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Detection of Mycobacterial DNA Using Nested Polymerase Chain Reaction of Pleural Biopsy Specimens: Compared to Pathologic Findings

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Background : Although there are many methods including AFB smear and culture, and the analysis of pleural fluid in the etiological diagnosis of pleural effusion, it is sometimes difficult to confirm a diagnosis especially in cases of incomplete pleural biopsies. Moreover, the high incidence of tuberculous pleuritis in young people caused confusion in the differential diagnosis of pleural effusion in Korea. The pathognomonic finding of tuberculous pleuritis in pleural biopsy is chronic granulomatous pleuritis (CGP) with caseous necrosis. But a biopsy does not always provide a definitive diagnosis, which shows in only 60~70% of all biopsies, because of either limitations in blind biopsies or inadequate specimens. An adequate biopsy also gives only limited information, such as chronic or nonspecific pleuritis.

Methods : We compared the clinical diagnosis, pathologic findings and detection of mycobacterial DNA using nested PCR of pleural biopsy tissues. We carried out the nested PCR for IS*6110* insertion sequence of *Mycobacterium tuberculosis* using outer primer IS-1/IS-2 (5'-AGGCGTTGGTTCGCGAGGG-3' / 5'-TGATGACGCCCTCGTTGCC-3') and inner primer IS-3/IS-4 (5'-CCAACCCGCTCGGTCTCAA-3' / 5'-ACCGATGGACTGGTCACCC-3') in 52 pleural biopsy tissues which were pathologically diagnosed as tuberculous pleuritis, malignant pleuritis or non-specific pleuritis.

Results : Five (71.4%) of 7 cases clinically and pathologically confirmed tuberculous pleuritis diagnosed as chronic granulomatous pleuritis (CGP) with caseous necrosis revealed positive in nested PCR for *M. tuberculosis*. Seven (36.8%) of 19 cases diagnosed as CGP without caseous necrosis were positive. However, only 3 (25%) of 12 cases diagnosed as non-specific chronic pleuritis were positive by PCR for *M. tuberculosis*. Neither congestive heart failure nor malignancies with pleurisy showed a positive reaction.

Conclusion : In this study, pathologic findings were significantly associated with the detection rate of mycobacterial DNA. And, even in patients with nonspecific or chronic inflammatory pleuritis, mycobacterial DNA could be detected by using nested PCR in pleural biopsy tissue with good specificity. Detection of mycobacterial DNA in pleural tissue might provide additional information for etiological diagnosis in patients with pleural effusion.

Key Words : Tuberculosis, Pleural biopsy, PCR

INTRODUCTION

Pleuritis is one of the most common forms of extrapulmonary tuberculosis. Tuberculous pleuritis can be diagnosed by clinical findings, rhoentgenography of chest, Mantoux test and analysis of pleural fluid, and confirmed only by pleural biopsy or

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mycobacterial stain and culture of pleural fluid[1]. However, a pleural biopsy does not always provide a definitive diagnosis because of a difficult biopsy technique, inadequate specimens and/or limited information, such as chronic or nonspecific pleuritis[2-4].

Recently, polymerase chain reaction (PCR) of mycobacterial DNA in pleural effusion has been used with variable sensitivity, 25~80% in the diagnosis of tuberculous pleural effusion[5-8]. We carried out the nested PCR in pleural biopsy tissue to compare the clinical diagnosis, the pathologic findings and the detection of mycobacterial DNA using nested PCR.

The purposes of this study are first, understanding the diagnosis and differential diagnosis of tuberculous pleuritis from other causes of pleural effusion, especially in cases of an inadequate pathologic diagnosis, such as chronic non-specific pleuritis; second, comparing the pathologic findings to the PCR results and other laboratory findings of pleural fluid; third, studying the correlation between the pathologic findings and the PCR results with or without radiological parenchymal infiltrations.

MATERIALS AND METHODS

Study population and pleural biopsy

Tissues were obtained from 52 patients who underwent diagnostic pleural biopsies with Cope needle from Jan 1994 through Dec 1995 at Seoul Paik Hospital, Inje University. Five slices of 7 μm-thick paraffin-embedded tissue specimens were taken from each pleural biopsy specimen. The blade of the microtome and the toothpick were changed every time to avoid cross-contamination of samples. The sections for PCR were collected in 1.5 mL Eppendorf tubes. Deparaffinization was done with xylene and octane 3 times. The study subjects were divided into 3 groups: subjects with chronic granulomatous pleuritis (CGP) with caseous necrosis (group A), CGD without caseous necrosis (group B) and subjects with chronic or nonspecific pleuritis (group C).

Control Subjects

Tissue was obtained from three patients who had congestive heart failure (group D) and eleven patients who had pleural effusion due to malignant tumors (group E), such as lung cancer, mesothelioma, mesenchymal tumor and metastatic lymphoma.

DNA Preparation

The modified technique for isolation of mycobacterial DNA described by Patel et. al.[9] was done. Briefly, the deparaffinized pellet was resuspended in a 200 μL digestion buffer, 500 μL of 1 M TRIS-HCl (pH=8) containing 100 mM EDTA, 150 mM NaCl, and 200 μg/mL of protease K (Sigma Chemical Co., St. Louis, MO) and then we disaggregated the tissue with a 23 gauge syringe.

Next, we incubated it for 1 hour at 55℃ and overnight at 37℃. Then we spun the tubes briefly and removed any liquid. We incubated it at 95℃ for 10 min to inactivate protease. Then the nucleic acid was purified by phenol/ chloroform extraction and precipitated with isopropanol. The precipitate was redissolved, digested with 4 U/mL RNase. DNA was re-extracted with phenol/chloroform and precipitated with isopropanol repeatedly 3 times. DNA precipitates were dissolved in 10 mM TRIS-HCl (pH=8).

Amplification of mycobacterial DNA

Oligonucleotides were synthesized using a DNA synthesizer (Perceptive) and purified by ethanol precipitation. The sequences for oligonucleotides used in this study are summarized in Table 1. The primers were selected by using the oligomer program to amplify a fragment of the IS*6110* insertion element present in the *M. tuberculosis* complex. DNA amplification was performed as previously described. All reaction contained 10 mM TRIS-HCl (pH 8.3, Sigma), 50 mM KCl, 100 μg/mL gelatin, 25 pmol each oligonuclotide primer, 0.3 mM each dNTP (Pharmacia), 25 to 50 U/mL Taq polymerase (Bioneer).

Detection of mycobacterial DNA

The presence of amplified mycobacterial DNA sequences was detected as previously described[10-15]. Briefly, aliquots of the amplification products were electrophoresed into 2% agarose gels. The gels were stained with ethidium bromide and illuminated with ultraviolet light and the presence of visible amplification products was detected by Polaroid camera. The positive control for detection of Mycobacterial DNA was H37Rv strain of *M. tuberculosis*, and negative control was *Mycobacterium avium*.

Radiologic findings

Chest PA and both lateral decubitus radiographs were obtained from all individuals. Two radiologists and one pulmonologist read the radiographs in a random manner by the diagnostic guideline of pulmonary tuberculosis according

Table 1. The sequences of primer pairs used in this study

outer primer	IS-1	5'-AGGCGTTGGTTCGCGAGGG-3'
	IS-2	5'-TGATGACGCCCTCGTTGCC-3'
inner primer	IS-3	5'-CCAACCCGCTCGGTCTCAA-3'
	IS-4	5'-ACCGATGGACTGGTCACCC-3'

to the Korean National Tuberculous Association criteria without clinical information. Pulmonary tuberculosis was considered when 2 or more investigators interpreted apical parenchymal infiltrations without a mention of activity.

Measurement of biochemical parameters

Blood and pleural fluid were obtained from all individuals at the same time as the pleural biopsy. Adenosine deaminase (ADA) was determined enzymetically using an analyzer (U/L, Hitach 7150); CA125 was measured directly by radioimmunoassay (U/mL, Cobra RIA/QC 5005, 5010). Total protein of serum and pleural fluid were measured by Biurep method (g/dL, Olympus A5400).

Statistical Analysis

The correlation between the pathologic findings, the pleural fluid analysis, the PCR result of mycobacterial DNA and the various clinicoradiologic findings were analysed for statistical significance by the *t*-test, 2 by 2 χ^2-test. The statistical software program used was Statistical Package for the Social Sciences (SPSS/PC+ 10.0, Chicago, IL). A *p*-value of less than 0.05 was accepted as statistically significant.

RESULTS

Correlation between nested PCR and the pathologic findings

Fifteen (39.5%) of 38 specimens revealed positive in nested PCR for IS*6110* insertion sequence of *M. tuberculosis*. Positive control was the H37Rv strain of *M. tuberculosis* (H37) and negative control was *M. avium* (MA) (Figure 1). Five (71.4%) of 7 cases, clinically and pathologically confirmed tuberculous pleuritis diagnosed as chronic granulomatous pleuritis (CGP) with caseous necrosis, revealed positive in nested PCR for IS*6110* insertion sequence of *M. tuberculosis*. Seven (36.8%) of 19 cases diagnosed as CGP without caseous necrosis were positive. However, three (25%) of 12 cases diagnosed as chronic or nonspecific pleuritis were positive in PCR for *M. tuberculosis*. None of congestive heart failure and malignancies-induced pleurisy showed a positive reaction (Table 2).

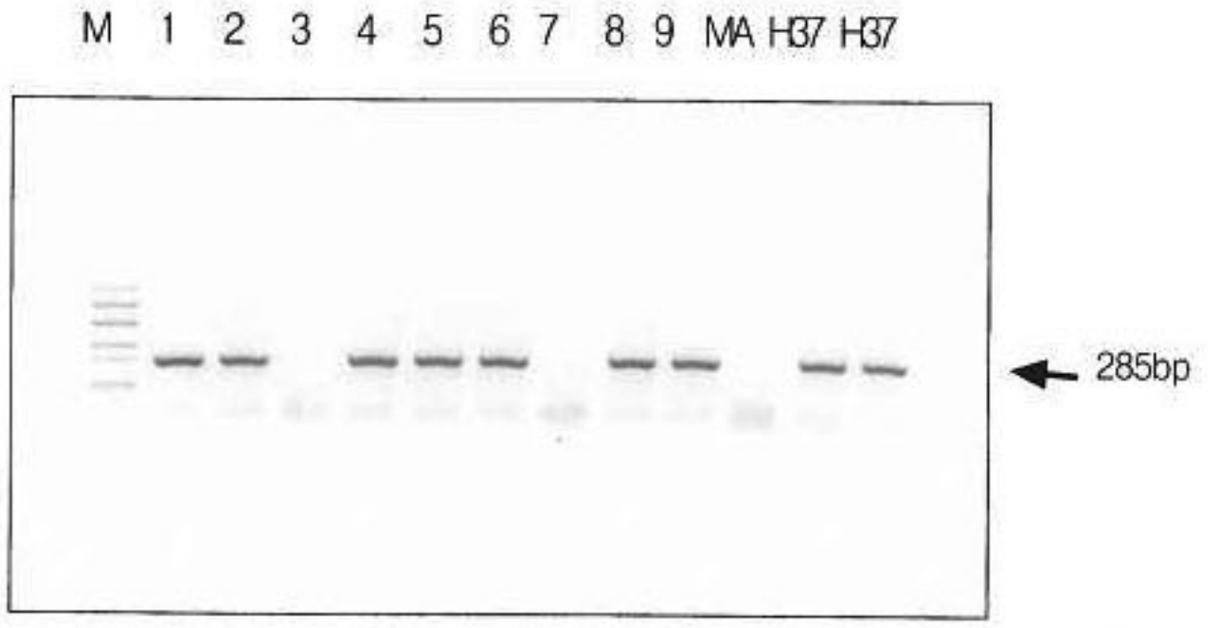

Figure 1. Polymerase chain reaction amplification of mycobacterial DNA in pleural tissues. Positive control was the H37Rv strain of *M. tuberculosis* (H37) and negative control was *M. avium* (MA). M; 100 bp DNA molecular weight marker (New England BioLabs, Inc. USA). All specimens were positive except 3rd, 7th and MA lane.

Table 2. Demographic data according to pathologic groups of study population

Group	Group A	Group B	Group C	Group D	#Group E
Pathologic Finding	CGP with caseation	CGP without caseation	Chronic or non-specific pleuritis	Congestive Heart Failure	Malignant diseases
Number of Patients (Male)	7 (5)	19 (9)	12 (11)	3 (3)	11 (5)
Age (years)	37.0±17.2	37.0±17.2	43.3±15.3	59.3±15.0	54.3±12.0
Positive Results in Nested-PCR (%)	5 (71.4)	7 (36.8)	3 (15.8)	0	0
Clinical Diagnosis	TP*	TP*	TP**	Congestive heart failure	Malignancies*

* TP, tuberculous pleurisy.

** Tentative clinical diagnosis with exclusion of other etiologies, therapeutic trial with anti-tuberculous medications and clinical follow-up.

Malignant diseases were lung cancer (7 cases), mesothelioma (2 cases), mesenchymal tumor (1 case) and lymphoma (1 case).

Correlation between positive result in nested PCR and the radiologic findings

When the parenchymal infiltrations in simple chest X-ray suspected of active or inactive pulmonary tuberculosis were combined, positive PCR reaction was higher than without radiological parechymal infiltraitons ($p<0.05$) (Table 3). This finding suggests that the pathogenesis of tuberculous pleurisy is more likely the rupture of a subpleural focus of tubercle bacilli into the pleural space than an immunologic response to mycobacterial antigen exposure. However, a negative PCR result was not correlated with negative radiological infiltrations. Because of the small number of patients in each group, we could not find any correlation between positive or negative PCR reaction and radiological infiltrations in each group.

Table 3. Correlation between PCR results and radiological infiltrations in tuberculous pleurisy including group A, B and C

	PCR (+)	PCR (-)
No. of patients with RI* (%)	13 (86.7%)	9 (39.1%)
No. of patients without RI* (%)	2 (13.3%)	14 (60.9%)

*RI, Radiological parenchymal infiltrations which indicate active or inactive pulmonary tuberculosis from a simple chest X-ray.

Relationship between the pathologic findings and the laboratory findings of pleural fluid

Ratio of pleural fluid and serum of CA125 level was higher in groups A and B than in groups C, D, E. However, it is not statistically significant (Table 4). Many of the laboratory analysis of pleural fluid did not provide statistically significant information for differential diagnosis of tuberculous pleuritis except the serum and pleural fluid ADA level ($p<0.05$ between Group B & E) (Table 4).

DISCUSSION

Tuberculous pleuritis is thought to result from the rupture of a subpleural focus of tubercle bacilli into the pleural space and a resultant local cell-mediated immune response to mycobacterial antigens[1-3]. The IS*6110* insertion sequence was more sensitive than the protocol used to detect the gene coding for the 65 kD mycobacterial antigen sequences because the IS*6110* is present in 10~15 copies in each mycobacterial genome, whereas the gene coding for the 65kD antigen is probably present in only a single copy[8, 16]. Many studies have shown that the primer has different sensitivity and specificity for the detection of mycobacterial DNA. We used the primer set for 285 bp in IS6110, which showed generally acceptable sensitivity and specificity because the yield of PCR in tissue paraffin block might be low[17, 18].

The sensitivity of the nested PCR in chronic granulomatous pleuritis with or without caseation would be increased with fresh pleural tissue and large amount of tissue. Ghossein *et al.* & Popper HH *et al.* insisted that mycobacteria might be missed in the usual 4-6 μm section[19, 20]. In this study, to increase sensitivity, we used 7 slices of 7 μm thick paraffin embedded tissue in each test.

Although many of the laboratory findings of pleural fluid are used in the diagnosis of pleural effusion, none of them provides a definitive diagnosis, except pleural biopsy. Our data also showed that lots of laboratory findings of pleural fluid, including CA125, protein, glucose and lymphocyte count, did not provide definitive clues to differential diagnosis, except

Table 4. Comparison between pathologic findings and laboratory findings of pleural fluid

	Group A	Group B	Group C	Group D	Group E
B-CA125	95.4±41.3	160.7±191.4	157.6±170.8	351.9±95.9	196.0±174.6
P-CA125	448.1±115.9	468.0±139.9	323.3±146.2	396.5±169.6	409.4±144.8
P/B CA125	5.8±2.0	5.4±2.8	3.2±2.3	1.1±0.2	3.4±2.3
B-ADA*	29.5±12.7	19.8±5.9	29.4±23.5	10.8±4.9	15.6±3.9
P-ADA*	61.4±21.3	64.3±16.3	30.9±29.6	4.1±2.8	19.9±7.3
P/B-ADA*	2.3±1.1	3.5±1.2	1.4±1.3	0.4±0.2	1.3±0.3
B-Protein	6.8±0.9	7.0±1.0	6.9±1.0	6.9±0.2	7.0±0.5
P-Protein	4.9±1.6	6.1±2.7	6.3±2.4	3.2±1.1	5.1±1.8
P/B-Protein	0.7±0.2	0.9±0.3	0.9±0.3	0.5±0.2	0.7±0.2
RBC	2486±1518	8327±2143	12683±2584	5436±2624	107484±42183

* Statistically significant ($p<0.05$) between Group B & E.

B, Blood; P, Pleural fluid; ADA, Adenosine deaminase (U/L, enzymetry, Hitach 7150); CA125 (U/mL, Radioimmunoassay, Cobra RIA/QC 5005, 5010).

Neutro, Neutrophil; Lympho, lymphocyte.

the ADA level between chronic granulomatous pleuritis without caseous necrosis and malignancy induced pleural effusion.

Even in patients with nonspecific or chronic inflammatory pleuritis (Group C), mycobacterial DNA was detected using nested PCR in pleural biopsy tissue. The reason why the sensitivity in Group C was quite low (25%) suggests that first, the effusion was not caused by tuberculosis even though clinically diagnosed by exclusion and a therapeutic trial; second, the pleural effusion responded to only local cell mediated immunologic reaction; third, an inadequate biopsy was performed or an incorrect process might have been done during a procedure; last, Group C has lesser content of the mycobacterial genome than Group A or B.

In this study, pleural effusion combined pulmonary infiltration which was suspected tuberculosis, was well correlated with a positive PCR reaction of mycobacterial DNA. This means that a cell-mediated immune response to a mycobacterial antigen-associated pleural effusion might have few tuberculous bacilli in the pleural tissue and resulted in a negative PCR reaction.

To avoid a false-positive diagnosis of tuberculosis, it may be necessary to quantify the amount of target DNA according to the type of specimens. Using pleural tissue is probably more specific than other specimens, such as sputum, because false positive were reported as less than 1%[21]. However, in control group E of this study, which was associated with malignancies, mycobacterial DNA was not detected at all. This result will be helpful to make a differential diagnosis with tuberculous pleurisy and malignant pleural effusion in elderly patients.

CONCLUSIONS

In this study, the pathologic findings are significantly associated with the detection rate of mycobacterial DNA. The nested PCR for *Mycobacterium tuberculosis* with pleural biopsy can be used as a rapid diagnostic or differential diagnostic method in not only chronic granulomatous pleuritis without caseous necrosis but also in patients with chronic or non-specific pleuritis.

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