

Rapid Identification of *Mycobacterium Leprae* by Polymerase Chain Reaction-restriction Fragment Length Polymorphism Analysis of the Heat Shock Protein 65 Gene from Skin Specimens

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INTRODUCTION

Leprosy caused by *Mycobacterium leprae* (*M. leprae*), is a chronic granulomatous disease affecting the skin and peripheral nervous system, which is transmitted through direct contact with nontreated or inadequate treatment patients.^[1] Diagnosis of leprosy depends on the clinical signs and symptoms and slit skin smear positivity. However, it's sometimes similar with other granulomatous disease caused by mycobacterial infection. Early stage leprosy is difficult to diagnose by clinical criterion alone because the sensitivity of acid-fast bacilli staining is quite low. The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) shows the great advantage in rapid identification and diagnosis for early cases and has a differentiation between leprosy and nonleprosy cases.

METHODS

Patients

Three leprosy patients included in this study visited our hospital between November 2011 and October 2012 [Table 1]. The three patients had typical clinical symptoms presenting from 2 months to 5 years and were proved *M. leprae* infection based on the clinical and histological features and skin smear acid-fast staining.

Primers and restriction enzymes

The nest PCR-RFLP assay was based on a previously published method^[2,3] using 2 outer forward and reverse primers (310 bp) and 2 inner primers (133 bp), and was

designed to detect the common to all mycobacteria in the heat shock protein 65 gene region. Three restriction enzymes (*Hha* I, *Bst*UI, and *Mbo* I) were used in restriction analysis.

DNA extraction

The DNA was extracted from three patients' paraffin-embedded skin biopsy samples using the TaKaRa DEXPAT kit (TaKaRaBio Company, Japan). Sterilized distilled water was used for negative control, while DNA extracted from bacillus Calmette–Guérin was used for positive control.

Nested-polymerase chain reaction

For the first round PCR, 5 µl of purified DNA was added to the PCR mixture (final volume of 50 µl) containing 3 µl of both outer primers (25 pmol/L), 10 µl 5×PCR buffer (including 1.5 mmol/L MgCl₂), 1 µl 10 mmol/L dNTPs (2.5 mmol/L of each dNTP), 1.5 U PromegaGoTaq polymerase, and water to 50 µl. The reaction was performed under the following conditions: Initial denaturation at 94°C for 3 min; 35 cycles at 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s and a final elongation at 72°C for 10 min.

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Table 1: Clinic data of three *Mycobacterium leprae* cases

Patient number	Gender	Age (years)	Residence (province)	Course of disease	Distribution of lesions	Paresthesia	Neural thickening	Acid-fast staining	Diagnosis
1	Female	25	Guangdong	5 years	Face	+	-	-	TT
2	Male	29	Sichuan	2 months	Legs, waist	-	-	+	BB
3	Male	52	Guangdong	5 months	Face, arm	+	-	+	BL

TT: Tuberculoid-tuberculoid leprosy; BB: Borderline-borderline leprosy; BL: Borderline-lepromatous leprosy.

For the second round, 3 μ l of the first PCR product was added to the PCR mixture (final volume of 50 μ l) containing 3 μ l of both inner primers, 10 μ l 5 \times PCR buffer (including 1.5 mmol/L MgCl₂), 1 μ l 10 mmol/L dNTPs (2.5 mmol/L of each dNTP), 1.5 U PromegaGoTaq polymerase, and water to 50 μ l. The reaction was performed under the following conditions: Initial denaturation at 94°C for 3 min; 35 cycles at 94°C for 30 s, 52°C for 30 s, and 72°C for 30 s and a final elongation at 72°C for 10 min. The amplified fragments were electrophoresed in 2.0% agarose gel and visualized under ultraviolet (UV) light.

Restriction analysis

The nested PCR products were digested by *Hha* I, *Mbo* I (Promega, Madison, USA), and *Bst*U I (Biolabs, New England). The 12 μ l of nested PCR product was added directly to a mixture (final volume of 25 μ l) containing 10 U of *Hha* I, *Mbo* I or *Bst*U I endonucleases, respectively, 2.5 μ l 10 \times restriction corresponding buffer and water to 25 μ l, and digested at 37°C for 4 h (*Hha* I and *Mbo* I) and 60°C for 1 h (*Bst*U I). The digestion products were electrophoresed in 2.0% agarose gel and visualized under UV light.

RESULTS

Bacillus Calmette–Guérin produced a single nest-PCR 133-bp band as we expected and showed a typical *Mycobacterium tuberculosis* pattern. All samples of three cases also had a single 133 bp band and were observed to produce 130 bp (*Hha* I) and 130 bp (*Mbo* I) and 120 bp (*Bst*U I) PCR/restriction enzyme pattern, which was a new pattern differing from other mycobacteria pattern published previously^[4] [Figure 1].

DISCUSSION

The incidence of leprosy is sporadic and rarely reported in intensive areas. The global prevalence rate is around 1.25 per 10,000 persons.^[5] According to the World Health Organization, the continents with the highest incidence of leprosy are Africa, South America, and Southeast Asia.^[1] In China, leprosy presented a lower epidemical status and was ignored easily by physicians.

Leprosy affects the skin, peripheral nervous system, respiratory system, and eyes, and it can cause nerve damage and deformity, so it is necessary to find an effective way to diagnose rapidly and correctly at early stage. Leprosy is defined as a spectral disease, showing a various types of clinical features, tuberculoid (TT), borderline tuberculoid (BT), midborderline (BB), borderline lepromatous (BL),

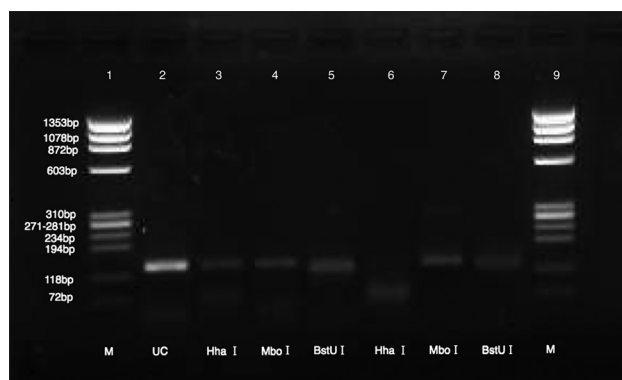


Figure 1: The pattern of *Mycobacterium leprae* by polymerase chain reaction (PCR)-restriction fragment length polymorphism analysis. Lane 3, lane 4, and lane 5: The nested PCR product of *M. leprae* sample showed the bands of 130 bp, 130 bp, and 120 bp after *Hha* I, *Mbo* I, and *Bst*U I digestion, versus 75 + 65 bp, 130 bp, and 120 bp (lane 6, lane 7, and lane 8) from bacillus Calmette–Guérin; lane 1 and lane 9: Φ 174 bp ladder DNA marker (Biolabs, New England); lane 2: The nest-PCR product.

and lepromatous (LL) corresponding to patients' immune response. The indeterminate form (I) included cases do not fit into any of the five groups. Multiple form skin lesions were observed with erythema, papules, plaques, nodules, and diffuse infiltration.^[1] The diagnosis of leprosy depends on the skin lesions, anesthesia (thermal, pain, and tactile), peripheral neural enlargement, histopathological features, and acid-fast staining positivity. The atypical clinical features at an early stage and a wide spectrum of clinical manifestations lead to misdiagnosis frequently.

Historically, high-performance liquid chromatography and DNA sequence analysis have been used to detect *M. leprae*, but these methods were difficult to implement in the clinical lab. Recently, PCR had been reported to use for rapid and accurate detection of *M. leprae*, which revealed to have an advantage as sensitive and specific molecular methods.

We studied three cases of leprosy using PCR-RFLP to detect *M. leprae* from their paraffin-embedded skin biopsy samples. The method involved restriction enzyme analysis of nested PCR products obtained with primers encoding for the 65-kDa protein, which was common to all mycobacteria.^[2] Using three restriction enzymes, the mycobacterial DNA from PCR product can be differentiated in the species levels. In a review, it was able to show unique patterns for *M. tuberculosis*, *Mycobacterium fortuitum*, *Mycobacterium kansasii*, *Mycobacterium chelonae*, *Mycobacterium avium-intracellulare*, and *Mycobacterium marinum*, which

could affect skin to cause the granulomatous diseases and smear acid-fast staining positivity.^[2] Unfortunately, *M. leprae* was not be involved.

Our study demonstrated that *M. leprae* produced a nested PCR 133 bp band as expected, and a new pattern differing from other mycobacteria patterns was observed. The new PCR/restriction enzyme pattern had not been reported previously.

In conclusion, *M. leprae* can be rapidly detected and identified using PCR-RFLP. The new PCR/restriction enzyme pattern would help to arrive at the differentiation between leprosy and other mycobacterial infectious cases. It also had shown an advantage to detect the clinical samples from paraffin-embedded skin biopsy and fresh tissues.

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Conflicts of interest

There are no conflicts of interest.

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