

Original Article

SNP Genotypes of *Mycobacterium leprae* Isolates in Thailand and Their Combination with *rpoT* and TTC Genotyping for Analysis of Leprosy Distribution and Transmission

Benjawan Phetsuksiri^{1*}, Sopa Srisungngam¹, Janisara Rudeeaneksin¹, Supranee Bunchoo¹,
Atchariya Lukebua¹, Ruch Wongtrungkapun², Soontara Paitoon³,
Rama Murthy Sakamuri⁴, Patrick J. Brennan⁴, and Varalakshmi Vissa⁴

¹National Institute of Health, Department of Medical Sciences,
Ministry of Public Health, Nonthaburi;

²Raj Pracha Samasai Institute, Department of Disease Control,
Ministry of Public Health, Nonthaburi; and

³The Office of Disease Prevention and Control Regional Center 5, Nakhon Ratchasima, Thailand; and

⁴Colorado State University, Fort Collins, Colorado, USA

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SUMMARY: Based on the discovery of three single nucleotide polymorphisms (SNPs) in *Mycobacterium leprae*, it has been previously reported that there are four major SNP types associated with different geographic regions around the world. Another typing system for global differentiation of *M. leprae* is the analysis of the variable number of short tandem repeats within the *rpoT* gene. To expand the analysis of geographic distribution of *M. leprae*, classified by SNP and *rpoT* gene polymorphisms, we studied 85 clinical isolates from Thai patients and compared the findings with those reported from Asian isolates. SNP genotyping by PCR amplification and sequencing revealed that all strains like those in Myanmar were SNP type 1 and 3, with the former being predominant, while in Japan, Korea, and Indonesia, the SNP type 3 was found to be more frequent. The pattern of *M. leprae* distribution in Thailand and Myanmar is quite similar, except that SNP type 2 was not found in Thailand. In addition, the 3-copy hexamer genotype in the *rpoT* gene is shared among the isolates from these two neighboring countries. On the basis of these two markers, we postulate that *M. leprae* in leprosy patients from Myanmar and Thailand has a common historical origin. Further differentiation among Thai isolates was possible by assessing copy numbers of the TTC sequence, a more polymorphic microsatellite locus.

INTRODUCTION

Leprosy is a chronic disease caused by *Mycobacterium leprae*, the uncultivated mycobacteria. The disease remains a health problem and is endemic in many countries. According to the World Health Organization (WHO) reports in 2010 from 141 countries and territories, the global registered prevalence of leprosy at the beginning of 2010 was 211,903 cases, while the number of new cases detected during 2009 was 244,796 cases (1). The detection rate of new cases each year is constant, reflecting the extent of leprosy transmission. In Thailand, the national prevalence of leprosy in 2008 was 0.14 per 100,000, although there is considerable variation in prevalence between districts. The number of reported new cases of leprosy has fallen, to 401 in 2008 and to 300 in 2009 (2). The slow decline of new case detection rate and the national prevalence reflects the continuing spread of leprosy. Similar to other regions in the world, despite the decline in leprosy transmission, stig-

matization and discrimination towards those affected by leprosy still continues to be a problem in Thailand.

One of the difficulties in studying the molecular epidemiology of leprosy is the homogeneity of leprosy genomic DNA (3,4). Genotyping tools were not available until two variable number tandem repeats (VNTRs) were detected in 2000 (5,6), followed by the discovery of short tandem repeats (STRs) with the potential for genotyping (7,8). STRs with polymorphisms have been examined for their potential in strain differentiation and were found to be useful in distinguishing isolates in areas with a high prevalence of leprosy (4,8-10). STRs with small ranges of variation, such as a 6-bp repeat of the *rpoT* gene, have proven to be valuable for analysis of global transmission of leprosy (5,8,11). Two *rpoT* genotypes, 3-copy type and 4-copy type, were identified in many Asian countries, and the 4-copy type is predominant in Japan and Korea (11). Recent studies reported four genotypes defined by three single nucleotide polymorphisms (SNPs). SNPs, which are rare in *M. leprae*, have been reported to be useful in tracing the global dissemination of leprosy (12). The purpose of this study was to expand the analysis of geographic distribution of *M. leprae* by investigating SNPs and *rpoT* genotypes in Thai clinical isolates. Comparing SNPs and *rpoT* genotypes with those from other Asian countries provided insights into the distribution and trans-

*Corresponding author: Mailing address: National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Tiwanond Rd., Nonthaburi, Thailand. 11000. Tel: +66-2-5801567, Fax: +66-2-9659700, E-mail: benjapask@health.moph.go.th

mission of leprosy in Thailand, and in this geographic region. Genotyping, based on variation number of TTC tandem repeats, was performed in an attempt to differentiate local strains of *M. leprae*.

MATERIALS AND METHODS

Skin specimens and processing of clinical specimens:

The study was approved by the Ethical Committee of the Ministry of Public Health of Thailand. DNA from *M. leprae*, stored in a sample bank, was used in genotyping. Extraction of DNA was performed previously from stored punch skin biopsies (6 mm³), all of which had been collected from newly diagnosed leprosy patients, most of them living in Northeastern Thailand. The leprosy patients were classified clinically as multibacillary (MB) ($n = 81$) or paucibacillary (PB) ($n = 4$), based on bacteriological findings and clinical appearance. With further classification according to the Ridley-Jopling system, there were 30 lepromatous leprosy (LL), 42 borderline lepromatous (BL), 5 borderline borderline (BB), and 4 borderline tuberculoid (BT) leprosy patients in this study. Collection of specimens was performed prior to treatment of the patients. Biopsy specimens were kept at -20°C until they were processed for DNA extraction.

M. leprae DNA templates were prepared from homogenized skin biopsy specimens and the resulting DNA was kept at -20°C in a DNA bank until subsequent analysis. Briefly, skin biopsies were cut into small pieces with sterile scissors and manually ground in glass tissue homogenizers in the presence of 300- μL deionized water (13). DNA was extracted from 100 μL of tissue homogenate using lysis buffer, as previously described (14), or DNeasy tissue kit, according to the manufacturer's instructions (Qiagen, Valencia, Calif., USA). DNA was finally recovered in a final volume of 100 μL of distilled water.

SNP genotype analysis: To amplify three SNP loci at positions 14676, 164275, and 2935685 in *M. leprae* genomic DNA, PCR was carried out using 2.5 U of *Taq* polymerase (Qiagen) in a final volume of 25 μL , containing 2 μL of DNA template, 10 pmol of each primer pair (12), 200 μM dNTP, and buffer supplied by the manufacturer. The mixture was denatured at 94°C for 5 min; this was followed by 45 PCR cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min, and a final extension at 72°C for 10 min in a thermal cycler (Eppendorf, Hamburg, Germany). Aliquots containing 1–2 μL purified PCR product were directly sequenced, using an ABI sequencer 3130 XL, at the National Institute of Health, Thailand core facilities, using BigDye Terminator v3.1 and forward primers. In brief, 4 μL of BigDye v3.1 buffer (Applied Biosystems, Foster City, Calif., USA) and 20 pmol of primer were added. The final volume was adjusted to 20 μL using deionized water. The initial denaturation was performed at 94°C for 1 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 25 s, and extension at 60°C for 4 min. In some instances, PCR products were purified using the QIAquick PCR purification kit (Qiagen) prior to sequencing.

***rpoT* genotyping:** A pair of primers, A (5'-ATGCC

GAACCGGACCTCGACGTTGA-3') and B (5'-TCG TCTTCGAGGTCGTCGAGA-3'), as described previously (5), was used to amplify a DNA fragment containing the 6-base tandem repeat of the *rpoT* gene. The PCR mixture had a total volume of 25 μL and was composed of DNA prepared from skin biopsy samples (1–2 μL), 1.5 mM MgCl_2 , 0.2 mM deoxynucleoside triphosphate, 2 U of *Taq* DNA polymerase, and 10 pmol of each primer. The amplification was carried out in a thermocycler (Eppendorf) using a protocol consisting of an initial denaturation step at 95°C for 5 min; 35 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 30 s, and extension at 72°C for 30 s; and a final extension step at 72°C for 10 min. The amplicons were detected by 3% agarose gel electrophoresis and the copy number of the DNA repeat in the amplified products was identified directly by DNA sequencing at the National Institute of Health, Thailand.

TTC genotyping: PCR amplification for TTC repeats was performed using the primers, TTC-A (5'-GGACCTAAACCATCCCGTTT-3') and TTC-B (5'-CTACAGGGGGCACTTAGCTC-3'), as described previously (6). Briefly, DNA templates and 10 pmol of each primer were added into a PCR mixture containing the PCR reagents in a final volume of 25 μL , as described above. The amplification was performed with an initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 59°C for 1 min, and extension at 72°C for 1 min; and a final extension step at 72°C for 10 min. PCR products were purified and sequenced according to the protocol described above.

RESULTS

Distribution of SNP *M. leprae* genotypes: The SNP variants detected in Thai clinical isolates were limited to SNP type 1, CGA and SNP type 3, CTC. In this study population, the SNP type 1 was identified in 68 isolates, while the SNP type 3 was detected in 17 samples. Since there has been a previous study on SNP types in Asian countries, the SNP genotypes of Thai clinical isolates could be compared to those of Asian isolates (11). SNP type 1 is predominant in Thailand, as it is in Myanmar. This pattern differs from that of other Asian countries, where SNP type 3 is more common than SNP type 1 (Table 1). Comparing the distribution of SNP types in Japan, there was no SNP type 1 isolated on the mainland, while SNP type 4 was conserved and found only in Japanese Brazilians (11). SNP type 2, which is infrequent in this Southeast (SE) Asian region, was not detected in Thai isolates (Table 1). The relationship between SNP genotypes and clinical forms of leprosy is shown in Table 2.

Genotyping of the *rpoT* gene: The 6-base repeat region of the *rpoT* gene was amplified by PCR and analyzed by electrophoresis. All isolates yielded amplification products of 91-bp, but not 97-bp. Sequencing revealed that the 91-bp amplified product contained 3 copies of the 6-base repeat, GACATC. Compared to the findings of a previous study, *M. leprae* isolates from Thailand had a single *rpoT* genotype, a 3-copy variant, similar to those isolated in Myanmar and Okinawa, Japan (11) (Table 1).

TTC repeat genotyping: The TTC genotype was de-

Table 1. Comparison of SNP and *rpoT* genotypes of *M. leprae* isolates in Thailand and other Asian countries

| Origin | SNP | | | | <i>rpoT</i> type | |
|----------------------------------|--------|--------|--------|--------|------------------|--------|
| | Type 1 | Type 2 | Type 3 | Type 4 | 3 copy | 4 copy |
| Thailand | + | * | — | + | — | + |
| Japan Mainland ¹⁾ | — | + | + | * | — | + |
| Japan Okinawa ¹⁾ | + | — | + | * | — | + |
| Japanese Brazilian ¹⁾ | — | — | + | * | + | — |
| Korea ¹⁾ | + | + | + | * | — | + |
| Indonesia ¹⁾ | + | + | + | * | — | + |
| Myanmar ¹⁾ | + | * | + | — | + | — |
| China ²⁾ | — | — | + | — | NA | NA |

SNP types 1, 2, 3, and 4 refer to genotypes CGA, CTA, CTC, and TTC at *M. leprae* TN genome nucleotides 14676, 164275, and 2935685, respectively.

rpoT types refer to 3 or 4 copies of 6-bp tandem repeat, GACATC, in the *rpoT* gene.

¹⁾: Report from Matsuoka et al., 2006 (11).

²⁾: Report from Weng et al., 2007 (10).

*, predominant genotypes; +, presence; —, absence; NA, not available.

Table 2. SNP genotypes and clinical forms of leprosy

| Clinical form | SNP genotype | | Total |
|---------------|--------------|--------|-------|
| | Type 1 | Type 3 | |
| PB | 4 | 0 | 4 |
| MB | | | |
| BT | 4 | 0 | 4 |
| BL | 34 | 8 | 42 |
| BB | 4 | 1 | 5 |
| LL | 22 | 8 | 30 |
| Total | 68 | 17 | 85 |

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

terminated by the copy number of TTC repeats. By sequence analysis of amplicons spanning this microsatellite locus, it was demonstrated that the copy number of TTC repeats varied considerably among Thai *M. leprae* strains, and 24 variants were identified (Table 3). The TTC copy number relative to SNP type 1 or 3 status is interesting, as shown in Table 3 and Fig. 1. The TTC repeats were highly variable, and therefore have the potential to be used as a marker for strain differentiation of *M. leprae* in local communities.

DISCUSSION

Thailand is situated in SE Asia on the Indochina Peninsula and is surrounded by leprosy-endemic neighbors, such as Myanmar, Cambodia, and Lao PDR. Even though the national prevalence rate of leprosy is less than 1.0 per 10,000 in the population, and the disease is considered to be under control according to WHO criteria, there are some regions where the actual prevalence is high. Therefore, surveillance-control systems and epidemiological studies for tracking leprosy transmission are required.

In order to more fully understand the transmission

Table 3. Strain differentiation among SNP type 1 and SNP type 3 of Thai clinical isolates

| Type no. | TTC variant | SNP | |
|----------|-------------|--------|--------|
| | | Type 1 | Type 3 |
| 1 | 10 | 3 | — |
| 2 | 11 | 1 | — |
| 3 | 12 | 4 | — |
| 4 | 13 | 3 | — |
| 5 | 14 | 5 | — |
| 6 | 15 | 11 | 1 |
| 7 | 16 | 8 | — |
| 8 | 17 | 7 | — |
| 9 | 18 | 6 | — |
| 10 | 19 | 8 | 4 |
| 11 | 20 | 3 | — |
| 12 | 21 | 4 | — |
| 13 | 22 | — | 1 |
| 14 | 23 | 2 | 1 |
| 15 | 24 | — | 2 |
| 16 | 25 | — | 1 |
| 17 | 26 | — | 1 |
| 18 | 27 | 1 | — |
| 19 | 28 | — | 1 |
| 20 | 29 | 2 | — |
| 21 | 30 | — | 2 |
| 22 | 33 | — | 1 |
| 23 | 35 | — | 1 |
| 24 | 42 | — | 1 |
| Total | 24 | 68 | 17 |

and epidemiology of leprosy, several investigators have searched for polymorphic markers within the genome of *M. leprae* and have evaluated the potential of these markers as epidemiological tools in strain differentiation (7,8,15,16). However, the variability pattern for each of these genomic markers should be experimentally evaluated in the patient populations of interest. SNP types were examined based on polymorphisms of nucleotides at positions 14676, 164275, and 2935685 of *M. leprae* genomic DNA. Four types of SNP: type 1, CGA; type 2, CTA; type 3, CTC; and type 4, TTC, were reported previously (12). In the present group of isolates, all strains belonged to SNP type 1 and 3 at a ratio of 68:17. In contrast to this high degree of conservation of SNP type, and also of the *rpoT* genotype (3 copies of 6-base tandem repeats), in Thai clinical isolates, polymorphism of TTC repeats was highly dynamic, and demonstrated usefulness in the genotyping of *M. leprae* in local settings. Differences in the number of allelic variants of TTC repeat loci between the present study and previous studies might be mainly influenced by the total number of *M. leprae* isolates studied and the composition of different isolate lineages in each study. Our findings in Thai patients are consistent with previous studies demonstrating that repeats in *rpoT* and SNPs are considerably conserved, and this is useful for studying the geographic distribution of *M. leprae* in the region or at a global level. In contrast, the extensive variation of TTC repeats enables differentiation of local *M. leprae* strains (11,14,17). SNP type 1 and 3 frequencies and the *rpoT* 3 copy allele are common between

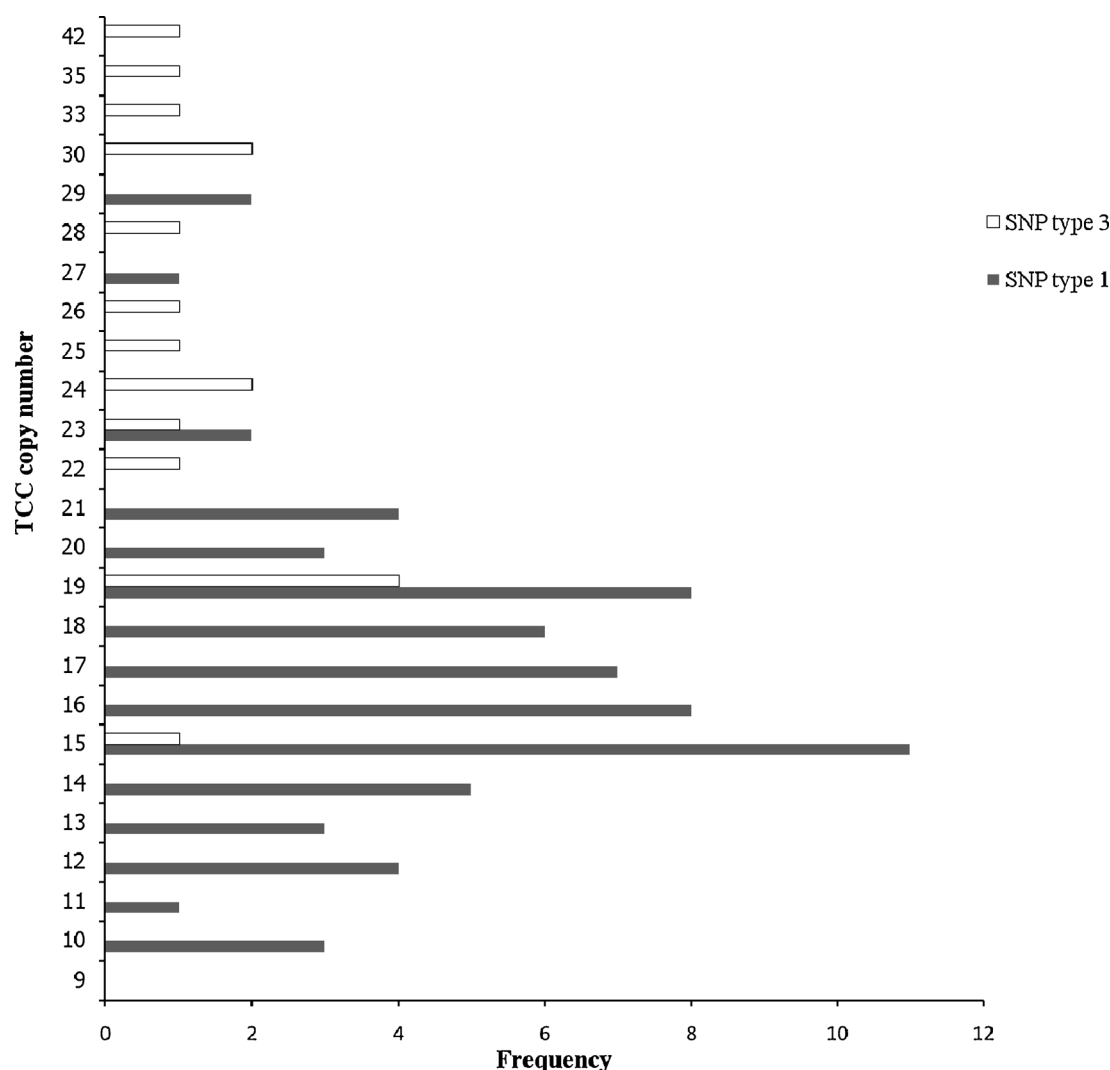


Fig. 1. TTC repeats of SNP type 1 and SNP type 3 in Thai clinical isolates of *M. leprae*. TCC copy numbers in SNP type 1 and type 3 were analyzed by PCR and sequencing.

Myanmar and the Philippines (11,15), while the SNP type 2 was identified in Myanmar but not in Thai or Philippino clinical isolates. Therefore, while some *M. leprae* lineages appear to have disseminated and become established among the people of neighboring and proximal SE Asian countries, others have not. Additional markers are required to confirm this transmission at regional and global levels.

Regarding the TTC genotyping, in some instances, it was observed that the exact endpoints in sequence tracings of TTC repeats were difficult to identify. The stutter phenomenon made a shift of copy reading into 1–2 copies was acceptable. The potential occurrence of a wide range of genetic variations between individuals based on the use of hypervariable repeats, such as TTC, points to a need for caution when using highly dynamic markers. Thus, for strain typing and differentiation, the use of multiple markers, including VNTRs and SNPs, is beneficial. Which markers are suitable should be determined after assessing their allelic diversity in the study population of interest.

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Conflict of interest None to declare.

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