

## Typing of clinical isolates of *Mycobacterium leprae* and their distribution in Korea

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**Summary** Although there is no genetic diversity in isolates of *Mycobacterium leprae*, the variance of tandem repeats in the *rpoT* gene was recently demonstrated. We have typed clinical isolates of *M. leprae* in Korea using difference of the tandem repeats in the *rpoT* gene. Among 69 patients, 65 Korean isolates (94.2%) demonstrated four copies of the 6 bp tandem repeat (GACATC) in the *rpoT* gene, and incidences of three copies were found in only two Koreans and two foreigners (2.9%, respectively).

### Introduction

The incidence of leprosy is decreasing in the Western Pacific Area, and the prevalence will be reduced to 1/10,000 population with effective campaigns and multiple drug therapy (MDT).<sup>1</sup> Most countries, such as Korea, have controlled leprosy successfully; however, there are more endemic countries in the Western Pacific region and the South East Asia Area Region of the WHO. Industrialization has produced an influx of foreign workers into Korea, some of whom have developed leprosy.

Studies with restriction fragment length polymorphism (RFLP) methods suggested that *M. leprae* occurs with minimal or no differences in isolates from geographically different areas, and it was impossible to detect the exact sources of infection.<sup>2,3</sup> *Mycobacteria* such as *Mycobacterium bovis* and *Mycobacterium tuberculosis* have been reported to have subtypes when analysed using RFLP,<sup>4</sup> spoligotyping,<sup>5</sup> and DNA finger printing methods.<sup>6</sup> These methods successfully detect transmission of specific strains between countries and among species of animals.<sup>7</sup> Various trials to type *M. leprae* with RFLP, based on constant

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patterns of restriction fragments and results from DNA probing,<sup>2,3,8</sup> did not show genetic diversity in geographically distinct isolates of *M. leprae*.

The sequence of a sigma factor that has a major role in transcription of RNA polymerase has been used to map the evolutionary relationships of bacteria.<sup>9</sup> A sigma factor of *M. leprae* known as *rpoT* has 1725 base pairs (GenBank accession, no. U15181) and is a candidate for subtyping of *M. leprae*.<sup>10</sup> Matsuoka *et al.* recently have introduced a new typing method to detect three copies (accession no. AB019193) or four copies (accession no. AB019194) of the six-base tandem repeats in the *rpoT* of *M. leprae* and have confirmed it with the sequencing of the *rpoT* gene.<sup>11</sup> This method provides a new tool in typing of *M. leprae* for molecular epidemiological purposes.

In their report of typing of *M. leprae* using the *rpoT* gene, it was impossible to detect three copies of the tandem repeats in *M. leprae* isolates from Korea because there were only 11 samples.<sup>11</sup>

The object of this study was to examine the proportion and distributions of the genotypes of *M. leprae* in Korea and to compare it with genotypes of *M. leprae* from foreign leprosy cases in Korea.

## Materials and methods

To prepare *M. leprae* DNA, 4 mm punch biopsies from 69 patients with a positive BI on their first clinic visit, including 67 from Korea, one from Bangladesh and one from Nepal, were collected from the Institute of Hansen's Disease in Seoul, the Affiliated Hospital of the Korean Leprosy Control Association in Euiwang City, the Catholic Skin Clinic and the Jesus Clinic in Taegu City, from 1996 to 2000. After mincing half of the biopsy with No. 10 and No. 15 disposable scalpels in Petri dishes, 300  $\mu$ l of PBS (phosphate buffered saline 10 mM, pH 7.2) was added and the homogenate was transferred to a microcentrifuge tube. With three glass beads of 3 mm diameter, homogenization was performed by vortexing for 3 min. To remove tissue debris, homogenates were centrifuged at 125 *g* for 10 min. After centrifugation of the supernatant at 15,000 *g* for 20 min, the pellet was resuspended in 50  $\mu$ l of lysis buffer that contains proteinase K (10 mg) and Tween 80 (0.5%), and incubated at 60°C for 2 h, and was inactivated at 95°C for 10 min. *M. leprae* DNA was further extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1), and then precipitated with 150  $\mu$ l of ethanol. Footpad granulomas were obtained from the rifampin resistant Airaku-2 strain (from Dr Matsuoka, Leprosy Research Center, National Institute of Infectious Diseases, Tokyo, Japan) and the Thai 53 strain (laboratory strain from a Thai patient) from infected nude mice in the Institute of Hansen's Disease. Both strains possess three copies type (Dr Matsuoka, personal communication). Foot pads were dissected, soaked in 1% iodine solution, and minced with scalpels. Homogenization was carried out in 2 ml of PBS with 25–30 glass beads by Mickle homogenizer (Mickle Laboratory Engineering Co., Surrey, UK). *M. leprae* DNA was isolated in the same manner as described above.

*M. leprae* specific 18 kDa gene product was amplified by the PCR method with a few modifications.<sup>12</sup> A total 10  $\mu$ l of mixture containing 1  $\mu$ l template DNA and 100 pmol of each primer was overlaid with mineral oil in a reaction tube and hot-started by heating at 94°C for 5 min in the PCR 9600 (Perkin Elmer Co., Branchburg, NJ, USA). A reaction mixture including 125  $\mu$ M dNTPs, 0.5 IU Taq DNA polymerase (Boehringer Mannheim, Mannheim, Germany), 50 mmol/l KCl, 10 mmol/l Tris-HCl (pH 8.0) and 1.5 mmol/l MgCl<sub>2</sub>

was added. The PCR programme consisted of 40 cycles of 30 s at 94°C, 30 s at 60°C, 90 s at 72°C, and a final extension at 72°C for 10 min. The amplified products were detected as a 360-bp band by electrophoresis in a 2% agarose gel. This PCR method, which amplifies a region of the 18 kDa *M. leprae*-specific protein, was sensitive enough to detect as few as 10 *M. leprae*.

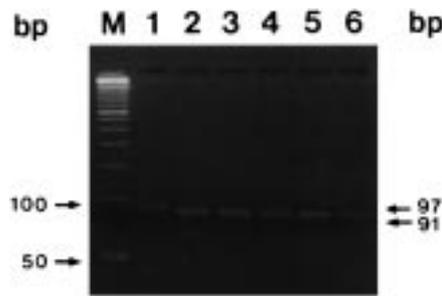
Typing of *M. leprae* with repetitive region in the *rpoT* gene was performed with the method reported by Matsuoka *et al.*,<sup>11</sup> a short fragment containing three or four copies of six base pair repeats (GACATC) was amplified. Briefly, PCR was carried out with 1 µmol/l of forward primer (5'-ATGCCGAACCGGACCTCGACGTTGA-3') and reverse primer (5'-TCGTCTTCGAGGTCGTCGAGA-3'), 1 µl of genomic DNA and reaction mixture (1 mmol/l dNTPs, 10×PCR buffer, 5 IU of Taq polymerase). PCR was performed under the condition of 30 s at 95°C, 2 min at 65°C and 4 min at 72°C for 45 cycles in the PCR 9600. The short fragments, 91 and 97 base pairs, were electrophoresed in a 4% MetaPhor agarose gel (FMC Bioproducts, ME, USA) using TBE (Tris/borate/EDTA, pH 8.0) buffer at 50 V for 2 h, and were stained with ethidium bromide solution. DNA samples for sequencing were recovered from agarose gel after electrophoresis using a Gene clean kit (Bio101, CA, USA). The DNA sequences were determined with a Model 377 automated DNA sequencer (Applied Biosystems, USA).

## Results

DNAs from 68 of the 69 patients showed the expected 360-bp band when the products of the PCR reactions targeting the 18 kDa *M. leprae* specific protein were analysed by electrophoresis (Figure 1). Among *M. leprae* isolated from Korean patients, 67 patients (94.2%) gave bands of 97 bp, corresponding to four copies of the tandem repeats, and only two patients gave bands of 91 bp, corresponding to three copies of the repeat. The Thai 53 strain and rifampin resistant Airaku-2 strain, and isolates from two foreign patients showed three copies of the tandem repeat, band of 91 bp (Figure 2). The two Korean patients who showed three copies were born at Chungcheongnamdo and Chollanamdo, the Southwest part of the Korean peninsula.



**Figure 1.** Detection of *M. leprae* specific 360-bp in agarose gel electrophoresis of the amplification product. Lane 1, negative control; lane 2, Thai 53 strain (wild type); lane 3, Airaku-2 strain (rifampin resistant strain, from Japan isolates), lane 4, isolate from case no. 33 (Bangladesh); lane 5, isolate from case no. 51 (Korean); lane 6, isolate from case no. 3 (Korean); lanes 7–11, isolate from case nos 2, 26, 39, 38 and 62 (Korean); M indicates 100-bp marker. Lane 5 did not show any positive band and lane 10 showed a very weak band in the gel, which does not show up well in the figure.



**Figure 2.** Difference of the three copies (91 bp product) and the four copies (97 bp product) of the tandem repeat of *rpoT* gene of *M. leprae* in 4% MetaPhor agarose gel with 50 V for 2 h. The case of four copies of tandem repeat was shown in lane 1, which was isolated from most Koreans, and the cases of three copies were identified from lanes 2–6. Lane 1, isolated from case no. 2; lane 2, *M. leprae*. This 53 strain; lane 3, *M. leprae* Airaku-2; lane 4, isolate from case no. 33 (Bangladesh); lane 5, isolate from case no. 51 (Korean); lane 6, isolate from case no. 3 (Korean). M indicates the DNA 50 bp ladder marker.

Sequences of the DNA fragments for 91 bp and 97 bp PCR product showed a single sequence variation in the *rpoT* gene (Table 1). Alignment of target region of the *rpoT* gene showed that the number of tandem repeats, composed of 6 bp repeats (GACATC), was different between 91 bp and 97 bp DNA fragment. 91 bp fragment contained three tandem repeats, whereas 97 bp contained four repeats in the target region of *rpoT* gene (Table 1).

**Discussion**

The results from the determination of the tandem repeat in the *rpoT* gene revealed four copies among 94.2% of Korean patients, but foreign workers from Bangladesh and Nepal who were diagnosed in Korea demonstrated three copies of the tandem repeats. Our results are consistent with the result of Matsuoka *et al.*, who reported finding four copies in patients

**Table 1.** Sequence alignment of DNA fragment corresponding from 544 to 579 (for four tandem repeats) or 544 to 573 (for three tandem repeats) of the *rpoT* gene in *M. leprae* from patients. Sequences from isolates of Figure 2 are shown. Korean-1 is from isolate of case no. 2 (four tandem repeats) and Korean-2 is of case no. 3 (three tandem repeats)

	544	I		II		II		IV		579		
Korea-1	-	GAG	GAC	ATC	GAC	ATC	GAC	ATC	GAC	ATC	GAA	GCC-AGC
	Ser	Glu	Asp	Ile	Asp	Ile	Asp	Ile	Asp	Ile	Glu	Ala
		I		II		III						
Thai-53	-	GAG	GAC	ATC	GAC	ATC	GAC	ATC	-	-	GAA	GCC-ACG
Airaku-2	-	GAG	GAC	ATC	GAC	ATC	GAC	ATC	-	-	GAA	GCC-ACG
Korea-2	-	GAG	GAC	ATC	GAC	ATC	GAC	ATC	-	-	GAA	GCC-AGC
Bangladesh	-	GAG	GAC	ATC	GAC	ATC	GAC	ATC	-	-	GAA	GCC-AGC
Nepal	-	GAG	GAC	ATC	GAC	ATC	GAC	ATC	-	-	GAA	GCC-AGC
	Ser	Glu	Asp	Ile	Asp	Ile	Asp	Ile	-	-	Glu	Ala

in the Main Island of Japan and Korea, and three copies from India, Indonesia, Nepal and Thailand.<sup>11</sup> However, we found three copies of tandem repeat in two Korean patients.

Because the incubation period of leprosy varies from 2 to 12 years, the exact source of the three copies of the tandem repeat in *rpoT* gene of *M. leprae* among Korean patients remains uncertain. They had family histories of contact with Korean cases, but not have any contact with foreign leprosy patients during the onset of their own leprosy in the past history.

Further studies to elucidate the origin and extent of the three copies in Korea are needed. When foreigners from South East Asia showed three copies, identical to patients in their home country, it is reasonable to conclude they became infected with *M. leprae* in their own countries.

The phenomenon of four copies of the tandem repeat predominating over Korea and the Main Island of Japan means that there were personal and cultural movements between the two nations. The transmission of leprosy seemed to go from Korea to Japan. When the typing of *M. leprae* in North Eastern China, the Mongolian area that is located outside of the Great Wall, is completed, a more detailed explanation can be possible regarding the transmission route of *M. leprae*. In the case of *M. tuberculosis*, it was reported that the IS6110 RFLP pattern of Korean isolates greatly resembled that of Japanese isolates, and was quite unlike those of China or Mongolia.<sup>13,14</sup>

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