Evaluation of Nested Polymerase Chain Reaction for Detecting Mycobacterial DNA in Pleural Fluid

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Abstract

A protocol based on the polymerase chain reaction (PCR) is the most sensitive method for detecting mycobacteria in clinical samples. However, few studies have assessed the usefulness of this method in the diagnosis of tuberculous effusion. We developed a highly sensitive and specific nested PCR method, that amplifies the bovine tuberculous MPB70 gene and the mycobacterial 16S rRNA gene for use in detecting *Mycobacterium tuberculosis* (*M. tuberculosis*) and mycobacteria, respectively, in clinical samples. We determined the sensitivity of this method for detecting mycobacteria in samples containing known amounts of mycobacterial DNA and in DNA extracted from pleural effusions obtained from 10 patients with pulmonary tuberculosis in whom standard microbiological techniques had detected mycobacteria in sputum but not in pleural effusion. The nested PCR method for the bovine tuberculous MPB70 gene and the mycobacterial 16S RNA gene was able to detect *M. tuberculosis* and mycobacterial genomes only if there were at least 2 copies per sample. Positive results for *M. tuberculosis* and the mycobacterial genomes were obtained by nested PCR in 2 of 10 and in 3 of 10 samples of pleural fluid, respectively but no mycobacteria were detected in malignant effusions obtained from 9 patients with lung cancer. The nested PCR method represents a rapid means for detecting mycobacteria in some pleural effusions previously found to be negative by culture. We speculate that the reaction of the host against mycobacteria is more important than the mycobacteria themselves in the pathogenesis of pleural effusion in which mycobacteria are not detected.

Introduction

Attempts at direct identification of acid-fast staining are usually unrewarding. Culture pleural fluid has a specificity approaching 100%, but the growth of mycobacteria *in vitro* entails a delay of 3 to 6 weeks, and gives negative results in a substantial number of cases. The polymerase chain reaction (PCR), a new technique for *in vitro* amplification of specific DNA segments^{1)~3)}, has been used to diagnose mycobacterial infection directly by detecting DNA of mycobacteria in clinical specimens^{4)~10)}. However, a few studies have evaluated the usefulness of PCR in the diagnosis of

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mycobacterial pleurisy when cultures were negative^{11)~15)}. We have already reported 2 PCR methods that amplify the bovine tuberculous MPB70 gene and the mycobacterial 16S rRNA gene, for detection of *M. tuberculosis* and mycobacteria, respectively, in sputum¹⁶⁾. In this study, we improved our methods and developed nested PCR, which is a two step amplification, first with outer primers and then with inner primers nested within the first primers, attempting to increase both the sensitivity and the specificity of the PCR¹⁷⁾. This study sought to determine the sensitivity of nested PCR for the detection of mycobacterium and its utility in the diagnosing of mycobacterial pleurisy when culture had failed to detect mycobacteria.

Materials and Methods

Case Material

Samples of pleural fluid from 10 patients with pulmonary tuberculosis and 9 patients with lung cancer were studied. The diagnosis of pulmonary tuberculosis was established by sputum culture for mycobacteria in all 10 cases, and that of lung cancer was established by the cytology of pleural effusion or by the histology of pleural biopsy specimens. Mycobacterial culture failed to detect mycobacteria in any pleural effusion. Two different samples of pleural fluid from each patient were studied by the nested PCR method.

DNA extraction

One milliliter of pleural effusion was mixed with 1 ml of distilled water and centrifuged at 10,000 g for 5 min; the aqueous phase was discarded. This step was repeated twice. The samples were heated for 30 min at 70°C and incubated for 15 min at 37°C with lysozyme, 10 mg/ml. The samples were homogenized with glass beads by vortexing and addition of 900 μ l of TE (10 mM Tris HCl, 1 mM EDTA) buffer, and incubated at 37°C overnight. After the addition of 100 μ l of 20% SDS in 0.2 N NaOH, this mixture was treated with phenol/CHCl₃/isoamylalcohol (25 : 24 : 1). After ethanol precipitation, the DNA was dried and dissolved in 100 μ l of distilled water.

PCR amplification

The nucleotide sequences of the primers are shown in Table 1. These primer pairs amplified the 560 bp fragment of the mycobacterial 16S rRNA gene and the 332 bp fragment of the bovine tuberculous MPB70 gene. Outer primers are the same as those used in conventional PCR methods which we described previously¹⁶⁾. Five microliters of heat-denatured DNA sample was added to a solution containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 200 μ M each of dNTPs, 0.2 μ M each of outer primers and 1.25 units of Amplitaq DNA polymerase (Perkin-Elmer Cetus Co., Ltd). The total volume of each tube was 50 μ l. The contents of the tube were mixed gently, covered with 30 μ l of mineral oil and subjected to 34 cycles of 1 min denaturation at 94°C, 1

Table 1 Sequences of oligonucleotides used in this study

Oligonucleotide	Sequence (5'-3')
Mycobacterial 16S rRNA gene amplification primers	
Outer primers	AGAGTTTGATCCTGGCTCAG GCCGTGAGATTTACGAACA
Inner primers	TAACAAATGCAAGTCGAACG GCCGTGAGATTTACGAACA
Tuberculous MPB 70 gene amplification primers	
Outer primers	GAACAATCCGGAGTTGACAA AGCACGCTGTCAATCATGTA
Inner primers	CGCTGACGGCTGCACTGTCTG CACCGTCGCTTGGCGGTAG

min to allow annealing at 62°C, and 1 min of extension at 72°C. During the last cycle, the extension time was increased by 7 min. After the first PCR, 2 μ l of the product was amplified for 20 cycles with the corresponding inner primers. The product of the second PCR was analyzed by 2% agarose gel electrophoresis with ethidium bromide staining. A sample was considered to be positive if a single band corresponding to an amplified product of the appropriate size was seen in the gel.

Results

The 2-step PCR with nested primers was more sensitive than the conventional PCR for detecting mycobacterial DNA sequences in serially diluted DNA samples (Fig. 1). Nested PCR was at least 10 times more sensitive than conventional PCR, and could detect 0.01 pg (two copies) of genomic DNA for *M. tuberculosis* and mycobacteria. Because of the increased sensitivity, we were able to use simple agarose gel electrophoresis and ethidium bromide staining.

All the pleural fluid specimens obtained from patients with pulmonary tuberculosis and lung cancer were negative for mycobacteria on direct microscopic examination and culture. The results of PCR and the representative clinical data of these patients are shown in Table 2. A patient was considered positive if 1 of 2 different samples gave a positive result. All samples were negative by conventional PCR. However, by nested PCR, 3 of the 10 patients with pulmonary tuberculosis were positive for 16S rRNA PCR, and 2 of these 3 were also positive for MPB70 PCR. The concentrations of lactate dehydrogenase and adenosine deaminase in pleural fluid were higher in the 2 patients who

Fig. 1 Comparison of conventional and nested PCR. The amount of serially diluted *M. tuberculosis* DNA was 10^4 , 10^3 , 10^2 , 10^1 , 1 and 0 fg. (A) Conventional PCR (1) and nested PCR (2) for the 16S rRNA gene. The expected sizes of the products were 600 and 560 base pairs, respectively. (B) Conventional PCR (1) and nested PCR (2) for the MPB 70 gene. The expected sizes of the products were 372 and 332 base pairs, respectively.

Table 2 Results of 16S rRNA PCR and MPB70 PCR and clinical data in 10 patients with pulmonary tuberculosis

Case	Results of rRNA PCR		Results of MPB70 PCR		Concentration in pleural effusion	
	conv	nested	conv	nested	LDH (IU/L)	ADA (IU/L)
1	0/2	1/2	0/2	1/2	8,760	138
2	0/2	1/2	0/2	1/2	2,220	110
3	0/2	1/2	0/2	0/2	995	30
4	0/2	0/2	0/2	0/2	285	38
5	0/2	0/2	0/2	0/2	905	78
6	0/2	0/2	0/2	0/2	480	43
7	0/2	0/2	0/2	0/2	258	42
8	0/2	0/2	0/2	0/2	1,180	57
9	0/2	0/2	0/2	0/2	822	62
10	0/2	0/2	0/2	0/2	509	17

Abbreviations: LDH, lactate dehydrogenase; ADA, adenosine deaminase; conv, conventional PCR; nested, nested PCR

were positive by MPB70 PCR than in the patients who were negative by that PCR. In samples from patients with lung cancer, no band was detected by PCR for either the MPB70 gene or the 16S rRNA gene.

Discussion

We previously described 2 PCR methods¹⁶⁾ that amplify the bovine tuberculous MPB 70 gene and the mycobacterial 16S rRNA gene for use in detecting *M. tuberculosis* and mycobacteria, in sputum, respectively. This combination of PCR abilities was available to differentiate *M. tuberculosis* from nontuberculosis mycobacteria. In the previous study, we investigated the correlation between results obtained by the conventional PCR and bacteriological methods in 311 samples of sputum from cases of suspected mycobacteriosis. The PCR method detected 12 cases of infection with *M. tuberculosis* and 4 cases of infection with atypical mycobacteria in 17 specimens that were positive by bacteriological methods, but failed to do so in one case. Among the 294 specimens that were found to be negative by bacteriological methods, the PCR method detected 13 and 8 cases of and atypical mycobacteria, respectively. These results were confirmed by use of commercial DNA probes or by investigation of the clinical background of the patients. The PCR method did not detect mycobacterial DNA in 197 samples of sputum from normal subjects not suspected of having mycobacteriosis. These results indicated that our previous methods of PCR are highly specific for *M. tuberculosis* and mycobacteria, respectively, and are also sensitive enough to detect small numbers of copies of mycobacterial DNA in sputum, which could not be detected by bacteriological methods. In this study, we extended our previous method of PCR and developed a more sensitive and specific PCR protocol, i.e., nested PCR, in which the outer primers were the same as those used in previous PCR methods. Figure 1 shows that nested PCR for both the rRNA and MPB70 genes was at least 10 times more sensitive than conventional PCR and could detect as few as 2 copies of mycobacterial DNA. therefore, the sensitivity of nested PCR in this study is at least 10 times more sensitive than that of the previous study. Using this very sensitive method, we investigated pleural fluids in which bacteriological methods did not detect mycobacteria for the presence of mycobacterial DNA, and evaluated the usefulness of nested PCR.

Table 2 shows that although conventional PCR did not yield positive results, nested PCR showed

3 patients to be positive for 16S rRNA, and that 2 of these 3 patients were also positive for MPB70. In each of these 2 patients, 1 of 2 different samples yielded a positive result by both MPB70 and 16S rRNA PCR, while the other sample gave a negative result with both methods of PCR. We propose 2 reasons for the discrepant results in 2 samples from the same patient, and for the low prevalence (20%) of *M. tuberculosis* DNA in pleural fluids from patients with pulmonary tuberculosis that was definitely diagnosed by sputum culture. The first reason is that most of the DNA in pleural effusion was already degraded and inappropriate for PCR. The second is that the reaction of the host against mycobacteria may be more important than the mycobacteria themselves in the pathogenesis of tuberculous pleuritis.

Using conventional PCR and Southern blot hybridization, Pao et al.¹¹⁾ found *M. tuberculosis* in 8 of 37 (22%) samples of pleural effusion, while mycobacterial culture failed to detect any mycobacteria in the samples. All the specimens they tested had been collected from patients with clinical signs of tuberculosis, including characteristic radiographs or typical clinical manifestations of the disease, or a positive history of exposure to tuberculosis, an actual history of tuberculosis, and/or clinical response to anti-tuberculosis chemotherapy. In the present study, the diagnosis of pulmonary tuberculosis were all confirmed by sputum culture. Although there may be some differences between this study and that of Pao et al. in the reliability of clinical diagnosis of pulmonary tuberculosis, we demonstrated almost the same rate of positivity.

Nested PCR showed that 1 of the 3 patients who were positive for 16S rRNA was negative for MPB70. Mycobacteria other than *M. tuberculosis* may be present in pleural effusion obtained from patients with pulmonary tuberculosis or the number of copies of *M. tuberculosis* DNA was too low for MPB70 PCR. It is unlikely that the positive results were caused by contamination, because negative controls run with each batch of samples failed to detect any contaminating DNA, and the results of PCR for 20 specimens from patients with lung cancer, including 8 specimens from 1 patient that were tested twice, were all negative.

In summary, we have shown that mycobacteria can be detected in pleural effusion by PCR carried out with 4 sets of nested primers and by simple visualization in agarose gels. For samples of pleural effusion, PCR analysis should be repeated or carried out on separate samples from the same individual, because the number of copies of mycobacterial DNA present is usually very low if the culture is negative. The sensitivity of this method may be improved by increasing the amount of pleural effusion from which DNA is extracted. We speculate that the reaction of the host against mycobacteria may be more important than the mycobacteria themselves in the pathogenesis of tuberculous pleuritis.

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胸水中抗酸菌 DNA の検出における nested polymerase chain reaction の有用性の検討

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要 旨

臨床検体における抗酸菌の検出においては polymerase chain reaction が最も鋭敏な検査である。しかし、結核性胸水の診断における PCR の有用性についての報告は少ない。我々は、bovine tuberculous MPB70 gene と mycobacterial 16S rRNA gene を、鋭敏で特異的な nested PCR 法を用いて増幅し、臨床検体における結核菌と抗酸菌の検出に用いた。まず、既知の量の抗酸菌の DNA を用いて nested PCR の検出感度を検討した結果、bovine tuberculous MPB70 gene, mycobacterial 16S gene 共に、少なくとも 2 コピーあれば検出可能であった。さらに、喀痰培養にて結核と診断した症例のうち、胸水の培養で菌

が検出されなかった 10 症例の胸水より抽出した DNA を用いて臨床的有用性について検討した。その結果 nested ではない通常の PCR は全く検出できないのに対して、nested PCR によって、mycobacterial 16S rRNA は 10 例中 3 例、bovine tuberculous MPB70 gene は 10 例中 2 例に検出された。また、肺癌の胸水にはいずれの PCR によっても全く検出されなかった。したがって、nested PCR は、培養が陰性であっても、胸水中の抗酸菌を迅速に検出することが可能である方法と思われる。また、結核性胸膜炎においては、結核菌自体の関与より、これに対する反応の方が重要である可能性が示唆された。