pneumocystis carinii* pneumonia: DNA amplification studies on bronchoscopic alveolar lavage samples

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Abstract

Three HIV positive subjects presented with symptoms and radiographic changes suggestive of *Pneumocystis carinii* pneumonia. Methenamine silver staining of bronchoscopic alveolar lavage (BAL) fluid was negative (from one sample in one patient and two samples in the other two patients). Open lung biopsy was performed because of uncertain clinical progress and diagnosis; all three patients were found to have multiple pulmonary granulomata encasing numerous *P carinii* organisms. DNA amplification, using *P carinii* specific oligonucleotides, was performed on stored bronchoscopic BAL samples. *P carinii* specific amplification product was detected by ethidium bromide staining after electrophoretic separation on agarose gel in one case, and by the more sensitive technique of oligohybridisation in all three cases. In granulomatous *P carinii* pneumonia organisms are rarely identified in bronchoscopic alveolar lavage samples using histochemical staining, but are detectable by DNA amplification, although not at levels which can be readily distinguished from low, subclinical infection.

Methods

**CASE 1**

A 44 year old HIV-1 antibody positive homosexual man presented with a four week history of malaise, fever, and non-productive cough; he had no prior HIV related illness and was not receiving zidovudine nor taking primary prophylaxis against *P carinii* pneumonia. Investigations showed PO$_2$ = 9.5 kPa breathing air and the chest radiograph showed bilateral perilobar and lower zone interstitial infiltrates typical of *P carinii* pneumonia. Treatment was started with nebulised pentamidine (8 mg/kg/day) via a Respigard II nebuliser. Fibreoptic bronchoscopy and BAL performed after one day of treatment were negative by routine histochemical staining for *P carinii* and other pathogens. After treatment for a further seven days the patient was still asymptomatic and was rebronchoscoped; BAL was repeated and transbronchial biopsies were performed. Routine histochemical investigations were again negative. Eight days later the patient proceeded to open lung biopsy, at which granulomatous *P carinii* pneumonia was diagnosed. Treatment was changed to intravenous high dose co-trimoxazole and the patient made an uneventful recovery.

**CASE 2**

A 45 year old homosexual man with no prior HIV history was admitted with a five week history of progressive dyspnoea and non-productive cough; he was not receiving *P carinii* prophylaxis. Investigations showed PO$_2$ = 13.0 kPa and the chest radiograph showed bilateral patchy interstitial shadowing. Hypertonic saline induced sputum and BAL were negative by routine histochemical staining for *P carinii* and other pathogens. Empirical treatment for *P carinii* pneumonia,
with nebulised pentamidine had produced little clinical response after 15 days. The patient was bronchosoped again and analysis of BAL fluid by methenamine silver staining was again negative for _P. carinii_. Open lung biopsy, 12 days later, showed granulomatous _P. carinii_ pneumonia. Treatment with intravenous pentamidine (the patient was hypersensitive to co-trimoxazole) produced a rapid clinical recovery.

CASE 3
A 45 year old HIV-1 antibody positive homosexual man presented with a 10 day history of increasing dyspnoea and unproductive cough. Four months previously he had had _P. carinii_ pneumonia, treated with daily nebulised pentamidine for 21 days; he subsequently developed cutaneous lesions of Kaposi’s sarcoma and had begun zidovudine and monthly nebulised pentamidine as secondary prophylaxis against _P. carinii_. On this presentation the PO2 was 9.1 kPa and the chest radiograph showed bilateral interstitial shadows, some of which had a nodular appearance. Histochemical analysis of induced sputum and BAL fluid were negative for _P. carinii_ and other pathogens; at fibroptic bronchoscopy endobronchial lesions of Kaposi’s sarcoma were seen. Because of persistent symptoms, BAL was repeated 10 days later; routine laboratory analysis was negative. An open lung biopsy sample, taken 15 days later, showed granulomatous _P. carinii_ pneumonia. The patient responded rapidly to intravenous high dose co-trimoxazole.

DNA AMPLIFICATION
A sample of BAL fluid was stored at −20°C for DNA amplification studies in each patient. DNA was extracted from 2 ml of BAL fluid by means of proteinase K digestion and phenol/chloroform extraction. The oligonucleotide primers pAZ102-E: 5′-GATGGAAGTCTTTCTCCAGGCTCA-3′ and pAZ102-H: 5′-GCTGThATGTAGGTGTGCAAAGTACTC-3′ were used in an amplification reaction mixture containing 50 mM KCl, 10 mM TRIS, pH 8.0, 0.01% (w/v) gelatin, 3 mM MgCl2, 400 μM dNTPs (Boehringer Mannheim), 1.0 μM oligonucleotide primer, and 0.25 units/μl Taq polymerase (Boehringer Mannheim). Amplification was carried out with denaturation at 94°C for one and a half minutes, annealing at 55°C for one and a half minutes, and extension at 72°C for two minutes for 40 cycles. Negative controls, with no added template, were tested with each clinical sample. Amplification products were separated by electrophoresis in 1.5% agarose gels, and the presence of a _P. carinii_ specific band (346 base pairs) was tested by: (i) visualisation by ultraviolet light after ethidium bromide staining; and (ii) oligoblotting after transfer to “Hybond N” (Amersham) filters and hybridisation with the 32P end-labelled internal primer, pAZ102-L2: 5′-ATAAGGTAGAT-GTCGAAAG-3′, specific to human _P. carinii_. High stringency washes were at 48°C and filters were exposed overnight to radiographic film at −80°C with intensifying screens. Each amplification and detection were repeated in two separate experiments.

Results
A total of five BAL samples were analysed by DNA amplification, two from case 1 (taken before and after 6 days of treatment with nebulised pentamidine), two from case 2 (taken before and after 17 days after the start of treatment with nebulised pentamidine), and one from case 3 (taken before treatment). Two different methods of detection of the amplification product were used—visualisation by ethidium bromide staining after electrophoretic separation on an agarose gel and the more sensitive technique of oligohybridisation. _P. carinii_ specific amplification product was detected in the samples from all three patients by oligohybridisation and a faint band was seen on the ethidium bromide stained gel in the first sample from case 2, taken before the start of treatment. A reduction in the amount of amplification product in the second sample from both case 1 and case 2 was observed. This change may have been a result of the effect of treatment on parasite load.

Discussion
DNA amplification using _P. carinii_ specific oligonucleotide primers is a highly specific and sensitive tool for the diagnosis of _P. carinii_ pneumonia from bronchoscopic alveolar lavage and induced sputum. In granulomatous _P. carinii_ pneumonia most organisms reside within a palisade of histocytes within the interstitium and are not accessible to bronchoscopic alveolar lavage. However, small numbers of parasites, undetectable by silver staining, may be present in the alveolar spaces. Using the highly sensitive technique of DNA amplification with the polymerase chain reaction, we detected _P. carinii_ DNA in bronchoscopic alveolar lavage samples from three patients with granulomatous _P. carinii_ pneumonia, although the amount of amplification product in these samples was significantly lower than that in samples from patients with typical presentation of the disease. These levels of _P. carinii_ specific amplification product, detectable only by oligohybridisation, indicate that relatively small numbers of organisms are present in the sample.

When interpreting the significance of these levels of _P. carinii_ specific amplification product, detected only by oligohybridisation which is at least 100-fold more sensitive than ethidium bromide staining of the agarose gel, it is important to consider the site of origin of the sample. Because in oropharyngeal samples very few organisms move from the alveolar space and are carried by the mucociliary escalator to the bronchi and trachea where they are expectorated, a positive _P. carinii_ amplification product, detected by oligohybridisation alone, is consistent with acute
Pneumocystis pneumonia. In samples of bronchoscopic lavage fluid we have already shown high levels of amplification product readily detected by ethidium bromide stained gel from patients with P. carinii pneumonia, and that low levels of amplification product represent asymptomatic, subclinical colonisation. The data in this study also suggest that in immunocompromised patients with signs of pneumonia, the presence of detectable but low levels of P. carinii DNA in bronchoscopic alveolar lavage can occur with atypical granulomatous P. carinii pneumonia.

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