

**Figure 7.** Phylogenetic trees of common pathogenic mycobacteria. A bold letter indicates the species which have been identified in our institute.

and *hsp65* gene would be needed for further consideration. Because quality control is not used in BLAST, care should be taken when it is used as a comparison tool. After the analysis of four different gene sequences, the most homologous bacteria can be chosen as phylogenetic mycobacteria. Sometimes, there is no match to the four different genes, which might be indicative of a novel species.<sup>38</sup> It is recommended that samples be sent to a specialized institution for accurate identification of rare strains.

**Phylogenetic trees analysis**

Approximately 30 species of mycobacteria have been reported as human pathogens in Japan, and it is assumed that these species cause disease throughout Asia as well. Figure 7 shows a phylogenetic tree of these 30 pathogens based on 16S rRNA sequence data. *M. leprae* is closely related to *Mycobacterium haemophilum*, while *M. ulcerans* subsp. *shinshuense* is a close relative of *M. ulcerans*, *M. marinum* and even *M. tuberculosis*, which are all only distantly related to the RGM species.

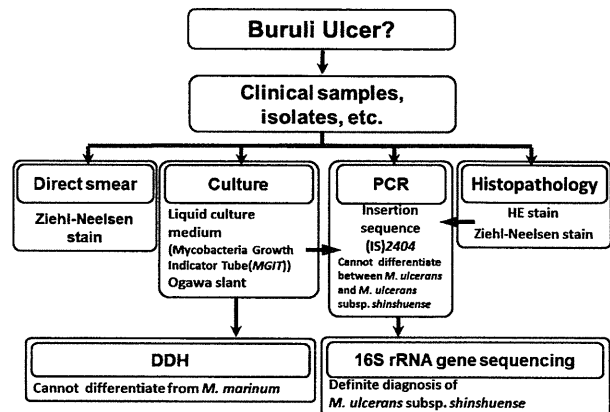
**Drug susceptibility test**

Antibiotic susceptibility profiles are key considerations in the choice of treatment options for mycobacterial infections. Cultured bacteria from cutaneous wounds can be used in susceptibility testing in order to choose antibiotics. The test is performed according to the microdilution method approved by the Clinical Laboratory and Standards Institute (CLSI).<sup>39</sup> In general, microdilution is an easy and reliable technique for this

**Table 6.** Drug resistance-related mutations in *Mycobacterium tuberculosis* and other mycobacteria

Drug	Related gene	Gene product
Rifampicin (RFP)	<i>rpoB</i>	DNA-dependent RNA polymerase β subunit <sup>40</sup>
Isoniazid (INH)	<i>katG</i> <i>inhA</i>	Catalase-peroxidase <sup>41</sup> NADH-dependent enoylacyl carrier protein reductase <sup>42</sup>
Ethambutol (EB)	<i>ahpC</i>	Alkyl hydroxyperoxidase <sup>43</sup>
Pyrazinamide (PZA)	<i>embB</i> , <i>embA</i> , <i>embC</i>	Arabinosyl transferase <sup>44</sup>
Streptomycin (SM)	<i>pncA</i>	Pyrazinamidase/nicotinamidase <sup>45</sup>
Kanamycin (KM)	<i>rpsL</i> <i>rRNA</i>	Ribosomal protein S12 <sup>46</sup> 16S rRNA <sup>47</sup>
Diaminodiphenyl sulfone (DDS, dapsone)	<i>folP</i>	Dihydropteroate synthase <sup>49</sup>
Fluoroquinolones	<i>gyrA</i> , <i>gyrB</i>	DNA gyrase A subunit, B subunit <sup>50</sup>
Clarithromycin (CAM)	<i>rRNA</i>	23S rRNA <sup>51</sup>

purpose. The approved method was revised recently to state that cation-adjusted Müller–Hinton broth (CAMHB) should be used as culture media for the assay. In particular, CAMHB without OADC supplementation should be used to determine the concentration of drugs against RGM isolates. Mutations in certain genes have been associated with antibiotic resistance (Table 6). Genotypic analysis of these genes can be performed,



**Figure 8.** Flow chart for the differential identification of Buruli ulcer. DDA, DNA–DNA hybridization; HE, hematoxylin–eosin; PCR, polymerase chain reaction.

but the process could prove to be very labor intensive in a clinical setting.

## CASE STUDY: IDENTIFICATION OF A CUTANEOUS NTM INFECTION

### Diagnosis of BU<sup>52-54</sup>

A PCR assay targeting IS2404 is frequently used to diagnose BU<sup>28</sup>. The PCR-amplified sequences of the IS2404 target are less than 200 bp (Table 4). At present, IS2404 has been found only in two strains of human pathogenic mycobacteria: *M. ulcerans* and *M. ulcerans* subsp. *shinshuense*. Moreover, it is present at more than 200 copies/genome, so the sensitivity is extremely high and it is suitable for screening and differential identification.<sup>55,56</sup> It is noteworthy that some groups of mycobacterium derived from fish and amphibians also carry IS2404,<sup>57-60</sup> therefore, caution must be exercised during environmental studies. Figure 8 shows the flow chart for the diagnosis of BU. It is principally the same as that found in the World Health Organization manual for the diagnosis of *M. ulcerans* disease.<sup>61</sup>

### Practical PCR assay using paraffin specimens

DNA should be fragmented when dealing with formalin-fixed paraffin-embedded clinical samples. The amplification of long DNA regions by PCR is often difficult, but assays targeting shorter regions of less than 200 bp are more feasible. The caveat is that detection sensitivity is very low with formalin-fixed paraffin-embedded samples; however, the transport and storage of specimens are relatively easy. In practice, the quality and integrity of DNA from these specimens should be confirmed. Amplification of the  $\beta$ -globin region of human genomic DNA should be used as a control. In Figure 5 (to the left of the marker), lanes 1 and 2 show positive signals for the human  $\beta$ -globin gene, demonstrating that the quality of DNA from the formalin-fixed paraffin-embedded specimens was sufficient for amplification. To the right of the marker lane are amplicons of IS2404. Lanes 1 and 2 show a 154-bp band, the right size of the IS2404 target in *M. ulcerans* and *M. ulcerans* subsp. *shinshuense*. No signal was observed in the negative control (NC) lane, while 154-bp and 205-bp bands appeared in the positive control (PC) lane.

## CONCLUSION

At present, 156 species and 13 subspecies of mycobacteria have been registered.<sup>62</sup> New mycobacterial species are being reported because liquid medium is broadly used as the isolation medium, and the ease of isolation from broth culture has increased significantly. Moreover, the progression of genotypic analysis has contributed to this increased rate of discovery. The increase in the number of NTM patients has highlighted the importance of rapid diagnosis of mycobacterial agents.

An elementary, but important, precautionary statement is that specimens should be collected before starting antibiotic treatment. For cases in which the mycobacterium species are rare or are difficult to diagnose, further analysis may be required by a specialized institution.

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## Characteristic mutations found in the ML0411 gene of *Mycobacterium leprae* isolated in Northeast Asian countries



M. Kai<sup>a,\*</sup>, N. Nakata<sup>a</sup>, M. Matsuoka<sup>a</sup>, T. Sekizuka<sup>b</sup>, M. Kuroda<sup>b</sup>, M. Makino<sup>a</sup>

<sup>a</sup>Department of Mycobacteriology, Leprosy Research Center, National Institute of Infectious Diseases, 4-2-1 Aoba-cho, Higashimurayama, Tokyo 189-0002, Japan

<sup>b</sup>Pathogen Genomics Center, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

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### ABSTRACT

Genome analysis of *Mycobacterium leprae* strain Kyoto-2 in this study revealed characteristic nucleotide substitutions in gene ML0411, compared to the reference genome *M. leprae* strain TN. The ML0411 gene of Kyoto-2 had six SNPs compared to that of TN. All SNPs in ML0411 were non-synonymous mutations that result in amino acid replacements. In addition, a seventh SNP was found 41 bp upstream of the start codon in the regulatory region. The seven SNP sites in the ML0411 region were investigated by sequencing in 36 *M. leprae* isolates from the Leprosy Research Center in Japan. The SNP pattern in 14 of the 36 isolates showed similarity to that of Kyoto-2. Determination of the standard SNP types within the 36 stocked isolates revealed that almost all of the Japanese strains belonged to SNP type III, with nucleotide substitutions at position 14676, 164275, and 2935685 of the *M. leprae* TN genome. The geographical distribution pattern of east Asian *M. leprae* isolates by discrimination of ML0411 SNPs was investigated and interestingly turned out to be similar to that of tandem repeat numbers of GACATC in the *rpoT* gene (3 copies or 4 copies), which has been established as a tool for *M. leprae* genotyping. All seven Korean *M. leprae* isolates examined in this study, as well as those derived from Honshu Island of Japan, showed 4 copies of the 6-base tandem repeat plus the ML0411 SNPs observed in *M. leprae* Kyoto-2. They are termed Northeast Asian (NA) strain of *M. leprae*. On the other hand, many of isolates derived from the Okinawa Islands of Japan and from the Philippines showed 3 copies of the 6-base tandem repeat in addition to the *M. leprae* TN ML0411 type of SNPs. These results demonstrate the existence of *M. leprae* strains in Northeast Asian region having characteristic SNP patterns.

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### 1. Introduction

Leprosy is a chronic infectious disease caused by infection with *Mycobacterium leprae*. Dr. Gerhard Hansen, GHA first observed this bacterium in samples derived from leprosy patients in 1873 (Irgens, 1984). At that time, leprosy was prevalent worldwide and the absence of effective drugs for treatment resulted in peripheral nerve damage, leading to severe deformity. Thereafter more effective drugs were used in the treatment of leprosy and deformity was reduced. The present strategy for leprosy treatment is based on the multi-drug therapy (MDT) recommended by the World Health Organization (WHO, 1982), which has successfully reduced the number of leprosy cases in the world (WHO, 2012). However, *M. leprae* cannot be cultivated in artificial medium and the detailed biological characterization of the bacterium still progresses slowly.

Due to advances in molecular biology, the unique characteristics of *M. leprae* are becoming clearer through whole-genome DNA sequencing and analysis. At present, two complete genome sequences of *M. leprae* strains, TN and Br4923, have been determined and are publically accessible on an Internet database (Cole et al., 2001; Monot et al., 2009). The genome size of *M. leprae* is approximately 3.3 Mb, which is smaller than that of *Mycobacterium tuberculosis* (4.4 Mb) (Cole et al., 1998). Massive gene decay was shown within the *M. leprae* genome relative to other mycobacteria and the number of ORFs was estimated at approximately 1600. A previous study using RFLP analysis showed no divergence in *M. leprae* isolate genomes, even when originating from different geographic locations (Clark-Curtiss and Docherty, 1989; Williams et al., 1990). Comparative genome analysis also confirmed the lack of divergence in *M. leprae* isolates. There is no significant difference between whole genomes of the two *M. leprae* strains, TN and Br4923 except SNPs subtyping, although they were isolated from countries that rank among those with the highest leprosy burden, yet are geographically remote (India and Brazil) (Monot et al., 2009). In contrast, single nucleotide polymorphisms (SNPs) and variable number of tandem repeat (VNTR) analysis indicated some

\* Corresponding author. Tel.: +81 42 391 8211; fax: +81 42 391 8807.

E-mail addresses: [mkai@nih.go.jp](mailto:mkai@nih.go.jp) (M. Kai), [n-nakata@nih.go.jp](mailto:n-nakata@nih.go.jp) (N. Nakata), [Matsuoka@nih.go.jp](mailto:Matsuoka@nih.go.jp) (M. Matsuoka), [sekizuka@nih.go.jp](mailto:sekizuka@nih.go.jp) (T. Sekizuka), [makokuro@nih.go.jp](mailto:makokuro@nih.go.jp) (M. Kuroda), [mmaki@nih.go.jp](mailto:mmaki@nih.go.jp) (M. Makino).

variability in the genomic DNA of *M. leprae* isolates (Groathouse et al., 2004; Matsuoka et al., 2004; Monot et al., 2005; Zhang et al., 2005).

*M. leprae* cannot be cultivated in artificial medium and thus animal models such as the armadillo or athymic nude mice are utilized to grow *M. leprae* bacilli for research. Importantly, the animal models take 1–2 years to establish infection, requiring a great deal of time, manpower, and cost. Moreover, to successfully culture *M. leprae* bacilli from a human biopsy sample, the biopsy materials should be taken from patients who are not undergoing drug treatment. Recently the number of leprosy patients has decreased globally and, although welcomed from a public health perspective, has resulted in a scarcity of biopsy samples for research. The Leprosy Research Center in Japan has established and maintained many *M. leprae* strains over the course of 5 decades (Matsuoka, 2010a,b). We therefore analyzed the genomes of Japanese *M. leprae* strains to identify any small differences as compared with strains TN and Br4923.

## 2. Materials and methods

### 2.1. Bacterial strains

*M. leprae* Thai-53 and Kyoto-2 strains were selected for genome analysis. The Thai-53 strain, derived from an multibacillary (MB) patient in Thailand, has been maintained in the Leprosy Research Center in Japan for a long period upon establishment in the nude mouse footpad system (Kohsaka et al., 1976), and supplied to researchers both foreign and domestic (Matsuoka, 2010b). The Kyoto-2 strain was established from biopsy sample obtained at skin clinic of Kyoto University, Japan (Matsuoka, 2010a). The *M. leprae* strains were passaged in the nude mouse footpad at every 10 months. In addition, a genotyping analysis was undertaken on 34 other isolates, comprised of 17 isolates from the Leprosy Research Center in Japan, 3 isolates from Thailand, 1 isolate from

Indonesia, 7 isolates from Korea, and 6 isolates from the Philippines.

### 2.2. Preparation and sequencing of genomic DNA

Genomic DNA was extracted from the isolates using the QIA-amp DNA mini kit (Qiagen). Shotgun DNA libraries were generated according to the manufacturer's sample preparation protocol for genomic DNA (Bentley et al., 2008). Briefly, genomic DNA was randomly sheared. Following ligation of a pair of adaptors to the repaired ends, the DNA was amplified using adaptor primers, and fragments were isolated. Sequencing analysis was done by Illumina GAllx and reads were mapped to the published complete genome sequence for *M. leprae* strain TN (<http://genolist.pasteur.fr/Leproma/>). Several gaps were detected and filled in by standard PCR and direct sequencing. The genome sequences of Thai-53 and Kyoto-2 were compared with those of strains TN and Br4923 using Mapview software for genome analysis, with the exception of dispersed repeat regions. In addition, a more limited analysis was done on other strains obtained from collaborators in the Southeast Asian countries.

## 3. Results

### 3.1. SNPs analysis

The whole genome sequence, with the exception of the dispersed repeat regions of *M. leprae* Kyoto-2, was determined and compared to that of other *M. leprae* strains. Some peculiar differences, relevant to the Kyoto-2 genome, were found. We focused on one of those interesting differences in the Kyoto-2 genome. The ML0411 gene of Kyoto-2 had six SNPs and five nucleotide differences compared to that of TN and Br4923, respectively. All six SNPs were non-synonymous mutations that result in amino acid replacement. Moreover, one base change was observed at 41 bp

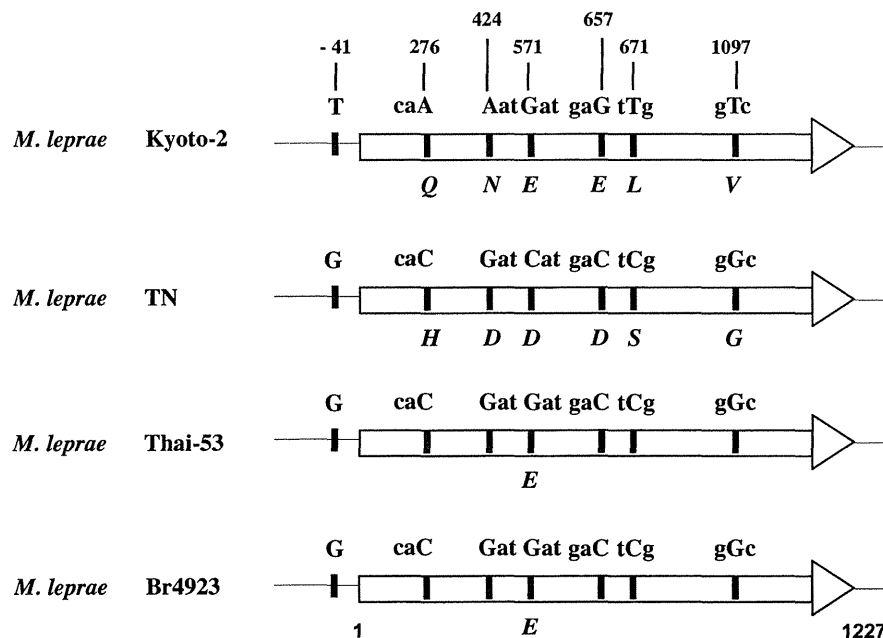


Fig. 1. Comparison of nucleotide sequences of *M. leprae* strains in gene ML0411 and the upstream region. ML0411 gene is indicated with arrows. The letters above arrows show nucleotide bases and set of the three bases indicate codons. SNPs sites of nucleotide bases are indicated with capital letters. The numbers show SNPs position on ML0411. The letters below arrows show amino acids by one letter notation.

upstream of the start codon of ML0411 in the regulatory region (Fig. 1). No other ORF has as many non-synonymous mutations in the *M. leprae* genome. Within the TN and Br4923 strains, there is only one SNP within 191st codon (at position 571st in base sequence) of ML0411.

### 3.2. Genotyping

The sequences of seven SNP sites in the ML0411 region were investigated in 36 *M. leprae* isolates including Thai-53 and Kyoto-2. The SNP pattern of 19 isolates (12 isolates from Honshu Island in Japan and 7 isolates from Korea) showed a pattern identical to that of Kyoto-2 except at nucleotide 657 (Table 1). On the other hand, all Japanese strains in this study belonged to SNP type III with the exception of one (Airaku-2), that contains a SNP type I involving nucleotides at positions 14676, 164275, and 2935685 of the *M. leprae* TN genome DNA. We compared the standard SNP typing (type I–IV) and the characteristic seven SNPs in Kyoto-2, with the geographical distribution of strains. The results show that isolates having the Kyoto-2 SNP pattern in gene ML0411, including all Korean isolates, were SNP type III, while the other Japanese isolates tested were SNP type III or I. Furthermore, the isolates having the Kyoto-2 SNP pattern turned out to be similar to that of the tandem repeat of six bases (GACATC) in the *rpoT* gene, which has been well-established as a tool for *M. leprae* genotyping. All seven

Korean *M. leprae* isolates tested in this study, as well as those 12 isolates derived from Honshu Island in Japan, showed 4 copies of the 6-base tandem repeats. On the other hand, isolates derived from Okinawa Island in Japan and from the Philippines only contained 3 copies of the tandem repeat (Fig. 2).

### 4. Discussion

Several genetic markers of *M. leprae* have been researched and evaluated for use as epidemiological tools for strain differentiation (Lavania et al., 2007; Matsuoka et al., 2000; Phetsuksiri et al., 2012). SNP typing using nucleotides polymorphisms at three sites of *M. leprae* TN genomic DNA were established (Monot et al., 2005). Four types of SNPs, C–G–A, C–T–A, C–T–C and T–T–C at positions, 14676, 164275, and 2935685 were proposed as Type I–IV in the SNP typing and adopted globally (Sakamuri et al., 2009; Watson and Lockwood, 2009). Recently, a more detailed subtyping system composed of 16 subtypes (subtypes A to P) from 84 SNPs was reported (Monot et al., 2009). Almost all of the Japanese isolates belong to SNP type I or III (Matsuoka, 2010b). The subtypes of the type III Japanese strains, Kyoto-2 and Hoshizuka-4, were determined to be K (unpublished data). Interestingly, six of the unique 7 SNPs in the ML0411 region found in Kyoto-2 (T, A, A, G, T, and T at positions, –41, 276, 424, 571, 671, and 1097, respectively) were not only specific for Kyoto-2 but also for the other Japanese

**Table 1**  
Genotyping of East Asian isolates of *M. leprae* by using several tools.

ML0411	Nucleotide position							Number of 6bp repeat in <i>rpoT</i>	SNPs type (L,IV)	SNPs sub-type (A,P)	Distribution*
	–41st	276th	424th	571st	657th	671st	1097th				
	Amino acid position										
	–	92nd	142nd	191st	219th	224th	366th				
TN	G	C	G	C	C	C	G	3	I	A	S
Br4923	G	C	G	G	C	C	G	3	IV	P	
Hoshizuka-4	T	A	A	G	C	T	T	4	III	K	N
Kanazawa	T	A	A	G	C	T	T	4	III	ND	
Keifu-4	T	A	A	G	C	T	T	4	III	ND	
Kitasato	T	A	A	G	C	T	T	4	III	ND	
Kusatsu-3	T	A	A	G	C	T	T	4	III	ND	
Kusatsu-6	T	A	A	G	C	T	T	4	III	ND	
Kyoto-1	T	A	A	G	C	T	T	4	III	ND	
<b>Kyoto-2</b>	<b>T</b>	<b>A</b>	<b>A</b>	<b>G</b>	<b>G</b>	<b>T</b>	<b>T</b>	<b>4</b>	<b>III</b>	<b>K</b>	
Oku-4	T	A	A	G	C	T	T	4	III	ND	
Tsukuba-1	T	A	A	G	C	T	T	4	III	ND	
Zensho-2	T	A	A	G	C	T	T	4	III	ND	
Zensho-4	T	A	A	G	C	T	T	4	III	ND	
Zensho-5	T	A	A	G	C	T	T	4	III	ND	
Zensho-9	G	C	G	G	C	C	G	3	III	ND	S
Airaku-2	G	C	G	G	C	C	G	3	I	ND	
Airaku-3	G	C	G	G	C	C	G	3	III	ND	
Amami	G	C	G	G	C	C	G	3	III	ND	
Ryukyu-2	G	C	G	G	C	C	G	3	III	ND	
Ryukyu-6	G	C	G	G	C	C	G	3	III	ND	
Thai-53	G	C	G	G	C	C	G	3	I	A	
Thai-237	?	A	?	G	C	C	G	3	I	ND	
Thai-311	G	C	G	G	C	C	G	3	II	ND	
Indonesia	G	C	G	G	C	C	G	3	II	ND	
Korea-1	T	A	A	G	G	T	T	4	III	ND	N
Korea-4	T	A	A	G	C	T	T	4	III	ND	
Korea-5	T	A	A	G	C	T	T	4	III	ND	
Korea-6	T	A	A	G	C	T	T	4	III	ND	
Korea-7	T	A	A	G	C	T	T	4	III	ND	
Korea-8	T	A	A	G	C	T	T	4	III	ND	
Korea-9	T	A	A	G	C	T	T	4	III	ND	
Cebu-1	G	C	G	G	C	C	G	3	II	A	S
Cebu-2	G	C	G	G	C	C	G	3	II	ND	
Cebu-3	G	C	G	G	C	C	G	3	II	A	
Cebu-4	G	C	G	G	C	C	G	3	II	ND	
Cebu-5	G	C	G	G	C	C	G	3	II	ND	
Cebu-6	G	C	G	G	C	C	G	3	II	ND	

\* S: type of southern islands of Japan, N: type of Honshu island of Japan.

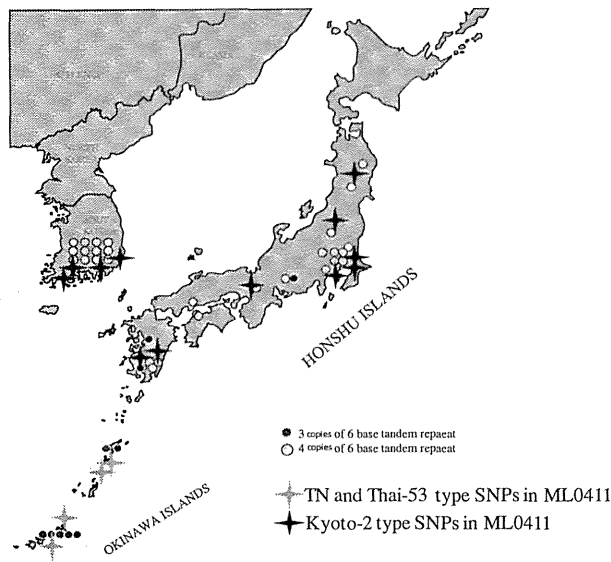


Fig. 2. Geographical distribution of *M. leprae* isolates derived from Japan and Korea having different genotypes.

isolates stocked in the Leprosy Research Center in Japan (Fig. 2). Some isolates derived from areas of the Philippines and Korea geographically nearest to Japan showed that the geographical distributions of the six SNP patterns in ML0411 were divided into two groups: those that contain the Kyoto-2 pattern (T-A-A-G-T-T) including others isolated in Honshu Island of Japan and Korean isolates, or the Thai-53 pattern (G-C-G-C-C-G) including others isolated in the Okinawa Islands of Japan, the Philippines and other southern countries (data not shown). The isolates having the Kyoto-2 SNP pattern in the ML0411 region were SNP type III whereas Thai-53 SNP patterns in the ML0411 region in the other Japanese isolates tested were SNP type I or III. Furthermore, the isolates having the Kyoto-2 SNP pattern turned out to contain 4 copies of the 6-base tandem repeat (GACATC) in the *rpoT* gene, which is well established as a tool for *M. leprae* genotyping (Lavania et al., 2007; Matsuoka et al., 2000). Therefore, these seven SNPs in ML0411 might be useful for studying the geographic distribution of *M. leprae* in the South and East Asian (SA & EA) regions or at a global level. The high frequencies of the standard SNP type I and III and the 3 tandem repeats in *rpoT* are common in SA & EA countries (Matsuoka et al., 2006). The results from this study seem to indicate that some *M. leprae* lineages, showing SNP type III, 4 copies of tandem repeats in *rpoT*, and the Kyoto-2 SNP pattern in ML0411, appear to have disseminated and become established among the people of neighboring and proximal SA & EA countries such as China, Korea and Japan. This linkage might have occurred by genetic linkage in the evolutionary process of *M. leprae*. There has been no report of the existence of such a special lineage in *M. leprae* globally, and these Northeast Asian (NA) strains of *M. leprae* should be further characterized. Recently, *Mycobacterium lepromatosis* was found and established to be closely related to *M. leprae*, but clearly distinct from *M. leprae* (Han et al., 2008; Vera-Cabrera et al., 2011). Although *M. lepromatosis* harbors 4 copies of 6-base tandem repeat in the *rpoT* gene, five nucleotides differ in the flanking region of 6-base tandem repeat and *M. lepromatosis* contains 3 repeats of 21 nucleotides, CGAGCCCAATACAGCATCT in *rpoT* gene but not *M. leprae*. All NA strains with 4 copies of 6-base tandem repeat in the *rpoT* gene showed identical nucleotide sequences at flanking region of 6-base tandem repeat with *M. leprae* and do not contain 21-base repeats, thus NA strains are clearly

belonging to the *M. leprae*. However, here we describe for the first time genotyping by using three indexes, namely, SNPs typing, copy number of 6 base repeats in *rpoT*, and SNPs pattern in ML0411. The grouping of the NA strain is the first report of a strain that has different genotype from that of *M. leprae* strain reported.

The locations of ML0411 and *rpoT* on the *M. leprae* genome are separated by approximately 100 kb. To confirm the possible genetic linkage between ML0411 and *rpoT*, three separate SNPs of the Kyoto-2 genome located between the two genes were selected (coordinates in the *M. leprae* TN genome are 12324, 34353, and 54342) in several *M. leprae* isolates. There was no indication that the SNP pattern can be separated in the Kyoto-2 and TN strains (Fig. S1). The results indicate that there was no genetic linkage between ML0411 and *rpoT*. Therefore, the non-synonymous mutations on ML0411 and variation of copy number of the 6-base tandem repeats in *rpoT* might be evolutionally independent and reflect regional characteristics of *M. leprae* strains. The nucleotide bases of the 191st codon (GAT) in strain TN are changed to CAT in strain Br4923. Combining analysis of the *rpoT* and ML0411 patterns might be useful for identifying not only the NA strain of *M. leprae* but also the detailed genotyping of *M. leprae* strains globally.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.meegid.2013.07.014>.

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## Invited Review

# Buruli Ulcer and Mycolactone-Producing Mycobacteria

Kazue Nakanaga<sup>1\*</sup>, Rie Roselyne Yotsu<sup>2</sup>, Yoshihiko Hoshino<sup>1</sup>,  
Koichi Suzuki<sup>1</sup>, Masahiko Makino<sup>1</sup>, and Norihisa Ishii<sup>1</sup>

<sup>1</sup>Leprosy Research Center, National Institute of Infectious Diseases, Tokyo 189-0002; and  
<sup>2</sup>Department of Dermatology, National Center for Global Health and Medicine,  
Tokyo 162-8655, Japan

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**SUMMARY:** Buruli ulcer (BU) is an emerging human disease caused by *Mycobacterium ulcerans*, which mainly affects the extremities. It is most endemic in sub-Saharan Africa; however, it has been reported worldwide, including in some non-tropical areas. “*M. ulcerans* subsp. *shinshuense*” is proposed as a subspecies of *M. ulcerans*, which have been reported from Japan and China. A total of 35 BU cases have been reported as of November 2012. Although *M. ulcerans* is categorized as nontuberculous mycobacteria, it has some unique characteristics that could only be observed in this bacterium. It possesses a giant virulent plasmid, composed of 174-kbp nucleotides, coding polyketide synthase to produce macrolide toxin called mycolactone. The discovery of such a linkage of plasmid and its pathogenesis has not been reported in other human disease-causing mycobacteria.

## 1. Introduction

Buruli ulcer (BU) is a necrotizing skin disease caused by *Mycobacterium ulcerans*, belonging to nontuberculous mycobacteria (NTM) family. It has a unique characteristic of producing a macrolide toxin called “mycolactone” (1-5), which has not been identified in any other species of mycobacteria including *Mycobacterium tuberculosis* and *Mycobacterium leprae*. BU has been reported in at least 33 countries, and *M. ulcerans* disease is probably the third most common mycobacterial disease after tuberculosis and leprosy in some endemic areas among immunocompetent individuals (6). BU is categorized as a neglected tropical disease by the World Health Organization (WHO). The most endemic areas include sub-Saharan Africa, and a control strategy has been implemented to minimize morbidity and disability associated with BU especially in these areas (Fig. 1). The mode of transmission remains unclear;

however, it is speculated that an aquatic environmental source is the origin of infection. Similarities in characteristics are found in other mycobacterial strains, which mainly infect frogs, fish, or turtles (7-9), and therefore, they must be differentiated when searching for *M. ulcerans* in environmental sources. In this review, the history of researches on *M. ulcerans* and other mycolactone-producing mycobacteria are described, focusing on mycolactone and their virulent plasmids.

## 2. Epidemiology and symptoms of BU

### 2-1. History

Historically, BU first gained notice from two foci. One focus was in Central Africa where the first related description was made by Sir Albert Cook. He described several cases of chronic large ulceration in Uganda in 1897. Later on, the disease was named after Buruli County in Uganda, where the first large epidemic was investigated in the 1960s (10,11). At present, sub-Saharan Africa, especially Cote d’Ivoire, Ghana, and Benin are the highest endemic countries to be controlled. The other focus is in southeastern Australia. In the first report, there was a series of unusual painless ulcers in a patient from Bairnsdale in 1935 (12). Thirteen years after the first report, MacCallum et al. made the first description of the mycobacterial infection in 1948 (13). The name *M. ulcerans* first appeared in a report by Fenner in 1952 (14). Currently, Bellarine Peninsula in Victoria is known to be the highest endemic area in Australia (15).

\*Corresponding author: Mailing address: Department of Mycobacteriology, Leprosy Research Center, National Institute of Infectious Diseases, Aoba-cho 4-2-1, Higashimurayama-shi, Tokyo 189-0002, Japan. Tel: +81-42-391-8211, Fax: +81-42-394-9092, E-mail: nakanaga@nih.go.jp

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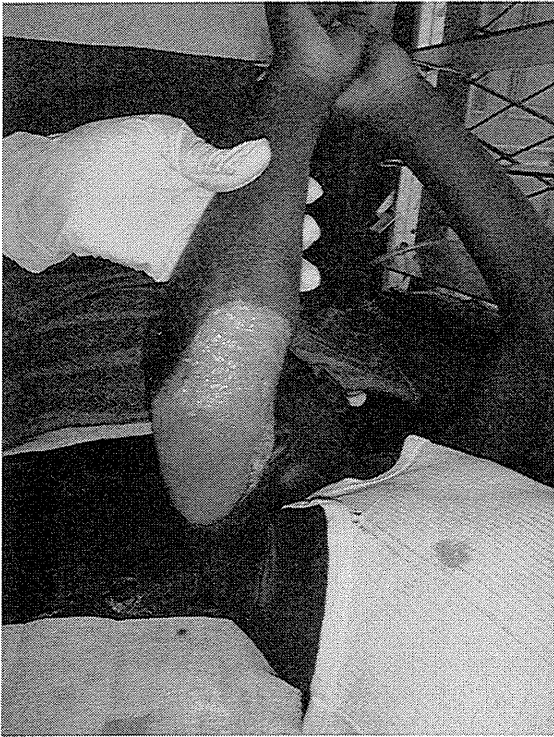


Fig. 1. A Buruli ulcer case in Amasaman Hospital (Accra, Ghana); a large ulcer on the left elbow extensor of an 8-year-old boy. The photograph was taken by Nakanaga, K. and Yotsu, R. R. at the Amasaman Hospital with the approval of the patient's parent, the director of the hospital, and the program manager of the National Buruli Ulcer Control Programme (Dr. Edwin Ampandou), during their visit to Ghana in November 2011 with support of Dr. Kingsley Asiedu from the Global Buruli Ulcer Initiative, Department of Control of Neglected Tropical Medicine, WHO, Geneva.

## 2-2. Symptoms

BU often starts as a painless papule or nodule, looking like an insect bite. However, it gradually leads to the destruction of skin and ulceration. Interestingly, despite their severity, the lesions are often painless. The ulcer usually presents with a yellow-whitish necrotic base with undermined borders and edematous surroundings. Major affected sites are the extremities and the face. If patients seek treatment during early stages, antibiotics (rifampicin and streptomycin as recommended by the WHO) can prove to be successful. Delayed treatment may cause irreversible deformity, long-term functional disabilities such as restriction of joint movement, extensive skin lesions, and sometimes life-threatening secondary infections (1-5).

## 2-3. BU in Japan

The first reported case of BU in Japan was a 19-year-old woman in 1980 (16). The causative agent was isolat-

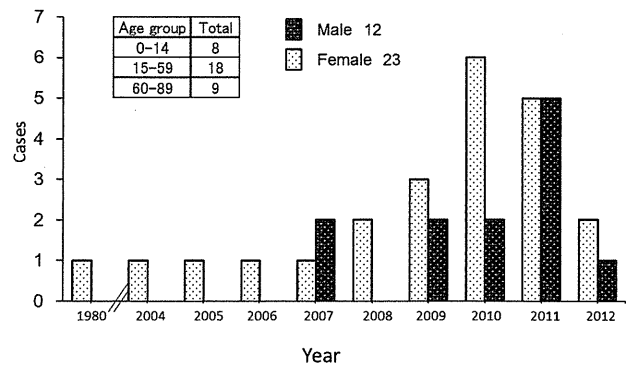


Fig. 2. Buruli ulcer cases in Japan by year diagnosed as of November 2012 (28,29).

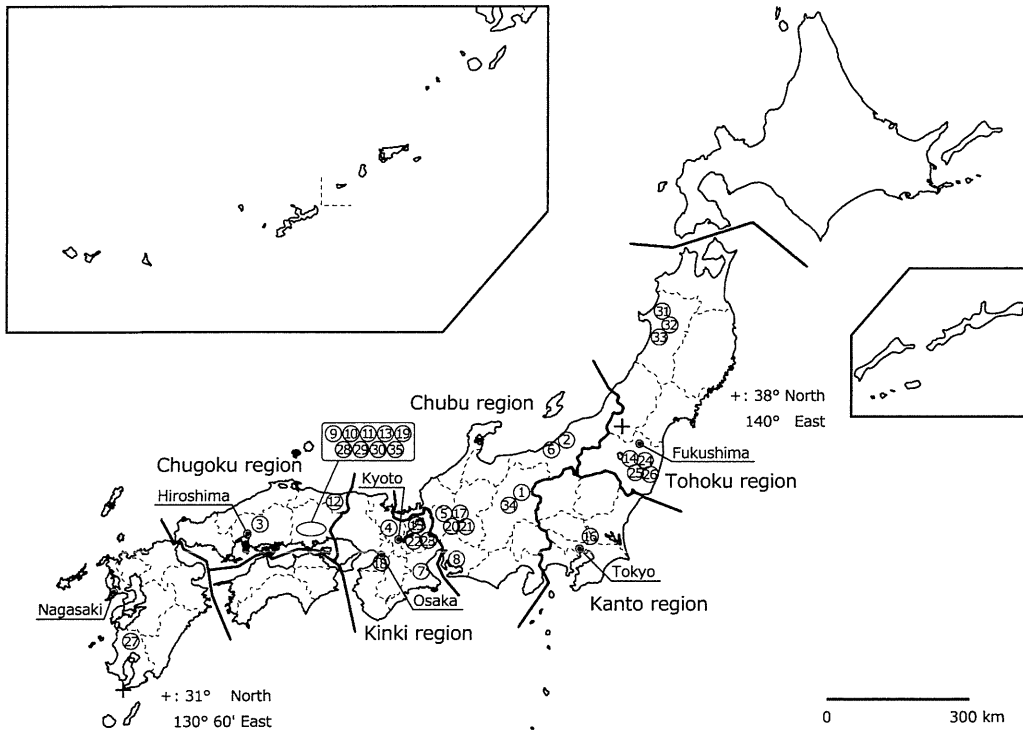


Fig. 3. Distribution of Buruli ulcer cases in Japan: a total of 35 cases as of November 2012 (28,29).

Table 1. Characteristics of cases reported in Japan as of November 2012 (28,29)

Known isolate	“ <i>Mycobacterium ulcerans</i> subsp. <i>shinshuense</i> ”
International traveling	None
Mode of transmission	Unknown, not clear with aquatic environment <sup>1)</sup>
Regional bias	Honshu island (northern limit, Akita Prefecture; southern limit, Kagoshima Prefecture; no case in Shikoku island)
Seasonal bias	Autumn and winter (unclear incubation period)
Age	2–84 years
male cases:female cases	12:23
Pain sensation	More outstanding in Japanese cases
Sensitivity against antibiotics	Sensitive
Affected regions	Usually extremities (legs, arms, or face)
Size of ulcer	Mainly < 5 cm (category I)

<sup>1)</sup>: Three cases in one family member often contact with tiny stream on their property.



Fig. 4. A Buruli ulcer case in the National Hospital Organization (NHO) Higashihiroshima Medical Center (Hiroshima, Japan); an ulcer on the right elbow extensor of a 20-year-old woman (19,20). The photograph was provided by the attending doctor (Dr. Hideaki Imada) in the NHO Higashihiroshima Medical Center.

ed and classified as “*M. ulcerans* subsp. *shinshuense*” because it was closely related to *M. ulcerans* (17). The second reported case was a 37-year-old woman in 2003 (18). After the second report, there has been a steady increase, summing up to a total of 35 cases as of November 2012 (16–26) (Fig. 2). Among all cases, 23 (65.7%) subjects were female and 12 (34.3%) were male. The average age of those affected was 39.7 years (range, 2–84 years) for females and 43.3 years (range, 5–81 years) for males. Eleven cases were found in the Chugoku region, 9 in the Chubu region, 7 in the

Tohoku region, 6 in the Kinki region, 1 in the Kanto region, and 1 in the Kyushu region (Fig. 3). Although there was no geographical focus in the distribution, 3 out of 4 cases in Fukushima Prefecture (Tohoku region) were family members. The highest number of cases (9 cases) was found in Okayama Prefecture (Chugoku region). There has also been one report from China of which *M. ulcerans* subsp. *shinshuense* was isolated from an ulcer (27). Hence, *M. ulcerans* subsp. *shinshuense* is now speculated to be an endemic species of *M. ulcerans* in east Asia (28,29). This subspecies has been isolated or at least confirmed through direct nucleotide sequencing in the identified cases. The characteristics of BU in Japan are shown in Table 1. Though there seem to be some differences in clinical presentation such as more painful cases in Japanese cases (Fig. 4), histopathological findings do not differ from those of other parts of the world. Unlike other mycobacterial infections, the histopathology of BU presents with poor formation of granulation tissue and little inflammatory cell infiltration surrounding the ulcerative lesion. Although there is extensive necrosis, the circumjacent epidermis is usually unaffected, and the lesions are usually deeper in the subcutaneous areas. Acid-fast bacilli are often evident on the lower dermis.

### 3. Mycolactone and mycolactone-producing mycobacteria

#### 3-1. Isolation of mycolactone

From the very beginning, pathologists were certain that some toxic substances from *M. ulcerans* relate to the unique characteristic of the skin lesions in BU because histopathological changes were observed distant from where massive acid-fast bacilli were detected (30–32). In 1999, mycolactone was isolated as the cause of cytopathicity and cell-cycle arrest in cultured L929 murine fibroblasts (33). Guinea pigs produced lesions similar to those of BU in humans following purified mycolactone injection (34). Mycolactone is not a protein but a smaller molecule, which is composed of a 12-membered ring (macrolide) to which two polyketide-derived side chains are attached. It structurally resembles immuno-suppressants such as rapamycin, FK506, and cyclosporin A. The characteristic mixture of mycolactone congeners varies in clinical isolates from geographically different areas (35). The structure that

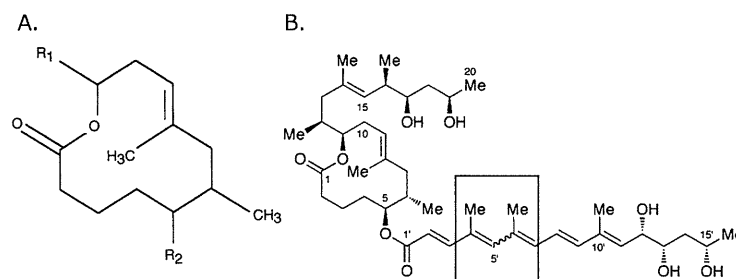


Fig. 5. Basic structure of mycolactone and complete structure of mycolactone A/B. Boxed site indicates isomers A (cis) and B (trans) (33,36).

has the highest activity in mammalian cell lines is mycolactone A/B, which exists as a 3:2 equilibrating mixture; the major and minor components are Z- $\Delta 4',5'$ - and E- $\Delta 4',5'$ -isomers, respectively, on the unsaturated fatty acid side chain (36) (Fig. 5).

### 3-2. Discovery of mycolactone synthetase bearing plasmid pMUM001

Attempts to identify genetic bases of the virulent factor in *M. ulcerans* started by comparing closely related mycobacterial strain *Mycobacterium marinum* (37). This idea of subtraction was thought of because these two strains were genetically related species but *M. marinum* produced no mycolactone. A type I polyketide synthase (pks) gene fragment was identified as an *M. ulcerans* specific gene based on genomic suppressive subtractive hybridization between *M. ulcerans* and *M. marinum* (38). In 2004, a giant plasmid, which bears mycolactone-producing enzymes, was identified using pks probes identified through subtractive hybridization. These probes were hybridized using pulsed-field gel electrophoresed DNA or a BAC library of the *M. ulcerans* Ghana isolate Agy99. The plasmid pMUM001 is composed of 174,155 bp, as a 62.8% GC content, and its 81 protein-coding DNA sequences bears a cluster of genes for complete mycolactone synthesis (39,40). The discovery of pMUM001 had profound implications for mycobacterial research because mycobacterial plasmids have never been directly linked to virulence. Moreover, the *M. tuberculosis* complex, the most virulent mycobacterium for humans, is reported to have no plasmids.

### 3-3. Role of mycolactone

The role of mycolactone in *M. ulcerans* survival in nature is not clarified. On the other hand, during infection of humans, which causes BUs, the function of mycolactone is thought to cause "painless lesions" and "poor acute inflammatory cellular infiltration." Although its mechanisms have not yet been revealed, painless lesions were successfully demonstrated by *M. ulcerans* infection and/or purified mycolactone injection in mice. Nerve degeneration occurs through invasion of bacilli or mycolactone at the perineural and endoneurial level, inducing loss of pain sensation or hypoesthesia (41,42). Mycolactone effectively suppresses the capacity of dendritic cells to secrete  $\beta$ -chemokines macrophage inflammatory protein (MIP)-1 $\alpha$ , MIP-1 $\beta$ , regulated on activation, normal T cell expressed and secreted, as well as monocyte chemoattractant protein 1 and interferon  $\gamma$ -inducible protein 10, which may limit initiation of primary immune responses. Moreover, it inhibits the

recruitment of inflammatory cells to the infection site (43).

### 3-4. Other mycolactone-producing mycobacteria

In 2001, there was an outbreak of a new granuloma-forming disease in a laboratory, which killed a number of experimental frogs (*Xenopus tropicalis*). Granulomas were identified systemically on the skin as well as in other areas; the causative agent was later identified as *Mycobacterium liflandii* (44). It formed orange-pigmented colonies after long cultivation, possessed giant plasmid pMUM002 similar to pMUM001, and had IS2404 and IS2606 like *M. ulcerans*. The product of pMUM002 has now been identified as mycolactone E (7). *M. liflandii* was initially thought to be the causative pathogen for BU; however, it is now known that it infects mainly frogs (45).

Some *M. marinum* strains isolated from fish are known to be *Mycobacterium pseudoshottsii*, which have IS2404, IS2606, and pMUM003 (similar to plasmid pMUM001), and produce mycolactone F (8). *M. pseudoshottsii* has been isolated in Japan, especially from various types of farm-raised fish such as yellow tail (*Seriola quinqueradiata*), greater amberjack (*Seriola dumerili*), sevenband grouper (*Epinephelus septemfasciatus*), striped jack (*Pseudocaranx dentex*), and yellow-tail amberjack (*Seriola lalandi*) (46). *M. pseudoshottsii* were isolated from the kidney of the fish that were lethally affected. The genomes of some of these mycolactone-producing mycobacteria were analyzed, and interestingly, it is now evident that these species are very closely related in their evolution. What is more striking, differences in the genome sequencing of the plasmids for pMUM001, pMUM002, and pMUM003 correspond to differences in the structures of the unsaturated fatty acid side chains for each type of mycolactone, namely, A/B, E, and F (47,48).

## 4. Conclusion

Some researchers have reported that mycobacterial strains that have pMUM001-like plasmids and produce mycolactone should be considered a single species (49). This conclusion emerged from a number of genomic studies. Nonetheless, infection of these mycobacteria in fish or frogs, especially in places with high risk of infection such as fish farms, is causing a considerable anxiety in people. Therefore, there is a need to understand the possibility of infection from animals to humans, a need for invention of easy diagnostic tools, and a need to raise awareness among people about the mycobacteria

with the right information.

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**Conflict of interest** None to declare.

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## Basophils Infiltrate the Skin Lesions in Lepromatous Leprosy

Atsushi Otsuka<sup>1</sup>, Motoaki Ozaki<sup>1,2</sup>, Yuji Horiguchi<sup>3</sup>, Yozo Murata<sup>4</sup>, Kimiko Kumano<sup>4</sup>, Reiko Nogami<sup>5</sup>, Masamichi Goto<sup>6</sup>, Andrew F. Walls<sup>7</sup>, Norihisa Ishii<sup>8</sup>, Yoshiki Miyachi<sup>1</sup> and Kenji Kabashima<sup>1\*</sup>

Departments of Dermatology, <sup>1</sup>Kyoto University Graduate School of Medicine, 54 Shogoin-Kawara, Sakyo, Kyoto 606-8507, <sup>2</sup>National Sanatorium Nagashima Aiseien, Okayama, <sup>3</sup>Osaka Red Cross Hospital, Osaka, <sup>4</sup>Hyogo Cancer Center, Akashi, <sup>5</sup>National Sanatorium Kikuchi Keifuen, Kumamoto, <sup>6</sup>National Sanatorium Hoshizuka Keinen, Kagoshima, Japan, <sup>7</sup>Immunopharmacology Group, University of Southampton, Southampton General Hospital, Southampton, UK, and <sup>8</sup>Leprosy Research Center, National Institute of Infectious Diseases, Higashimurayama, Japan. E-mail: otsukam@kuhp.kyoto-u.ac.jp, \*kabash@kuhp.kyoto-u.ac.jp

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Leprosy is caused by *Mycobacterium leprae* and is manifested by damage to the skin and peripheral nerves. The disease is characterized by a spectrum of histologically different granulomatous skin lesions that reflects the immune response, varying from predominantly epithelioid cells with an absence or only occasional presence of bacilli at the tuberculoid end of the spectrum (TT) to an abundance of bacilli-filled foamy macrophages at the lepromatous leprosy (LL) end (1). While TT is characterized by strong Th1 immunity in the form of a systemic antigen-specific Th1 cellular immunity, LL is characterized by a high number of bacilli disseminated throughout the skin, with lesions expressing high Th2-type cytokines. The immunologically unstable borderline patients are classified as borderline tuberculoid (BT), mid-borderline (BB), and borderline lepromatous (BL). These types of leprosy depend on the host immune balance between Th1 and Th2.

It is well established that basophils exert effector functions during allergic responses through the cross-linking of FcεR1 during immediate- or late-phase reactions following allergen exposure. In addition, recent studies have shown that basophils promote Th2 skewing by antigen presentation in helminth infection and in response to protease allergens, and that dendritic cells are not necessary for this process to occur (2, 3). However, it has not been fully determined whether basophils play a role in the control of leprosy infection.

The monoclonal antibody BB1 recognizes a unique granule constituent of basophils. Taking advantage of this antibody, recent studies have demonstrated that basophils infiltrate inflammatory skin diseases (4, 5). It is not known whether basophils actually infiltrate the skin lesions of leprosy, and it is therefore necessary to analyse and compare each type of leprosy. We demonstrate here that basophils are significantly increased in number in the skin lesions of LL.

### MATERIAL AND METHODS

Skin biopsies from 45 patients with leprosy (33 males, 12 females; mean age 54.9 years; LL 24 patients; BL 11 patients; BT 7 patients; TT 3 patients; diagnosed by dermatopathologists) were examined by immunostaining, as reported previously (5). BB1 and 2D7 antibodies were purchased from BioLegend, San Diego, CA, USA.

### RESULTS

BB1-antibody-positive cells were observed to infiltrate only the skin lesions of LL (Fig. 1A). Consistently, anti-human basophils 2D7-positive cells (Fig. 1B, E) were observed only in the skin lesions of LL, but not in the lesions of BL, BT and TT (Fig. 1D). As a control, isotype-matched control mouse IgG1 were used (clone: MOPC-21), which showed negative in the skin lesions of LL (Fig. 1C). The number of 2D7<sup>+</sup> basophils infiltrating the skin lesions of LL range 0–18 (mean 2.5, standard error of the mean (SEM) 0.87) (Fig. 1F), was significantly higher than that of BL, BT and TT.

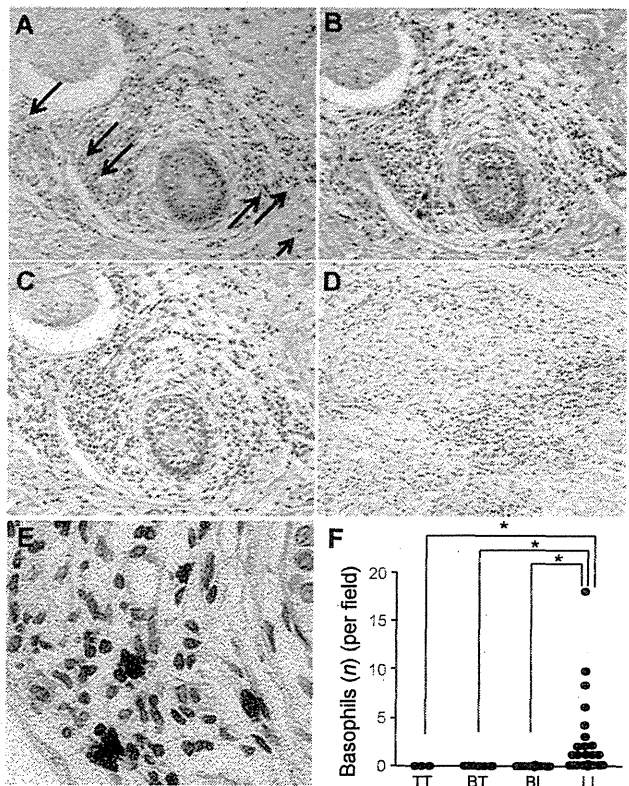


Fig. 1. Immunohistochemistry of basophils in leprosy skin lesions. Positive cells for (A) BB1 and (B) 2D7, but not for (C) isotype-matched control antibodies, were observed in the skin lesions of LL, but (D) not in the skin lesion of TT (E) High-power field of B. (F) The number of 2D7 cells was calculated in five high-power fields ( $\times 20$  objective). Data were analysed with the paired *t*-test. A *p*-value of less than 0.05 was considered statistically significant. (A, B, C, D  $\times 20$ .)



## DISCUSSION

In this study we evaluated for the first time the presence of basophils in the skin lesions of patients with leprosy. Interestingly, basophils infiltrated only into LL but not into BL, BT and TT. The recruitment of basophils to LL only might be due to the expression of the chemokine receptor CCR3 on basophils. Eotaxin 1 and 3, which are ligands of CCR3, are produced by dermal fibroblasts in response to Th2-type cytokines. Previous reports have shown that eotaxin 1 was identified as a plasma marker of LL and BL (1). In our study, however, basophils were not detected in the skin lesions of BL. One possible explanation for this discrepancy might be the different eotaxin levels of skin lesions in LL and BL cases. *M. leprae* antigens normally elicit Jones-Mote hypersensitivity, peaking at 48–72 h, which is in contrast to Th1-induced delayed-type hypersensitivity, peaking at 24 h (6). Since the characteristic of Jones-Mote hypersensitivity is a massive basophil infiltration, the infiltration of basophils into the skin lesions of LL might be due to Jones-Mote hypersensitivity induced by *M. leprae* antigens. Although the role of basophils in the development of leprosy is still unknown, the presence of basophils in LL skin lesions raises the possibility

that basophils may play a role in the pathogenesis of the LL type of leprosy and in the characterization of the subtypes of leprosy.

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# Simple Multiplex PCR Assay for Identification of Beijing Family *Mycobacterium tuberculosis* Isolates with a Lineage-Specific Mutation in *Rv0679c*

Chie Nakajima,<sup>a</sup> Aki Tamaru,<sup>b</sup> Zeaur Rahim,<sup>c</sup> Ajay Poudel,<sup>a</sup> Bhagwan Maharjan,<sup>d</sup> Khin Saw Aye,<sup>e</sup> Hong Ling,<sup>f</sup> Toshio Hattori,<sup>g</sup> Tomotada Iwamoto,<sup>h</sup> Yukari Fukushima,<sup>a</sup> Haruka Suzuki,<sup>a</sup> Yasuhiko Suzuki,<sup>a,i</sup> Takashi Matsuba<sup>l</sup>

Division of Global Epidemiology, Hokkaido University Research Center for Zoonosis Control, Sapporo, Hokkaido, Japan<sup>a</sup>; Osaka Prefectural Institute of Public Health, Osaka, Japan<sup>b</sup>; Tuberculosis Laboratory, International Centre for Diarrhoeal Disease Research, Bangladesh, Dhaka, Bangladesh<sup>c</sup>; German Nepal Tuberculosis Project (GENETUP), Kathmandu, Nepal<sup>d</sup>; Immunology Research Division, Department of Medical Research (Lower Myanmar), Ministry of Health, Yangon, Myanmar<sup>e</sup>; Department of Microbiology, Harbin Medical University, Harbin, China<sup>f</sup>; Laboratory of Disaster-Related Infectious Diseases, International Research Institute of Disaster Sciences, Tohoku University, Sendai, Japan<sup>g</sup>; Department of Microbiology, Kobe Institute of Health, Kobe, Japan<sup>h</sup>; JICA/JST, SATREPS, Tokyo, Japan<sup>i</sup>; Division of Bacteriology, Department of Microbiology and Immunology, Faculty of Medicine, Tottori University, Yonago, Tottori, Japan<sup>l</sup>

**The Beijing genotype of *Mycobacterium tuberculosis* is known to be a worldwide epidemic clade. It is suggested to be a possibly resistant clone against BCG vaccination and is also suggested to be highly pathogenic and prone to becoming drug resistant. Thus, monitoring the prevalence of this lineage seems to be important for the proper control of tuberculosis. The *Rv0679c* protein of *M. tuberculosis* has been predicted to be one of the outer membrane proteins and is suggested to contribute to host cell invasion. Here, we conducted a sequence analysis of the *Rv0679c* gene using clinical isolates and found that a single nucleotide polymorphism, C to G at position 426, can be observed only in the isolates that are identified as members of the Beijing genotype family. Here, we developed a simple multiplex PCR assay to detect this point mutation and applied it to 619 clinical isolates. The method successfully distinguished Beijing lineage clones from non-Beijing strains with 100% accuracy. This simple, quick, and cost-effective multiplex PCR assay can be used for a survey or for monitoring the prevalence of Beijing genotype *M. tuberculosis* strains.**

The *Mycobacterium tuberculosis* Beijing genotype, first identified by van Soolingen et al. (1), is known to be a worldwide epidemic clade (2–4). Its possible resistance to BCG vaccination, in addition to its tendency to have a multidrug-resistant (MDR) phenotype, might give a selective advantage to the wide geographic distribution of the Beijing genotype strains (3, 5–7). Although some of the Beijing genotype strains show hypervirulence in animal infection models (7–9), neither the virulence factor nor the phenotypically specific factor of this lineage has been elucidated. The origin of the Beijing lineage is thought to be east Asia, where the prevalence of this clade is from around 40% to >90% (1, 3, 4, 10–13). However, in some other global areas, i.e., countries in the former Soviet Union and South Africa, the prevalence of the Beijing lineage has increased markedly in a short period, and some increases were suggested to be related to MDR (4, 11, 14). In those areas, higher clonality of the circulating strains was suggested, and most were categorized as being in the modern or typical Beijing clade, which is defined as a strain having one or two IS6110 insertions in the noise transfer function (NTF) chromosomal region (11, 15). On the other hand, a higher variety of strains can be observed in east Asian countries. Especially in Japan and Korea, the majority of the strains belong to another cluster called the ancient or atypical Beijing clade (12, 16). Details regarding the higher pathogenicity of the Beijing lineage are controversial. Some studies have suggested that the modern Beijing clade is more prone to be pathogenic, tends to be drug resistant, and is likely able to escape from BCG vaccination (4, 8, 11, 14); however, some of the ancient Beijing clones were also shown to have higher pathogenicity (17) or a tendency toward acquiring drug resistance (16).

Since Beijing lineage prevalence has a great impact on the tu-

berculosis (TB) control program, several methods to distinguish this clade have been developed. First, van Soolingen et al. (1) identified this clade by its specific IS6110 restriction fragment length polymorphism (RFLP) signatures. Soon after, these strains were shown to have a specific spoligotype pattern lacking spacer numbers 1 to 34, and this has been proposed as the definition of the clade (18, 19), since IS6110 RFLP genotyping is time-consuming, and comparing results between laboratories is difficult. The deletion of spacers observed in the Beijing spoligotype is caused by the insertion of IS6110 in the direct repeat (DR) region (18). Since this typical spoligotype pattern has become a specific marker of the Beijing genotype, some PCR methods to detect this specific deletion, named region of difference 207 (RD207), have been developed (20–22). In addition to RD207, another deleted region named RD105 was also shown to be a good marker for discrimination of the Beijing genotype, although this deletion is common for all the east Asian lineages, including the non-Beijing strains (10, 23); however, most of these published detection methods require expensive real-time PCR equipment and high-cost reagents (24). The conventional PCR assay targeting RD207 still seems to be at a disadvantage, since it relies on an unstable inser-

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Address correspondence to Takashi Matsuba, matsubat@med.tottori-u.ac.jp, or Yasuhiko Suzuki, suzuki@czc.hokudai.ac.jp.

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tion sequence that is likely to be a target of homologous recombination.

Instead of unstable repetitive structures, single nucleotide polymorphisms (SNPs) were recently considered to be a robust target for defining the accurate position of a strain on the phylogenetic tree, since horizontal gene transfer or gene recombination between different strains is rare in the *M. tuberculosis* complex (MTC) (12, 24, 25). Filliol et al. (26) drew phylogenetic trees of the MTC using several typing methods and showed that the dendrogram drawn with SNPs most accurately reflected the true evolution of the MTC. Some of those SNPs are suggested to be specific to the Beijing or east Asian lineages. In a search for membrane proteins that are suitable for vaccine antigens and/or are targets for the specific detection of the MTC, we found a candidate protein encoded by the *Rv0679c* gene. This protein was expressed on the cell surface as a lipoarabinomannan-associated protein (27, 28), and the coding sequence has an SNP that seems to be specific to the Beijing clade. In this study, we confirmed the lineage specificity of this SNP and developed a simple and low-cost multiplex PCR assay to distinguish the Beijing lineage strains.

## MATERIALS AND METHODS

**Preparation of genomic DNA from *M. tuberculosis* isolates.** *M. tuberculosis* was isolated from the sputa or other clinical specimens of patients by conventional procedures using *N*-acetyl-L-cysteine (NALC)-NaOH. A total of 619 isolates obtained in Japan ( $n = 145$ ), Bangladesh ( $n = 122$ ), Nepal ( $n = 110$ ), Myanmar ( $n = 198$ ), and China (Heilongjiang Province,  $n = 44$ ) were used in this study. Some of these isolates were the same as those in previous studies, and the details are described elsewhere (13, 29–31). Colonies grown on egg-based medium (either Ogawa or Löwenstein-Jensen medium) were resuspended in distilled water and boiled for 20 min, and the supernatant was used in the Bangladeshi and Myanmar samples. In the Japanese and Nepalese samples, colonies were suspended in 0.5 ml of 10 mM Tris-HCl, 1 mM EDTA (Tris-EDTA [TE] buffer [pH 8]), and 0.5 ml chloroform; 0.5 g glass beads of 0.17-mm diameter was added; and they were disrupted with a bead beater (MicroSmash; Tomy Seiko Co. Ltd., Tokyo, Japan). After centrifugation at  $10,000 \times g$  for 5 min, DNA in the supernatant was precipitated by ethanol, and the precipitated genomic DNA was resuspended in TE buffer for further use. In China, bacteria grown in a BACTEC *Mycobacterium* growth indicator tube (MGIT) (Becton, Dickinson and Company, Franklin Lakes, NJ) were used, and DNA was extracted by lysozymes and the phenol-chloroform method (13). All the DNA samples extracted in each country were brought to Japan, and the following steps were carried out in the Hokkaido University Research Center for Zoonosis Control. To determine the specificity of the method, DNAs extracted from five reference MTC strains (i.e., *M. tuberculosis* H37Rv, *Mycobacterium africanum* ATCC 25420, *Mycobacterium oryzae* Z0001, *Mycobacterium microti* TC 89, and *Mycobacterium bovis* BCG Tokyo 172) and 30 nontuberculous mycobacterial (NTM) species, including *Mycobacterium avium*, *Mycobacterium intracellulare*, and *Mycobacterium kansasii*, were used.

**Gene sequencing and comparison.** A subset of 197 *M. tuberculosis* samples, 68 from Japan, 92 from Bangladesh, and 37 from Nepal, were chosen from the total 619 clinical isolates, and the *Rv0679c* gene fragment was amplified by PCR. The PCR mixture contained GoTaq PCR buffer (Promega Co., Madison, WI), 0.2 mM each deoxynucleoside triphosphate (dNTP), 0.3  $\mu$ M each primers og0001 and og0002 (Table 1), 0.5 M betaine, 1 ng genomic DNA from *M. tuberculosis*, and 0.5 units of GoTaq polymerase. Amplification was carried out by applying 35 cycles of denaturation at 95°C for 10 s, annealing at 57°C for 10 s, polymerase reaction mixture at 72°C for 40 s, and a final extension at 72°C for 5 min. The amplified DNA fragment was subjected to sequence analysis with BigDye Terminator v3.1 (Life Technologies Co., Carlsbad, CA) reagents by a sequencer, the 3130 genetic analyzer (Life Technologies

Co.), according to the manufacturer's protocol. The *Rv0679c* sequence was also compared with those of 80 whole-genome sequenced MTC strains registered in the GenBank (<http://www.ncbi.nlm.nih.gov/GenBank/>) or TB (<http://genome.tdb.org/annotation/genome/tbdb/MultiHome.html>) (32) databases by the BLASTn algorithm (<http://blast.ncbi.nlm.nih.gov/>).

**Genotyping.** The spoligotype of *M. tuberculosis* clinical isolates was determined as described previously (33). Briefly, the DR region was amplified with a primer pair, and the PCR products were hybridized to a set of 43 spacer-specific oligonucleotide probes, which were covalently bound to the membrane. The spoligo-international type (SIT) was determined by comparing spoligotypes against the international spoligotyping database (SpolDB4) (3).

The detection of an RD105 deletion was performed by multiplex PCR in Beijing clones and by conventional PCR in east Asian strains other than those of the Beijing type, since the deletion pattern is different between those two groups (10). The reaction mixture consisted of GoTaq PCR buffer (Promega), 0.2 mM each dNTP, 0.3  $\mu$ M (each) two or three primers (Table 1), 0.5 M betaine, 1  $\mu$ l extracted DNA sample, and 0.5 units of GoTaq polymerase. The target was amplified by 35 cycles of denaturation at 95°C for 10 s, annealing at 55°C for 10 s, and extension at 72°C for 40 s, with a final extension at 72°C for 5 min. RD207 deletion was detected by two PCR assays described by Warren et al. (22), and TbD1 was detected by PCR using the Huard et al. (25) protocol (Table 1). The amplified DNA fragment was subjected to agarose gel electrophoresis with ethidium bromide (EtBr) to see the size of the band under a UV transilluminator.

The multilocus sequence type (MLST) was determined with 9 SNPs, which were described by Filliol et al. (26) and were selected for Beijing subtyping by Iwamoto et al. (16). Each locus was amplified with a primer pair (Table 1), and the product was subjected to sequencing. SNPs were detected by comparing the sequences with those of H37Rv (34). The sequence type (ST) was identified according to Filliol et al. (26).

**Beijing lineage identification by multiplex PCR.** Multiplex PCR for the identification of the Beijing lineage was performed under the following conditions. The PCR mixture, in a final volume of 15  $\mu$ l, contained 1 $\times$  PCR buffer (1.5 mM Mg; TaKaRa Bio, Inc., Shiga, Japan), 0.5  $\mu$ l dNTP solution mix (10 mM each dNTP; New England Biolabs, Inc., Ipswich, MA), 0.5  $\mu$ l each of Fw and R1 primers, 0.2  $\mu$ l R2 primer (primer solutions in 10  $\mu$ M; Table 1), 1.5  $\mu$ l of 5 M betaine, 0.45  $\mu$ l of 25 mM MgCl<sub>2</sub> (to make a final Mg concentration of 2.25 mM), 1 ng of sample DNA, and 0.5 units of TaKaRa Hot Start Taq polymerase (TaKaRa). Amplification was carried out with the first denaturation at 95°C for 1 min followed by 35 cycles of denaturation at 95°C for 10 s, annealing at 66°C for 10 s, extension at 72°C for 15 s, and the final extension at 72°C for 3 min. The amplicon was subjected to electrophoresis in a 2% agarose gel that included EtBr. DNA samples extracted from the isolate BCG Tokyo 172 and a well-characterized clinical isolate (Beijing OM-9) were used as controls for the non-Beijing and Beijing banding patterns, respectively. Sensitivity was determined with serially diluted genomic DNA obtained from these BCG and Beijing control strains. A specificity study was performed with genomic DNA samples (2 ng/ $\mu$ l each) from the MTC and NTM strains described above.

## RESULTS

**Spoligotyping and MLST.** A total of 619 clinical isolates were subjected to spoligotyping, and 393 were identified as being in the Beijing lineage and 226 as a non-Beijing group (Table 2). The non-Beijing group consisted of a variety of strains belonging to the following lineages: east African-Indian (EAI), central Asian (CAS), Latin American Mediterranean (LAM), Haarlem, S, T, X, and non-Beijing east Asian (3). Ninety-four of the Beijing isolates were subjected to MLST analysis and were subtyped into 8 sequence-type classes, namely, ST26, ST3, STK, ST25, ST19, ST10, ST22, and ST8, which are listed in evolutionary order from ancient to modern Beijing types (16, 26).

TABLE 1 Primers used in the study

Target	Primer name	Nucleotide sequence	Purpose	Reference
Rv0679c	og0001	CCGGGAAGCTAGGAATGGTAA	Sequencing	This study
	og0002	AGCAACCTCGCAATCTGAC	Sequencing	This study
	ON-1002 (Fw)	GTCACCTGAACGTGGCCGGCTC	Multiplex PCR for Beijing type identification	This study
	ON-1258 (R1) <sup>a</sup>	<u>TCGGTACCCGTTTTTGTAGGTGACCGTC</u>	Multiplex PCR for Beijing type identification	This study
	ON-1127 (R2)	AGCAACCTCGCAATCTGACC	Multiplex PCR for Beijing type identification	This study
RD105	RD105-F (-239~-218)	GGAAAGCAACATACACACCACG	Multiplex PCR for east Asian type determination <sup>b</sup>	This study
	RD105-R	AGGCCGCATAGTCACGGTCCG	Multiplex PCR for east Asian type determination <sup>b</sup>	This study
	RD105-M (+304~-323)	TCCTGGGTGCCGAACAAGTG	Multiplex PCR for east Asian type determination <sup>b</sup>	This study
	RD105EA-F (-80~-60)	TCGGACCCGATGGCTTCGGTG	PCR for east Asian type determination <sup>c</sup>	This study
	RD105EA-R (61~-42)	TGATCACGGTTCGCCCGCAG	PCR for east Asian type determination <sup>c</sup>	This study
RD207	RD207-1F (Warren)	TTCAACCATCGCCGCTCTAC	PCR for Beijing type identification (set 1)	22
	RD207-1R (Warren)	CACCCTACTCTGCGCTTGG	PCR for Beijing type identification (set 1)	22
	RD207-2F (Warren)	ACCGAGCTGATCAAACCCG	PCR for Beijing type identification (set 2)	22
	RD207-2R (Warren)	ATGGCACGGCCGACCTGAATGAACC	PCR for Beijing type identification (set 2)	22
TbD1	TbD1F	CGTTCAACCCCAACAGGTA	PCR for ancestral <i>M. tuberculosis</i> determination	25
	TbD1R	AATCGAAGCTCGTGGAAACAC	PCR for ancestral <i>M. tuberculosis</i> determination	25
797736 <sup>d</sup>	Beijing ST-1F	GACGGCCGAATCTGACACTG	MLST for Beijing lineage	This study
	Beijing ST-1R	CCATTCCGGGTGGTCACTG	MLST for Beijing lineage	This study
909164 <sup>d</sup>	Beijing ST-2F	CGTCGAGCTCCCACTTCTTG	MLST for Beijing lineage	This study
	Beijing ST-2R	TCGTGGAAGTGGACGAGGAC	MLST for Beijing lineage	This study
1477596 <sup>d</sup>	Beijing ST-3F	GTCGACAGCGCCAGAAAATG	MLST for Beijing lineage	This study
	Beijing ST-3R	GCTCCTATGCCACCCAGCAC	MLST for Beijing lineage	This study
1692067 <sup>d</sup>	Beijing ST-5F	GATTGGCAACTGGCAACAGG	MLST for Beijing lineage	This study
	Beijing ST-5R	TGGCCGTTTCAGATAGCACAC	MLST for Beijing lineage	This study
1892015 <sup>d</sup>	Beijing ST-6F	GCTGCACATCATGGGTTGG	MLST for Beijing lineage	This study
	Beijing ST-6R	GTATCGAGGCCGACGAAAGG	MLST for Beijing lineage	This study
2376133 <sup>d</sup>	Beijing ST-7F	TCTTGCAGCCGATGTGAAC	MLST for Beijing lineage	This study
	Beijing ST-7R	GAGCGCAACATGGGTGAGTC	MLST for Beijing lineage	This study
2532614 <sup>d</sup>	Beijing ST-8F	CCCTTTTCTGCTCGGACACG	MLST for Beijing lineage	This study
	Beijing ST-8R	GATCGACCTTCGTGCACTGG	MLST for Beijing lineage	This study
2825579 <sup>d</sup>	Beijing ST-9F	CCTTGGAGCGCAACAAGATG	MLST for Beijing lineage	This study
	Beijing ST-9R	CTGGCCGACGATTTTGAAG	MLST for Beijing lineage	This study
4137829 <sup>d</sup>	Beijing ST-10F	CGTCGCTGCAATTGTCTGG	MLST for Beijing lineage	This study
	Beijing ST-10R	GGACGCAGTCGCAACAGTTC	MLST for Beijing lineage	This study

<sup>a</sup> Beijing-type specific mutation-detection primer. Underlined 2-base sequences at the 5' end are not complementary sequences.

<sup>b</sup> This assay was used for Beijing genotype strains.

<sup>c</sup> This assay was used for non-Beijing genotype strains.

<sup>d</sup> This SNP nucleotide position on the *H37Rv* genome is according to references 26 and 34.

**Sequence analysis of the Rv0679c gene of *M. tuberculosis* isolates.** Nucleotide sequences of the full-length *Rv0679c* gene obtained from 197 clinical *M. tuberculosis* isolates collected in Japan, Bangladesh, and Nepal were compared with the *Rv0679c* sequence in *M. tuberculosis* H37Rv (34). Only a single nucleotide difference of cytosine to guanine at position 426, which leads to an amino acid change at codon 142 from Asn (AAC) to Lys (AAG), was detected in 87 isolates, all of which were identified as being in the Beijing lineage by spoligotyping and, supportively, by RD207 PCR (22) (data not shown). One Bangladeshi isolate showed a mixed peak of C and G at position 426 and was revealed as a mixed

culture of Beijing and another strain by RD105 and RD207 detection PCR (Table 2). None of the non-Beijing isolates had the mutation, and vice versa. In public databases, 14 strains reported from several countries were revealed to have this mutation, and all were confirmed as being in the Beijing lineage by checking for the RD207 deletion *in silico* (18). None of the other 66 MTC strains, which were determined to be non-Beijing, had this mutation. The 498-bp *Rv0679c* sequence was well conserved among the MTC strains, and the following three strains in the database showed alterations: *M. tuberculosis* strains C and T17 and *Mycobacterium canettii* CIPT 140010059.