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Diagnosis and Species Identification of Mycobacterial Infections by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism Analysis of Sterile Body Fluids

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Background/Aims: The development of effective, accurate, and rapid diagnostic methods for *Mycobacterium* infection and mycobacterial species identification is required. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) is an easy, rapid and inexpensive technique for identifying *Mycobacterium* spp.
Methods: We performed PCR-RFLP to detect and identify *Mycobacterium* spp. from 10 sterile body fluids, including ascites, cerebrospinal fluid, pleural fluid, synovial fluid, and peritoneal dialysis fluid. Clinical samples were collected from patients with diagnoses of definite, probable or suspected mycobacterial infection. The conserved RNA polymerase genes of *Mycobacterium* spp. were amplified by PCR.
Results: The amplified 360-bp region of *rpoB* was digested with the restriction enzyme *Msp*I or *Hae*III. The PCR-RFLP results for the clinical samples were identical to those for *M. tuberculosis, M. fortuitum, M. intracellulare*, and *M. avium*. In addition, the results of the PCR-RFLP were identical to those obtained by DNA sequencing.
Conclusions: PCR-RFLP analysis of sterile body fluids may be a useful method for the diagnosis of mycobacterial infections and for the differentiation of mycobacterial species. **(Korean J Intern Med 2009;24:135-138)**

Keywords: *Mycobacterium* infections; Polymerase chain reaction; Restriction fragment length polymorphism

INTRODUCTION

Identification of *Mycobacterium* spp. in clinical specimens is based primarily on culturing and biochemical tests. These conventional tests take several weeks and sometimes fail to provide precise identification [1]. Mycobacterial identification at the species level is a critical step in patient management, as the results obtained influence decisions on both the optimal therapy and the need for patient isolation. Therefore, the development of effective diagnostic methods that allow accurate and rapid diagnosis of mycobacterial infections and differentiation among species are urgently required. Although molecular diagnostic methods, high-performance liquid chromatography, and DNA sequence analysis have been investigated, these methods require substantial effort and are difficult to implement in the clinical setting [2]. Molecular biological tests using DNA amplification and probe hybridization have also been developed. Using these methods, the diagnosis and differentiation of bacterial species can be rapidly accomplished. However, the high costs associated with these methods make them less attractive in practice [2-7].

In contrast to these techniques, polymerase chain

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Table 1. Results of the culture, biopsy, PCR-RFLP, and sequencing analyses of sterile body fluids from patients

Clinical diagnosis	Specimens	Culture	Biopsy	PCR-RFLP	Sequencing
Definite TB	Synovial fluid	*M. tuberculosis*	Not done	*M. tuberculosis*	*M. tuberculosis*
NTMI	Pleural fluid	Atypical mycobacteria	Not done	*M. avium*	*M. avium*
Probable TB	Pleural fluid	No growth	Granulomatous inflammation	*M. tuberculosis*	*M. tuberculosis*
Suspected TB	Cerebrospinal fluid	No growth	Not done	*M. tuberculosis*	*M. tuberculosis*
NTMI	Peritoneal dialysis fluid	*M. fortuitum*	Not done	*M. fortuitum*	*M. fortuitum*
NTMI	Cerebrospinal fluid	*M. avium*	Not done	*M. avium*	*M. avium*
NTMI	Pleural fluid	Atypical mycobacteria	Granulomatous inflammation	*M. intracellulare*	*M. intracellulare*
Probable TB	Ascites	No growth	Granulomatous inflammation	*M. tuberculosis*	*M. tuberculosis*
Suspected TB	Pleural fluid	No growth	Not done	*M. tuberculosis*	*M. tuberculosis*
NTMI	Synovial fluid	Atypical mycobacteria	Not done	*M. fortuitum*	*M. fortuitum*

TB, tuberculosis; NTMI, non-tuberculous mycobacterial infection.

reaction-restriction fragment length polymorphism (PCR-RFLP) offers an easy, rapid, and inexpensive way to identify *Mycobacterium* spp [7,8]. Therefore, we attempted to diagnose mycobacterial infections at the early clinical stages and to differentiate between *Mycobacterium* species using PCR-RFLP.

METHODS

In the present study, we performed PCR-RFLP to detect and identify *Mycobacterium* spp. in sterile body fluids, including ascites, cerebrospinal fluid, pleural fluid, synovial fluid, and continuous ambulatory peritoneal dialysis (CAPD) fluid. Clinical samples were collected from patients who were diagnosed with mycobacterial infections. A definite tuberculosis (TB) case was defined as a positive result for *M. tuberculosis* on culture testing. A probable TB case was defined as a positive result in an acid-fast bacilli (AFB) smear, chronic granulomatous inflammation seen on histopathology, and/or other positive findings consistent with TB (*i.e.*, a high level of adenosine deaminase or a positive TB PCR result for appropriate samples). A suspected TB case was defined as having typical TB findings by radiology or clinical features of TB, and showing improvement after treatment. A non-tuberculous mycobacterial infection (NTMI) was defined as repeatedly positive results for non-tuberculous mycobacteria and clinical features associated with mycobacterial infection.

Between May, 2001 and July, 2003, sterile specimens from synovial fluid (n=2), CAPD fluid (n=1), ascites (n=1), pleural fluid (n=4), and cerebrospinal fluid (n=2) were obtained. The clinical diagnoses of those patients were: definite TB (n=1); probable TB (n=3); suspected TB (n=1); and NTMI (n=5).

An aliquot (5-20 mL) of each specimen was centrifuged at 14,000 rpm for 5 min and the supernatant was used for bacterial DNA extraction. Bacterial genomic DNA was extracted from clinical specimens using the DNeasy Tissue Kit (Qiagen, Hilden, Germany). The 360-bp *rpoB* gene was amplified by PCR using the following primers: forward, 5'-TCAAGGAGAAGCGCTACGA-3'; and reverse, 5'-GGATGTTGATCAGGGTCTGC-3'. The PCR consisted of 35 cycles of preheating at 94˚C for 5 minutes, denaturation at 94˚C for 1 minute, annealing at 60˚C for 1 minute, and extension at 72˚C for 1 minute, followed by a final extension step of 72˚C for 7 minutes. The 360-bp amplified product of *rpoB* was detected by 2.5% agarose gel electrophoresis; the gel samples included a 100-bp DNA ladder, a negative control (distilled water), and a positive control (360-bp amplified product of *rpoB* of *M. tuberculosis*). The amplified products were digested separately with *Msp*I (Boehringer Mannheim Biochemicals, Mannheim, Germany) and *Hae*III (Boehringer Mannheim Biochemicals) at 37˚C for 2 hours. The digested products

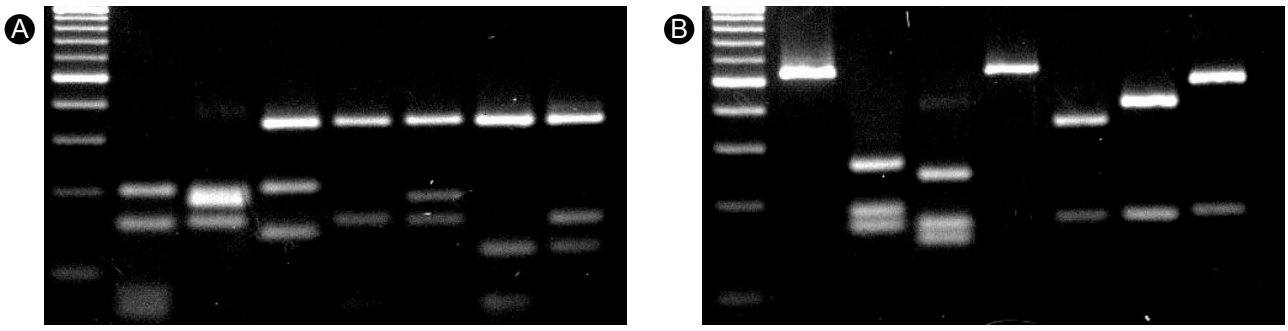

Figure 1. PCR-RFLP analyses of mycobacterial species. (A) PCR-RFLP patterns with *Msp*I. (B) PCR-RFLP patterns with *Hae*III. Lane 1, DNA size marker (50-bp ladder); lane 2, *M. avium*; lane 3, *M. chelonae*; lane 4, *M. fortuitum*; lane 5, *M. gordonae*; lane 6, *M. intracellulare*; lane 7, *M. kansasii*; lane 8, *M. tuberculosis*.

were separated by 4% agarose gel electrophoresis at 50 V for 2 hours; the gel samples included a 50-bp DNA ladder, a negative control (distilled water), and the digested products of *M. avium*, *M. chelonae*, *M. fortuitum*, M. gordonae, *M. intracellulare*, *M. kansasii*, and *M. tuberculosis* (Fig. 1). Reference strains that had been identified by conventional biochemical testing and *rpoB* sequencing were provided by the Department of Microbiology, Yonsei University College of Medicine [9]. For each specimen, the results of the culture tests, histologic tests, and clinical diagnoses were compared with the results of the PCR-RFLP analysis.

The DNA sequence of the PCR-amplified *rpoB* gene was analyzed using an *rpoB* primer and a DNA sequence analyzer. Species identification by DNA sequence analysis was compared with that achieved by PCR-RFLP.

RESULTS

PCR-RFLP results using 10 clinical samples such as ascites, cerebrospinal fluid, pleural fluid, synovial fluid, and CAPD fluid from subjects who were suspected of having mycobacterial infections were identical to the PCR-RFLP results of *M. tuberculosis*, *M. fortuitum*, *M. intracellulare* or *M. avium* (Table 1). PCR-RFLP results using the three culture-proven samples coincided with the results of culture test including *M. tuberculosis*, *M. fortuitum* and *M. avium*. In the other seven samples that were not identified by culture, the PCR-RFLP results provided the precise identification of each *Mycobacterium* spp. All of 2 probable and 2 suspected TB cases showed PCR-RFLP results consistent with *M. tuberculosis*. PCR-RFLP results of 3 NTMI cases known partially as atypical mycobacterium by culture test were consistent with *M. avium*, *M. intracellulare* or *M. fortuitum* respectively. In all cases the results of PCR-RFLP were also identical with those from sequencing.

DISCUSSION

Currently, laboratory diagnosis of *Mycobacterium* spp. infection is made primarily using smear tests and culturing. The advantage of the smear test is that the results can be obtained rapidly. However, since mycobacterial concentrations of 5×10^3 to 1×10^4/mL are required for the isolation procedure, the sensitivity of this test is low and the results may vary depending on the examiner [8-11]. It has been reported that 30-50% of culture-positive sputum samples show positive results [8-11]. Although the sensitivity of the *Mycobacterium* spp. culture test is relatively high, it takes 4-8 weeks to obtain the results, which means that treatment may be delayed and the bacteria may be transmitted to other people. Other shortcomings of these tests are that: 1) during the pretreatment process, *Mycobacterium* spp. may be killed; and 2) the pathogen isolation rate is relatively low in treated patients. In recent years, molecular methods for identifying bacteria (*e.g.*, PCR) have been introduced. These methods overcome the shortcomings of the smear and culture tests, while maintaining high levels of sensitivity and specificity [8-11].

In the present study, we examined whether PCR-RFLP using the *rpoB* gene could detect and differentiate between mycobacterial strains present in sterile body fluids. PCR-RFLP was performed on 10 clinical specimens, which included synovial fluid, CAPD fluid, ascites, pleural fluid, and cerebrospinal fluid. All of the patients were diagnosed as having or suspected of having *Mycobacterium* infection, as confirmed by PCR of the *rpoB* gene. With regard to the differentiation of mycobacterial strains, for

some patients who were diagnosed by culture testing, the results of the culture and PCR-RFLP were in agreement. Therefore, the detection and differentiation of strains by PCR-RFLP is believed to be clinically useful. However, as the PCR-RFLP method may produce a high number of false-positives, the overall clinical profile should be considered when assessing the results for patients.

There have been some reports on the use of PCR-RFLP for the diagnosis of mycobacterial infections. Kim et al. [5] reported that by analyzing the *rpoB* gene, 49 mycobacterial strains could be differentiated. In addition, PCR-RFLP analysis of bronchoalveolar lavage specimens enabled early diagnosis of mycobacterial infection, and the mycobacterial strains could be differentiated from each other. Similarly, Kontos et al. [12] reported that *Mycobacterium* spp. infection could be diagnosed effectively by culturing clinical specimens in the Bactec MGIT 960 system, followed by PCR-RFLP analysis. However, to our knowledge, there have been no studies using sterile body fluids to evaluate the usefulness of PCR-RFLP for the diagnosis of patients with mycobacterial infections and mycobacterial species identification.

The present study has some limitations. The number of subjects used was low. Therefore, we could not test the sensitivity, specificity, and predictive values of the assay. Moreover, culture-positive results were obtained for only six patients, and the infecting mycobacterial species was identified in only three patients. We cannot exclude the possibility of contamination by environmental mycobacteria. Furthermore, the false-positive rate of PCR-RFLP may be high. Finally, the positive control used for PCR-RFLP did not contain all the mycobacterial species that could be present in clinical specimens. In spite of these limitations, to our knowledge, this is the first investigation to focus on the usefulness of PCR-RFLP using various sterile body fluids.

In conclusion, PCR-RFLP analysis using sterile body fluids may be a useful method for diagnosing mycobacterial infections and for differentiating among mycobacterial strains. In the present study, the number of specimens and strains was low, which meant that the usefulness of PCR-RFLP analysis could not be compared in a statistically meaningful way with that of culture testing. Nonetheless, further studies on the usefulness of PCR-RFLP using more specimens and species are warranted.

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