Papers

Simple detection of the 5S ribosomal RNA of *Pneumocystis carinii* using in situ hybridisation

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Abstract

Aims—To investigate the effectiveness of digoxigenin incorporated double stranded DNA probes produced by the polymerase chain reaction (PCR), for the detection of *Pneumocystis carinii* using in situ hybridisation (ISH).

Methods—Formalin fixed, paraffin wax embedded sections of 26 human lung samples from 11 patients with *P carinii* pneumonia (PCP), and 15 with various types of fungal and viral pneumonia, were obtained during necropsy or transbronchial lung biopsy. Three additional PCP induced rat lung samples were also tested. PCR probes were prepared using the digoxigenin labelling mixture, and they were amplified from the DNA of a PCP induced rat lung after administration of dexamethasone, on the basis that 5S ribosomal RNA sequences are identical in human and rat *P carinii*. ISH was performed using this probe, and visualised using the digoxigenin nucleic acid detection kit. An immunohistochemical study using anti-human *Pneumocystis* monoclonal antibody was also carried out in parallel.

Results—ISH positively stained eight (of eight) lung necropsy specimens from patients with PCP, three (of three) transbronchial lung biopsy specimens from patients with PCP, and none of the three PCP induced rat lung specimens. In contrast, none of the specimens from patients with pneumonia caused by *Aspergillus* sp (n = 5), *Candida* sp (n = 4), *Cryptococcus* sp (n = 2), mucormycosis (n = 2), or cytomegalovirus (n = 2) were positive on ISH or immunohistochemistry.

Conclusions—Using a digoxigenin labelled PCR probe for the entire 5S rRNA sequence in conjunction with conventional staining, ISH is highly reactive and specific for the diagnosis of PCP.

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Keywords: *Pneumocystis carinii*, in situ hybridisation, PCR probe.

Pneumocystis carinii pneumonia (PCP) is one of the most dangerous complications in those who are immunosuppressed, including patients with AIDS. Nearly 60% of such patients

sustain a PCP attack. PCP must be diagnosed rapidly, because there is currently an 80% probability of recovery from a single episode for patients with AIDS.¹ Bronchoalveolar lavage, with or without a transbronchial lung biopsy specimen, as well as induced sputum, provide important diagnostic material.

Both Grocott's methenamine silver nitrate stain and toluidine blue O stain are used to visualise *P carinii* cysts in clinical samples. In contrast, Giemsa stain stains trophozoites and internal sporozoites, but leaves cyst walls unstained with a clear halo. These conventional stains are non-specific and require careful evaluation. More recently, monoclonal antibodies directed against *P carinii* have become available, permitting the immunohistochemical staining of both cysts and trophozoites.²⁻⁴

Due to developments in DNA technology, in situ detection of micro-organisms, especially viral agents, has been successfully used in recent years.⁵ Several papers have reported in situ detection of *P carinii* organisms.⁶⁻⁸ Previous in situ hybridisation (ISH) studies used a mixture of three biotin labelled antisense oligonucleotides as probes for the organism.^{7,8} Biotin labelling of oligonucleotides requires some technical skill, and the signal intensity is usually weak when used as a single probe.

Preparation of a PCR probe is rather simple if an appropriate set of primer pairs for amplification of the target is available.⁵ We selected the 5S ribosomal RNA (rRNA) of *P carinii* as the target sequence, because *P carinii* 5S ribosomal sequences are identical in humans and rats.⁹ The entire sequence is 120 base pairs long and its whole sequence can be amplified easily by standard PCR; it is also the appropriate size for tissue permeability. Furthermore, rRNA is an optimal target for ISH, because rRNA abounds in individual cells.

To date, *P carinii* has not been cultivated efficiently in vitro.¹⁰ Thus we used a PCP induced rat lung as the starting material for the preparation of digoxigenin labelled probe. Our method has the potential for widespread use as a routine test.

Methods

HISTOLOGY SPECIMENS

Eight formalin fixed, paraffin wax embedded lung tissue samples from patients with PCP,

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three transbronchial lung biopsy samples from patients who had had a bronchoscopy, and three *P. carinii* infected rat lung tissue samples were tested. Haematoxylin and eosin staining, and conventional Grocott's methenamine silver nitrate staining, were carried out on all the samples. These sections were reviewed, and the diagnosis of PCP confirmed by two of the authors (HS and YO).

For controls, formalin fixed, paraffin wax embedded necropsy samples from patients with *Aspergillus* pneumonia (n = 5), *Candida* pneumonia (n = 4), *Cryptococcus* pneumonia (n = 2), pulmonary mucormycosis (n = 2), and cytomegalovirus (CMV) pneumonia (n = 2) were examined in parallel.

PREPARATION OF SLIDES

Sections (4 µm) of formalin fixed, paraffin wax embedded tissue samples were mounted on glass slides precoated with 3-aminopropyl-methoxysilane (Dako, Japan). They were dewaxed in xylene, washed with absolute ethanol, and dried in air. Slides were then heated at 95°C for 30 minutes in target retrieval solution (Dako, Carpinteria, USA), to expose their rRNA. Before hybridisation, they were rehydrated in 50 mM Tris buffered saline, pH 7.6 (TBS).

PCR PROBES

Kitada *et al* showed that 5S ribosomal sequences are identical in human and rat *P. carinii* infection.⁹ PCP was induced in three Sprague-Dawley rats by administration of dexamethasone twice a week for six weeks, as described before.⁴ These animals were killed and their lungs were removed and stored at -70°C until used. We amplified the whole sequence of PC 5S rRNA, a 120 base pair PCR product, from a PCP induced rat lung. DNA was extracted from stored *P. carinii* infected rat lung tissue. Briefly, minced rat lung tissue was frozen in liquid nitrogen and vortexed with 6 mm diameter glass beads several times. Then DNA was extracted with phenol/chloroform.

This PCR probe encompasses the entire sequence of the 5S rRNA, which was sequenced by Watanabe *et al*.¹¹ The upper primer was 5' AGT TAC GGC CAT ACC TCA AG 3', and the lower primer was 5' AAA GCT ACA GCA CGT CGT AT 3'. To prepare the digoxigenin labelled PCR probe, a two step PCR was carried out using a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, USA) as follows. The first round PCR amplification reaction was performed for 30 cycles in a 100 µl solution containing 10 mM Tris-HCl at pH 8.3, 50 mM KCl, and 1.5 mM MgCl₂ with 100 pmol of both primers, and 2.5 U TaKaRa Taq DNA polymerase (Takara Shuzo Co, Tokyo, Japan). Denaturation was performed at 94°C for 1.5 minutes, annealing was performed at 55°C for 2.5 minutes, and extension was performed at 72°C for 1.9 minutes. Second round PCR reactions were carried out in the same manner using digoxigenin DNA labelling mixture (Boehringer Mannheim GmbH, Germany) instead of dNTP solution for the incorporation of multiple digoxigenin molecules.

As a control probe, we prepared an irrelevant PCR probe based on the 5S ribosomal RNA sequence of *Aspergillus flavus*.¹² The upper primer was 5' ACA TAC GAC CAT AGG GTG TG 3', and the lower primer was 5' ACA TAC AAC AGG AGG GAT TC 3'. DNA was extracted from mycelial mats cultured in Sabouraud's agar medium.¹³ The amplified product encompassing the whole 5S ribosomal sequence was 121 base pairs long. Thermal cycling conditions used were 35 cycles at 94°C for 30 seconds, at 56°C for 45 seconds, and at 72°C for two minutes.

ISH

The PCR probe was dissolved in 6 × SSC containing 5 × Denhardt's solution and 10% dextran sulphate to an appropriate dilution, and applied to the glass slides. The slides were covered with a glass coverslip and heated at 95°C for three minutes, then kept at room temperature for one minute, and incubated at 40°C for 10 minutes. They were washed in six times with SSC at 37°C for 10 minutes, then in twice with SSC at room temperature for 30 minutes. Digoxigenin labelled hybrids were detected with anti-digoxigenin-alkaline phosphatase conjugated antibody (Boehringer Mannheim GmbH, Germany) diluted 1 in 500 for several hours, and signals were visualised by incubation overnight with a chromogen solution containing nitroblue tetrazolium salt (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). The tissues were finally washed with distilled water and mounted with Crystal mount (Biomed, Foster City, USA).

In order to confirm the specificity of ISH, negative controls including: prior RNase treatment; use of an irrelevant probe (PCR probe encompassing 5S rDNA of *Aspergillus flavus*); and no probe, were analysed. RNase was purchased from Sigma Chemical Co. (St Louis, Missouri, USA) and diluted to 50 µg/ml in solution. Mounted sections were treated with the solution at 37°C for 30 minutes.

IMMUNOHISTOCHEMISTRY

Anti-human *Pneumocystis* mouse monoclonal antibody was purchased from Dako (Glostrup, Denmark). Dewaxed tissue sections were immersed in methanol containing 0.3% hydrogen peroxide to block endoperoxidase activities. The sections were incubated with a 1 in 40 dilution of antibody for 30 minutes at room temperature, washed three times in TBS buffer for five minutes, and stained by the streptavidin-biotin method using the Histofine SA-PO (MO) kit (Nichirei, Tokyo, Japan) according to the manufacturer's instructions. Sections were visualised by incubation with diaminobenzidine and hydrogen peroxide as the substrate solution. The slides were subsequently counterstained with methyl green, dehydrated, cleared and mounted with Eukit (O Kindler, Germany).

Results

ISH

Dark purple reaction products indicating the presence of *P. carinii* organisms were observed in all of the lung tissue specimens from patients

diagnosed with PCP. ISH seems to highlight not only the cyst forms but also the trophozoites. In the necropsy tissue specimens numerous *P carinii* organisms filled the alveolar space, as shown in a typical photomicrograph (fig 1A). When compared with Grocott's methenamine silver nitrate stain (fig 1B), both cysts and trophozoites were easily seen. *P carinii* organisms were also stained in the tissue sections from patients with PCP obtained by transbronchial lung biopsy and in those from *P carinii* infected rat lung (figs 2A and 3A). Grocott's methenamine silver nitrate stain stained *P carinii* organisms black in the corresponding areas (figs 2B and 3B). Clusters of ring-shaped organisms stained brown, demonstrating the localisation of antigen on the surface of *P carinii*, were seen on consecutive slides in the necropsy lung and transbronchial lung biopsy tissue of patients with PCP (figs 1C and 2C). However, *P carinii* organisms could not be detected in *P carinii* infected rat lung tissues by immunohistochemistry (fig 3C).

Prior treatment with RNase (50 µg/ml) at 37°C for 30 minutes completely abolished the positive reaction (fig 1D). Likewise, when slides were reacted with either an irrelevant probe derived from *Aspergillus flavus*, or a hybridisation solution lacking the probe, no reaction products were detected (figs 1E and 1F). ISH was carried out on consecutive sections in an identical manner. ISH studies showed that *P carinii* was present in all the necropsy lung tissue samples, transbronchial lung biopsy samples, and *P carinii* infected rat lung tissue samples. In contrast, immunohistochemical studies produced rings of organisms stained brown, demonstrating cell surface localisation of the *P carinii* antigen, in eight out of eight necropsy samples, in three out of three transbronchial lung biopsy samples, and in none of the three *P carinii* infected rat lung tissue samples. These results are shown in table 1. *P carinii* organisms of human origin were recognised by both ISH and immunohistochemistry (figs 1A and 1C). *P carinii* organisms were even identi-

Figure 1 ISH for detection of *P carinii* in necropsied lung tissue. *P carinii* organisms were stained a dark purple colour in the alveolar space (A). Grocott's methenamine silver nitrate stain showed black stained scattered *P carinii* cysts (B). Immunohistochemistry using anti-human *Pneumocystis* monoclonal antibody resulted in brown-coloured ring-like staining, corresponding to *P carinii* organisms (C). Previous treatment with RNase (50 µg/ml) abolished the positive reaction in ISH (D). No reaction products were detectable when the slides were reacted with an irrelevant probe: digoxigenin labelled PCR probe against *Aspergillus flavus* (E), or when the hybridisation solution contained no PCR probe (F).

fied by ISH in the small tissue samples obtained from transbronchial lung biopsies (figs 2A and 2C), as well as the relatively large necropsy lung tissue samples. However, *P. carinii* of rat origin were recognised by ISH, but not by immunohistochemistry (figs 3A and 3C).

In other human tissues containing various fungal and viral infections, no positively stained products were seen with ISH or immunohistochemistry. Necropsy lung tissue sections from patients with *Aspergillus* pneumonia (n = 5), *Candida* pneumonia (n = 4), pulmonary cryptococcosis (n = 2), pulmonary mucormycosis (n = 2), and CMV pneumonia (n = 2) all gave negative results.

Figure 2 ISH showed *P. carinii* organisms in the transbronchial lung biopsy tissue from a patient with adult T cell leukaemia/lymphoma complicated by PCP (A). Grocott's methenamine silver nitrate stain showed *P. carinii* cysts in the same area (B). Immunohistochemistry showed *P. carinii* organisms in a consecutive section (C).

Table 1 Reactivity of in situ hybridisation (ISH) and monoclonal antibody based immunohistochemistry (IHC)

Pathogen	No. positive	ISH positive	IHC positive
<i>Pneumocystis carinii</i>			
Necropsy	8	8	8
Transbronchial lung biopsy	3	3	3
(Infected rat lung)	(3)	(3)	(0)
<i>Aspergillus</i>	5	0	0
<i>Candida</i>	4	0	0
<i>Cryptococcus</i>	2	0	0
Mucor	2	0	0
CMV	2	0	0

Discussion

Molecular diagnosis has been made possible by the development of ISH and PCR techniques. In the early phase of ISH cloned fragments of complementary or genomic DNA were used in DNA-DNA or DNA-RNA hybridisation after incorporating label by nick translation. Owing to recent advances in DNA synthesis technology, oligonucleotide sequences complementary to target DNA or RNA can be easily synthesised, labelled, and used as probes, but their use in conjunction with non-isotopic detection systems has been limited. This is due to a lack

Figure 3 ISH showed *P. carinii* organisms in the alveolar septa of *P. carinii* infected rat lung tissue, without alveolar involvement (A). Grocott's methenamine silver nitrate staining revealed *P. carinii* cysts in the same area (B). However, immunohistochemistry showed nothing (C).

of sensitivity which results from the low amounts of label in these short sequences. To use a synthetic oligonucleotide successfully in non-isotopic methods, probe cocktails containing several sequences are required.¹⁴

Previous studies used either biotin labelled cloned DNA fragments derived from rat *P. carinii*⁶ or biotin labelled probe cocktails for ISH.⁷⁻⁸ Montone *et al* had a different approach; they elegantly synthesised terminally biotin labelled oligonucleotide probes with six biotin moieties, based on the 5S rRNA sequence, and successfully detected *Aspergillus* species in formalin or Bouin's fixed, paraffin wax embedded tissue specimens using ISH.¹⁵ Preparing this kind of probe, however, is not easy. With the advent of PCR technology, amplifying and labelling single stranded and double stranded DNA probes can be done relatively easily, without using bacterial culture with plasmids, insert preparation, and conventional labelling protocols.⁵⁻¹⁴

We have shown the superiority of digoxigenin labelled PCR probes, which can contain many digoxigenin molecules and be synthesised easily. This resulted in high signal intensity in our ISH study. Our results also seem to confirm the specificity of the ISH method for use in the histopathological diagnosis of PCP, and we have clearly shown that ISH is comparable with immunohistochemistry without any reduction in specificity and sensitivity. Although production of monoclonal antibodies requires skills, time, and labour, PCR probes can be easily prepared even from unpurified material, if DNA or RNA sequences are known. Consequently, our ISH method is ideal for the detection of *P. carinii* organisms, and could be used in the detection of other micro-organisms in formalin fixed, paraffin wax embedded human tissue sections.

Another advantage of the present method over immunohistochemistry using monoclonal antibodies is that it can also be applied to animal infection studies, as we and other groups have shown.⁶⁻⁸ Kitada *et al* directly sequenced PCR products which were amplified from material from patients, mice, and rats.⁹ They

found that the deduced sequences were all identical, suggesting that the *P. carinii* 5S rRNA sequence is conserved in these hosts. These advantages will clearly make it easier to diagnose PCP histopathologically using ISH in any general laboratory environment.

- 1 Hopkin JM. *Pneumocystis carinii*. Oxford: Oxford University Press, 1991:65-6.
- 2 Kovacs JA, Ng VL, Masur H, Leoung G, Hadley WK, Evans G, *et al*. Diagnosis of *Pneumocystis carinii* pneumonia: improved detection in sputum with use of monoclonal antibodies. *N Engl J Med* 1988;318:589-93.
- 3 Linder E, Lundin H, Vorm H. Detection of *Pneumocystis carinii* in lung derived samples using monoclonal antibody to a 82 kDa parasite antigen. *J Immunol Methods* 1987;98:57-62.
- 4 Kobayashi M, Moriki T, Uemura Y, Takehara N, Kubonishi I, Taguchi H, *et al*. Immunohistochemical detection of *Pneumocystis carinii* in transbronchial lung biopsy specimens: Antigen difference between human and rat *Pneumocystis carinii*. *Jpn J Clin Oncol* 1992;22:387-92.
- 5 Finckh U, Lingenfelter PA, Henne KW, Schmidt CA, Siegert W, Myerson D. In vitro amplification and digoxigenin labeling of single stranded and double stranded DNA probes for diagnostic in situ hybridization. In: Rolfs A, Schuller I, *et al*, eds. *PCR: clinical diagnostics and research*. Berlin: Springer Verlag, 1992:A37-43.
- 6 Wakefield AE, Hopkin JM, Burns J, Hipkiss JB, Stewart TJ, Moxon ER. Cloning DNA from *Pneumocystis carinii*. *J Infect Dis* 1988;158:859-61.
- 7 Edman JC, Kovacs JA, Masur H, Santi DV, Elwood HJ, Sogin ML. Ribosomal RNA sequence shows *Pneumocystis carinii* to be a member of fungi. *Nature* 1988;334:519-22.
- 8 Hayashi Y, Watanabe J, Nakata K, Fukayama M, Ikeda H. A novel diagnostic method of *Pneumocystis carinii*: In situ hybridization of ribosomal ribonucleic acid with biotinylated oligonucleotide probes. *Lab Invest* 1990;63:576-80.
- 9 Kitada K, Oka S, Kimura S, Shimada K, Serikawa T, Yamada J, *et al*. Detection of *Pneumocystis carinii* sequences by polymerase chain reaction: animal models and clinical application to non-invasive specimens. *J Clin Microbiol* 1991;29:1985-90.
- 10 Armstrong MYK, Cushion MT. In vitro cultivation. In: Walzer PD, ed. *Pneumocystis carinii pneumonia*. 2nd edn. New York: Marcel Dekker Inc, 1994:3-24.
- 11 Watanabe J, Hori H, Tanabe K, Nakamura Y. Phylogenetic association of *Pneumocystis carinii* with the 'rhizopoda/myxomycota/zygomycota group' indicated by comparison of 5S ribosomal RNA sequences. *Mol Biochem Parasitol* 1989;32:163-8.
- 12 Chen M-W, Anne J, Volckert G, Huysmans E, Vandenberghe A, De Wachter R. The nucleotide sequences of the 5S rRNAs of seven molds and a yeast and their use in studying ascomycete phylogeny. *Nucleic Acids Res* 1984;12:4881-92.
- 13 Tang CM, Cohen J, Holden W. An *Aspergillus fumigatus* alkaline protease mutant constructed by gene disruption is deficient in extracellular activity. *Mol Microbiol* 1992;6:1663-71.
- 14 Wilkinson DG. The theory and practice of in situ hybridization. In: Wilkinson DG, ed. *In situ hybridization: a practical approach*. Oxford: Oxford University Press, 1992:61-73.
- 15 Montone KT, Litzky LA. Rapid method for detection of *Aspergillus* 5S ribosomal RNA using a genus specific oligonucleotide probe. *Am J Clin Pathol* 1995;103:48-51.