

TYPING OF THAI CLINICAL ISOLATES OF *MYCOBACTERIUM LEPRAE* AND ANALYSIS OF LEPROSY TRANSMISSION BY POLYMORPHISM OF TANDEM REPEATS

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Abstract. *Mycobacterium leprae* isolates from Thai leprosy patients were typed for strain differentiation and analysis of leprosy transmission using the six base tandem repeat, GACATC, in *rpoT* gene and TTC repeat as genetic markers. *M. leprae* DNA was isolated from skin biopsies of new untreated leprosy patients living in remote areas or in suburban regions of Thailand where leprosy is in low prevalence. In *M. leprae* strains of 100 patients, TTC alleles exhibited variations in length with 10 to 30, 33 and 35 repeats, the most common alleles being 15, 16, 17 and 19 repeats. All isolates contained three copies of the six base repeat in *rpoT* gene. Application of TTC repeats in tracking leprosy transmission in two families with multi-cases identified a single (but different) strain of *M. leprae* in each family.

INTRODUCTION

Despite the reduction of registered numbers, leprosy is still a major health problem in several countries of Asia, Latin America and Africa. In Southeast Asia, leprosy is endemic in many countries that are considered as being sources of transmission. The incidence of leprosy is declining in Thailand and the prevalence is less than 1 per 10,000 population as a result of an effective leprosy control program and intensive multidrug therapy (MDT). However, immigration and movement of population may facilitate the transmission of leprosy.

Leprosy is caused by *Mycobacterium leprae* and manifests itself as damage to skin and peripheral nerve (Lockwood and Suneetha, 2005). Most people incubate the infection for at least 3 to 5 years before developing clinical symptoms and only a fraction of the people exposed to *M. leprae* ever develop clinical leprosy (Britton and Lockwood, 2004). The delay in presentation of leprosy symptoms also results in long transmission period. It is believed that the major reservoir and dominant source of infection of the disease are untreated leprosy patients. Even though MDT, with effective bactericidal antibiotics such as rifampin, can reduce infection and consequently interrupts further transmission, the number of new cases has not declined suggesting an ongoing transmission (Matsuoka *et al*, 2004; WHO, 2002, 2006).

In order to prevent the spread of leprosy,

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it is necessary to identify the source of infection and to differentiate *M. leprae* strains (Groathouse *et al*, 2004). In addition, strain typing methods for *M. leprae* isolates would be useful in distinguishing relapse and re-infection after the completion of leprosy chemotherapy. Fingerprint by restriction fragment length polymorphism has been used to discriminate *Mycobacterium tuberculosis* isolates but it is not applicable for leprosy since there is no insertion element and few restriction sites generating genetic diversity (William *et al*, 1990). Recently, two studies reported the application of polymorphism in copy number of two repetitive sequences, TTC and a six-base tandem repeat in the *rpoT* gene, to differentiation of *M. leprae* strains (Matsuoka *et al*, 2000; Shin *et al*, 2000). Thus, in this study, polymorphisms in *M. leprae rpoT* gene and TTC tandem repeat were analyzed to determine the local distribution of *M. leprae* in Thailand. The predominant genotypes may indicate the distribution of some *M. leprae* specific strains in this geographic locale. Comparing the genotypes of *M. leprae* will elucidate the origin and transmission of leprosy.

MATERIALS AND METHODS

Skin biopsy specimens

The majority of skin biopsy specimens were collected from newly diagnosed, untreated multibacillary (MB) leprosy patients since paucibacillary (PB) specimens contain low bacterial loads and give poor PCR product (Matsuoka *et al*, 2005). Patients were residents of rural villages and suburban areas where leprosy is still a problem. The classification of leprosy was determined based on host immune response, bacterial examination and skin lesions (Ridley, 1964; Ridley and Jopling, 1966). With these standard criteria, a MB patient was defined as one presenting with five or more leprosy skin lesions regardless of bacterial index (BI), which was deter-

mined based on the number of detectable acid-fast bacilli (AFB) in slit skin smears. All patients positive for BI in slit skin smear were also classified as MB patients. According to the Ridley-Jopling scale, MB patients were further classified into borderline tuberculoid (BT), borderline lepromatous (BL) and lepromatous (LL) leprosy (Ridley and Jopling, 1966). For the BT type, patients were defined as to BT (-) or BT (+) depending on the presence and absence of AFB. Punch skin biopsies (6 x 6 mm) were collected by experienced leprosy clinicians at the time before starting WHO multidrug therapy according to standard procedures (WHO, 1987). Upon collection, biopsy specimens were frozen immediately at -20°C prior to shipping on ice to Sasakawa Research Building, Ministry of Public Health, Nonthaburi, Thailand. A total of 100 biopsy specimens obtained from geographically different regions of Thailand were genotyped.

Preparation of *M. leprae* DNA

Skin biopsy punch specimens were cut into small pieces with sterile scissors and manually ground in glass tissue homogenizer in the presence of 300 µl of deionized water. *M. leprae* DNA was then extracted from 50 µl of the homogenate by addition of 100 µl of lysis buffer containing 60 µg/ml proteinase K (Amersham Bioscience, Alameda, CA), 0.05% Tween 20 and 100 mM Tris-HCl, pH 8.5. The mixture was incubated for 18 hours at 60°C. After the inactivation of proteinase K at 85°C for 15 minutes, lysed cells were removed by a brief centrifugation step. Isopropanol was added to precipitate DNA. After centrifugation, DNA pellet was washed in 70% ethanol, dried and resuspended in 10 mM Tris buffer, pH 8.5.

PCR amplification

A pair of primers, A (5'-ATGCCGAACC GGACCTCGACGTTGA-3') and B (5'-TCGT CTTCGAGGTCGTCGAGA-3') (GenBank Accession No. AB01914) was used to amplify a DNA fragment containing the six-base tandem

repeat of the *rpoT* gene (Matsuoka *et al*, 2000). A total volume of 25 μ l of PCR mixture was composed of DNA prepared from skin biopsy samples, Q-solution (Qiagen, Valencia, CA), 15 mM MgCl₂, 0.2 mM each of the four deoxynucleoside triphosphate, 2 U of *Taq* DNA polymerase (Qiagen) and 10 pmol of each primer. Amplification was carried out in a thermocycler (Model 9600; Perkin-Elmer, Applied Biosystems, Norwalk, Connecticut, USA) with an initial denaturation at 95°C for 5 minutes and 35 amplifying cycles consisting of denaturation at 95°C for 1 minute, annealing at 60°C for 30 seconds, and extension at 72°C for 1 minute. The final extension was conducted at 72°C for 10 minutes.

PCR amplification for TTC repeats was performed using primer TTC-A (5'-GGACC TAAACCATCCCGTTT-3') and TTC-B (5'-CTACAGGGGGCACTTAGCTC-3') (Shin *et al*, 2000). DNA template prepared for the *rpoT* genotyping and 10 pmol of TTC-A and TTC-B primers were added to PCR mixture as described above at a final volume of 25 μ l. After heating at 95°C for 5 minutes, amplification was conducted as follows: 35 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 30 seconds followed by final extension at 72°C for 10 minutes.

For *rpoT* genotyping, amplicons in a volume of 10 μ l were analyzed by electrophoresis in 3% agarose or 12% polyacrylamide gel. For analysis of TTC repeats, the amplicons were separated by electrophoresis in 3% agarose or 8% polyacrylamide gel in TBE buffer (90 mM Tris-base, 90 mM boric acid, 2.5 mM EDTA, pH 8.0) at 50 volts.

DNA sequencing

Amplicons of six-base repeats of *rpoT* gene and tandem repeats of TTC were subjected to direct sequencing using BigDye Terminator Cycle Sequencing FS Ready Reaction kit (Perkin-Elmer) and ABI Prism 310 XL

genetic analyzer (Perkin-Elmer). Forward primers were used in all sequencing reactions.

Ethical approval

The Institutional Ethics Committee of Ministry of Public Health, Nonthaburi, Thailand, approved the study. Formal consent was obtained from all subjects and skin specimens were collected only when informed consents were obtained.

RESULTS

Sequencing of the six-base repeats of *rpoT* gene

M. leprae isolates were obtained from leprosy patients residing in various provincial areas (Table 1). DNA was extracted and subjected to PCR amplification. Amplified prod-

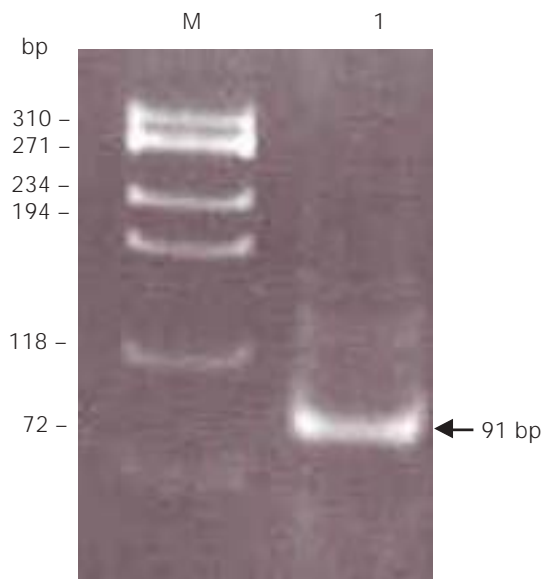


Fig 1—Detection of *rpoT* genotype by agarose gel electrophoresis. Skin biopsies from each leprosy patient were subjected to DNA extraction by proteinase K lysis and PCR amplification of a specific portion of *rpoT* gene. The 91 bp amplified DNA containing three copy of GACATC repeat in the *rpoT* gene was resolved by 3% agarose gel electrophoresis. Lane M, DNA marker; lane 1, 3 copies.

Table 1

Number and frequency of TTC genotypes in *M. leprae* Thai clinical isolates from 100 patients.

No. of TTC repeat	No. of patients					Total	Province
	MB				PB		
	LL	BL	BB	BT	BT		
10	-	1	-	-	1	2	Samut Prakan(1), Chaiyaphum(1)
11	-	1	-	1	1	3	Khon Kaen(1), Udon Thani(1), Samut Prakan(1)
12	-	2	2	-	-	4	Samut Prakan(1), Loei(1), Kalasin(1), Khon Kaen(1)
13	1	2	-	-	-	3	Chanthaburi(1), Chaiyaphum(1), Maha Sarakham(1)
14	3	4	-	2	-	9	Nakhon Ratchasima(2), Khon Kaen(3), Chaiyaphum(2), Bangkok(1), Nong Khai(1)
15	6	4	2	1	1	14	Samut Sakhon(1), Buri Ram(1), Nonthaburi(1), Nong Khai(1), Surin(1), Khon Kaen(2), Nong Bua Lum Phoo(1), Nakhon Sawan(3), Songkhla(1), Chaiyaphum(2)
16	4	4	1	-	1	10	Kalasin(1), Udon Thani(1), Khon Kaen(4), Nong Khai(1), Nakhon Pathom(1), Samut Prakan(1), Nakhon Sawan(1)
17	3	6	-	-	-	9	Sa Kaeo(2), Bangkok(1), Nong Bua Lum Phoo(1), Roi Et(1), Udon Thani(1), Khon Kaen(1), Chaiyaphum(2)
18	2	2	-	-	2	6	Nong Khai(1), Udon Thani(1), Bangkok(1), Khon Kaen(2), Maha Sarakham(1)
19	5	5	-	-	-	10	Maha Sarakham(2), Nakhon Ratchasima(1), Chaiyaphum(1), Bangkok(2), Kalasin(2), Udon Thani(1), Khon Kaen(1)
20	2	2	2	1	-	7	Bangkok(2), Phetchabun(1), Samut Prakan(2), Maha Sarakham(1), Chaiyaphum(1)
21	4	2	-	-	-	6	Khon Kaen(4), Kalasin(1), Chaiyaphum(1)
22	2	1	-	-	-	3	Chaiyaphum(1), Samut Prakan(1), Maha Sarakham(1)
23	1	-	-	-	-	1	Khon Kaen (1)
24	1	1	-	-	-	2	Udon Thani (1), Khon Kaen (1)
25	1	1	-	-	-	2	Khon Kaen (2)
26	-	1	-	-	-	1	Nakhon Phanom (1)
27	-	1	-	-	-	1	Nakhon Ratchasima (1)
28	1	-	-	-	-	1	Chaiyaphum (1)
29	1	1	-	-	-	2	Khon Kaen (1), Maha Sarakham(1)
30	1	1	-	-	-	2	Khon Kaen (2)
33	-	1	-	-	-	1	Khon Kaen (1)
35	-	1	-	-	-	1	Maha Sarakham (1)

MB, multibacillary leprosy; PB, paucibacillary leprosy; LL, lepromatous leprosy; BL, borderline lepromatous leprosy; BB, borderline leprosy; BT, borderline tuberculoid leprosy

ucts of 91 bp only were obtained from *rpoT* gene (Fig 1). There were three copies of the six-base repeat, GACATC, in the 91-bp amplified products with no variations among *M. leprae* Thai isolates (data not shown) indicat-

ing this was the single dominant genotype of *M. leprae* strains in Thailand.

Genotyping of TTC repeat

The copy number of TTC repeats showed

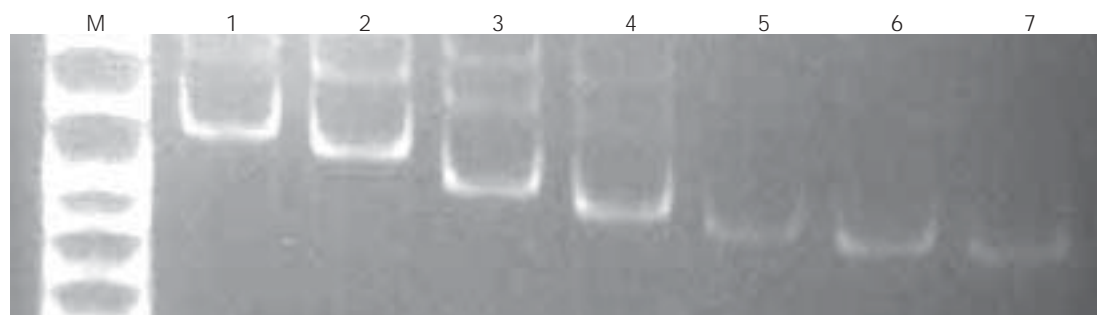


Fig 2—The polymorphism of TTC repeats in *M. leprae* isolates from Thai leprosy patients. *M. leprae* DNA was extracted from skin biopsies and subjected to PCR amplification for TTC repeats. The polymorphisms of TTC repeats were analyzed by 8% polyacrylamide gel electrophoresis. Lane M, DNA marker; lane 1, 33 copies; lane 2, 29 copies; lane 3, 22 copies; lane 4, 19 copies; lane 5, 15 copies; lane 6, 12 copies; lane 7, 10 copies.

Table 2
TTC genotypes of *M. leprae* isolates obtained from multi-household leprosy cases.

Family no.	Patient no.	Status	Date of diagnosis	Leprosy type	TTC genotype
1	1	Father	2006	LL	15
	2	Mother	2006	BL	15
	3	Daughter	2006	BL	15
2	1	Uncle	2005	BL	19
	2	Nephew	2005	BT	19

LL, lepromatous leprosy; BL, borderline lepromatous leprosy; BT, borderline tuberculoid leprosy

variation among Thai *M. leprae* strains, with 10 to 30, and 33 and 35 copies (Fig 2). *M. leprae* strains with 15 TTC repeats were most frequent and were found in 14 patients (Table 1). This was followed by strains with 16 and 19 TTC repeats, found equally in 10 patients. Of 6 paucibacillary leprosy patients, there were 5 *M. leprae* strains each with a different number of TTC repeats.

Genotyping of *M. leprae* isolated from multi-case family

In accordance with the low prevalence of leprosy in Thailand, multi-case families are rare. However, 5 patients from two multi-case household families were recruited for TTC typing. *M. leprae* genotypes obtained from members of the same household showed identical copy numbers of the TTC repeat (Table 2).

DISCUSSION

Genotyping of *M. leprae* isolates is essential for epidemiological analysis of leprosy transmission. In addition, it is a useful tool to distinguish between relapse and re-infection. The discovery of polymorphism of short tandem repeats in *M. leprae* resulted in major advance in typing of *M. leprae* (Abe *et al*, 1990; Matsuoka *et al*, 2000; Shin *et al*, 2000; Groathouse *et al*, 2004; Saroj *et al*, 2004; Truman *et al*, 2004; Zhang *et al*, 2005). DNA fragments containing the tandem repeats can be amplified and copy number can be determined by electrophoresis and sequencing. This present study demonstrated polymorphism of TTC repeats among *M. leprae* in Thai patients. However, *rpoT* gene of Thai clinical isolates contained only one *rpoT* genotype,

namely three copies of six-base tandem repeat, GACATC.

It has been reported previously that *M. leprae* isolates can be divided into two genotypes based on polymorphism in the *rpoT* gene that contain either three or four copies of GACATC repeat. Unlike those from Thailand, *M. leprae* isolates from East Asia, Korea and mainland Japan, contain four copies of the *rpoT* tandem repeat (Matsuoka *et al*, 2000). The implication is that should the genotype containing three copies of this tandem repeats be found in latter regions, it is feasible that this strain originated from elsewhere.

Shin *et al* (2000) showed that using polymorphism of TTC repeats 34 *M. leprae* isolates can be divided into 15 subspecies. Polymorphisms of TTC repeats could be divided into 23 subtypes among 100 Thai *M. leprae* isolates. The difference in the numbers of allelic variants of the TTC locus between our study and others might be influenced by the number of *M. leprae* isolates and composition of the different lineages of the isolates in each study. Typing by TTC repeats obviously shows discriminatory capacity over typing using the repeats in the *rpoT* gene suggesting the potential of the former method in discriminating *M. leprae* local strains, whereas the six-base tandem repeat of *rpoT* gene is more suitable for global epidemiology study of leprosy (Matsuoka *et al*, 2000, 2005).

Many reports indicated human beings as a major reservoir of leprosy but other sources are possible (Cho *et al*, 1992; Klaster *et al*, 1993; Ramaprasad *et al*, 1997; Izumi *et al*, 1999; Matsuoka *et al*, 2000). The port of exit and entry of leprosy is the nasal mucosa (Pedley and Geater, 1976; Fine *et al*, 1997), and hence the primary transmission mode of leprosy is by direct contact with patients (Noordeen, 1994). In two multi-case families studied, TTC repeats of *M. leprae* isolates showed identical pattern in each family implicating transmission had occurred among fam-

ily members. The present *rpoT* and TTC typing systems do not define all unique *M. leprae* isolates. Strains with identical number of six-base repeats in the *rpoT* gene or TTC repeats may consist of other subtypes. Therefore, other typing methods or other genetic markers are still required to be developed.

ACKNOWLEDGEMENTS

We are grateful to Professor Dr Patrick J Brennan from Colorado State University Fort Collins, Colorado, USA for valuable advice and discussion. This work was supported by a grant of Sasakawa Memorial Health Foundation, Japan, and the Department of Disease Control, Raj Pracha Samasai Institute, Ministry of Public Health, Thailand. Support from Thai NIH, Department of Medical Sciences, Ministry of Public Health, is also acknowledged.

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