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Clinical evaluation of rapid detection of *Mycobacterium tuberculosis* using a polymerase chain reaction method

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ABSTRACT

The polymerase chain reaction (PCR) for identification of *Mycobacterium tuberculosis* in uncultured clinical specimens was evaluated prospectively. Two oligonuleotide primers were derived from the nucleotide sequence of IS 6110, which amplified a 123-bp sequence found in members of the *M. tuberculosis* complex. The detection limit of *M. tuberculosis* DNA was 10 fg by gel electrophoresis and ethidium bromide staining after a forty cycle amplification. A total of 100 clinical specimens (from 70 patients) were investigated using the PCR method. Twenty-one samples were found to be positive and seventy-nine were negative. Compared with the conventional culture method, sensitivity and specificity of the PCR method's were 94.0% and 94.1%, respectively. Cases with PCR repeatedly positive revealed definite tuberculosis. Nineteen of the positive samples were from active culture positive tuberculosis cases, and one from a patient with improved condition after anti-tuberculosis treatment. Seventy-nine negative samples were from various respiratory diseases, including old inactive tuberculosis or active tuberculosis which had already been treated with anti-tuberculosis drugs. In this study, PCR results could be obtained within two days. The PCR method was found to be rapid, sensitive, and reliable test for clinical consideration. *Ryukyu Med. J., (3)127~131, 1995*

Key words: Polymerase chain reaction, *Mycobacterium tuberculosis*, clinical diagnosis

INTRODUCTION

Tuberculosis is a chronic infectious disease caused by mycobacteria of the tuberculosis complex, mainly *Mycobacterium tuberculosis*[1]. Despite great progress in treatment and control measures, tuberculosis is still a serious medical problem world-wide. There are at least 8 million new cases and about three million tuberculosis related deaths a year[2]. During the past few years, there has been an upsurge in the number of new cases reported, a fact that is thought to be partially related to the association of tuberculosis with the acquired immunodeficiency syndrome[3].

Diagnosis of tuberculosis is a long and tedious process: Identification and antibiotic sensitivity testing may last several weeks because of the slow growth rate of mycobacteria. Although direct microscopy is rapid and convenient, it is complicated by the fact that samples usually contain only a few organisms. Consequently, treatment may be delayed, or inappropriate empiric therapy for *M. tuberculosis* may be given to patients without mycobacterial infections or to patients who are infected with atypical mycobacteria that are unresponsive to the usual treatment for *M. tuberculosis*. However a probe system for identification of several species of mycobacteria in culture is commercially available, no direct probe for detecting mycobacteria in clinical specimens is currently available[4].

The need for a sensitive, reliable, and rapid test for the laboratory diagnosis of tuberculosis has long been acknowledged. DNA amplification by polymerase chain reaction (PCR) has already been used for diagnosis of genetic diseases and infections[5], and also holds great potential to become a rapid and sensitive method for detection of *M. tuberculosis* complex directly in clinical specimens.

MATERIALS AND METHODS

DNA extraction of bacterial strains

Suspensions of clinical isolates of *M. tuberculosis*, *M. bovis*, *M. avium*, *M. kansasii*, *M. intracellulare* and *M. fortuitum* were prepared in 0.5 McFarland standard. Mycobacterial DNA was extracted by a slightly modified version of Marmur's method[6]. Briefly, lysozyme (Wako Pure Chemicals, Osaka, Japan) was added to the mycobacterial suspensions and incubated at 37℃ for 10 min. Glass beads were added to the suspension and then vortexed

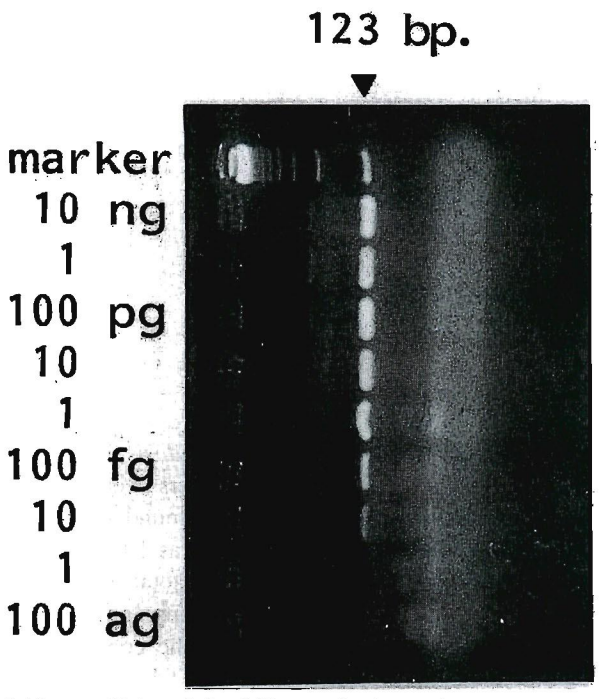

Fig.1 The sensitivity of the PCR examination evaluated with purified chromosomal DNA from *Mycobacterium tuberculosis*, a clinical isolate. lane 1; 100 ag, lane 2; 1 fg, lane 3; 10 fg, lane 4; 100 fg, lane 5; 1 pg, lane 6; 10 pg, lane 7; 100 pg, lane 8; 1 ug, lane 9; 10 ug of purified DNA. M; 123 bp ladder marker.

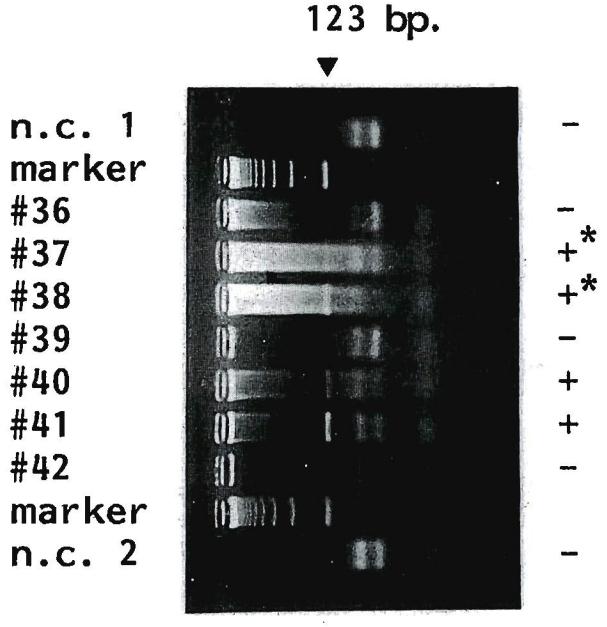

Fig.2 Representative results of PCR examination with clinical specimens. On the left side of the figure the specimen numbers are shown, on the right side the results of PCR are shown; (+) expresses PCR positive and (−) expresses PCR negative. Results with asterics were confirmed by PCR exam. of a 10-fold dilution of the original samples.

for 2 min. Sodium dodecyl sulfate (SDS; final concentration 1% w/v) was added, and reincubated at 60℃ for 10 min. After the incubation, mycobacterial DNA was extracted by phenol, followed by ethanol precipitation. The precipitated DNA was lysed in saline EDTA, digested with proteinase K (10 mg/l; Wako) and RNase I (Wako). The suspension was once extracted by phenol, precipitated by ethanol, and then washed with 70% cold ethanol. The residue was dried, and dissolved in a appropriate volume of TE buffer (10 mM Tris HCl [pH 8.0] and 1 mM EDTA). The concentration and the purity of the DNA suspension were determined by measuring the optical density at 260 nm and 280 nm, respectively. The suspension was used to evaluate the sensitivity and specificity of the PCR.

Sample preparation from clinical specimens

A total of 100 clinical samples from 70 inpatients with symptoms of respiratory malfunctioning and/or abnormal findings in their chest roentgenograms were tested. Prior to extraction, 1 ml of the sputa and bronchial washing were lysed with the same volume of dithiothreitol (20 mM; BRL Life Technologies, Inc., Geithusberg, MA), incubated at 70℃ for 20 min, and centrifuged at 7300 g for 10 min. The pellet was suspended in 900 μL of TE buffer. Clinical samples other than sputa and bronchial washing were mixed with the same volume of TE buffer, and treated as follows: Glass beads (2g) and 100 μL of lysozyme (10 mg/ml) were added to each sample, incubated in a sonicator at 37℃ for 45 min, and later vortexed for 1 min. Proteinase K was added to the suspension and then incubated at 55℃ for 45 min. 20% SDS-phenol mixture was added to the suspension and shaken for 30 min. After centrifugation at 7300 g for 10 min, the supernatants were again treated with phenol and ethanol. Precipitated DNAs were harvested by centrifugation, washed with 80% cold ethanol, dried, and resuspended in 50 μL of distilled water. 10 μL of the DNA suspension was used for PCR examination.

Oligonucleotide primers

In this PCR study, a primer pair was used whose specificity had been determined by Eisenach *et al*[7]. The oligonucleotides IS1 5'-CCTGCGAGCGTAGGCGTCGG-3' and IS2 5'-CTCGTCCAGCGCCGCTTCGG-3', which were derived from the nucleotide sequence of IS6110, amplified a 123 bp sequence found in members of *M. tuberculosis* complex.

PCR

The reaction mixture consisted of 10 mM Tris hydrochloride (pH 8.3), 50 mM potassium chloride, 1.5 mM magnesium chloride, 0.01% gelatin, 0.01% Triton X-100, 200 μM of each dNTP, 0.4 μM of each primer and 2.5 U of AmpliTaq polymerase (Perkin Elmer-Cetus, Nowalk). Mycobacterial DNA or clinical specimen DNA were subjected to a 30 cycle amplification reaction. Each cycle consisted of three phases: 94℃ for 1 min, 68℃ for 2 min

Table 1 Comparative results of polymerase chain reaction and conventional examination for *Mycobacterium tuberculosis*

Specimen (n)	PCR results	smear (+) culture (+)	smear (−) culture (+)	smear (+) culture (−)	smear (−) culture (−)
Sputum	+	9	5	0	2
(52)	−	1	0	1 [b]	34
Bronchial	+	0	2	0	2
washing (35)	−	0	0	0	31
Pleural fluid	+	0	0	0	0
(5)	−	0	1	0	4
Gastric juice	+	0	0	0	0
(3)	−	0	0	0	3
Bone marrow	+	0	0	0	1 [c]
aspirate (2)	−	0	0	0	1
Others [a]	+	0	0	0	0
(3)	−	0	0	0	3

a. Cerebrospinal fluid, urine and ascites.
b. *M.intracellulare* was isolated.
c. Pathological examination suggested tuberculous granuloma.

Table 2 Results of polymerase chain reaction and clinical diagnosis

Diagnosis	PCR		positive rate (%)
	positive	negative	
Bacteriologically confirmed tuberculosis			
active	10	1	91
inactive (post-treatment)	0	4	0
Clinically compatible tuberculosis			
active	1	0	
inactive	0	3	25
Non-tuberculous respiratory diseases	1	49	2

Table 3 Comparative results of repeated PCR examinations and the culture method

Cases with PCR positive	Culture method		positive predictive value (%)
	positive	negative	
Repeated test	5	0	100
Single test	5	2	71

and 72℃ for 2 min. Negative controls were included in each amplification experiment. Ten percent of the reaction products was applied to electrophoresis in a 6% agarose gel. The gels were stained with ethidium bromide, and photographed under a 305 nm UV transilluminator. Samples were considered to be *M. tuberculosis* complex positive when a single band of 123 bp was present. When shaggy bands appeared on electrophoresis, a 10-fold dilution of the samples was subjected to an amplification reaction in order to verify the result.

RESULTS AND DISCUSSION

The DNA amplification with the primer pair we selected, was specifically for *M. tuberculosis* complex (data not shown), as described by Eisenach *et al*[7]. Sensitivity testing using a serial dilution of purified mycobacterial DNA showed that this PCR assay could detect 10 fg of the mycobacterial DNA (Fig.1), corresponding to about three mycobacterial genome equivalents[8]. Therefore we concluded that this PCR assay was specific and sensitive

enough for clincal application. Many different mycobacterial genes have been proposed as targets for the detection of *M. tuberculosis* in clinical samples[7,9-13], however, the optimal target DNA has yet to be defined and the relation between positive identification of *M. tuberculosis* DNA and the clinical disease is uncertain. Several research groups have used the primer pair derived from IS6110 DNA and concluded that IS6110 DNA detection is superior to some other PCR assay systems in the detection of *M. tuberculosis*[14,15]. Insertion sequence (IS) elements are present as multiple repeats in mycobacterium genome[16], which enhances the sensitivity of this PCR method.

We evaluated this PCR method using 100 clinical specimens (Table 1, Fig.2). These specimens were tested in parallel by conventional methods, direct microscopic examination (Ziehl-Neelsen staining), and culture (1% and 3% Ogawa agar). PCR results were compared with the culture results (Table 1) and the former's specificity and sensitivity was 17/18 (94.1%) and 77/82 (94.0%), respectively. Positive predictive value was 16/21 (76.2%) and negative predictive value was 78/79 (98.6%). We also compared the PCR results with the clinical status of the cases from which the samples were taken (Table 2). PCR positive results were obtained in 12 cases. Ten cases were definite tuberculosis cases and culture positive. One case showed culture negative, though anti-tuberculosis treatment improved the clinical course of the patient. The final positive case turned out to be pneumonia caused by *Streptococcus pneumoniae*. A cephem antibiotic improved the symptoms of the patient, at a result the PCR tended to be positive. It was difficult to differentiate the existance of a few contaminated mycobacteria during the tests.

In five cases, we performed repeated PCR examinations and the cases which tested positive more than twice were all definite culture tuberculosis positive (Table 3). Though this is a preliminary study, it is worth noting that for diagnosing definite tuberculosis cases, repeated PCR positive tests may be more reliable than a single PCR positive test.

The PCR negative cases were seen in various respiratory diseases, including old inactive tuberculosis and active tuberculosis cases which had been treated with anti-tuberculosis drugs. One sample of pleural effusion with culture positive tuberculous pleuritis showed false negative. PCR examination of samples other than sputum would provide better opportunities for processing the specimens.

There have been many reports describing PCR method for the diagnosis of tuberculosis[17-20]. We evaluated the efficacy of repeated PCR examination of the same patient for the detection of *M. tuberculosis* infection.

In summary, our results suggest that the amplification of the IS6110 may represented a specific, sensitive and rapid means for detecting *M. tuberculosis*. Repeated PCR examination may virtually ensure the reliability.

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