58 Mve-Obiang A, Lee RE, Umstot E *et al.* A newly discovered mycobacterial pathogen isolated from laboratory colonies of *xenopus* species with lethal infections produces a novel form of mycolactone, the *Mycobacterium ulcerans* macrolide toxin. *Infect Immun* 2005; **73**: 3307–3312.
59 Rhodes M, Kator H, McNabb A *et al. Mycobacterium pseudoshottsii* sp. nov., a slowly growing chromogenic species isolated from Chesapeake Bay striped bass (*Morone saxatilis*). *Int J Syst Evol Microbiol* 2005; **55**: 1139–1147.
60 Ranger BS, Mahrous EA, Mosi L *et al.* Globally distributed mycobacterial fish pathogens produce a novel plasmid-encoded toxic macrolide, mycolactone F. *Infect Immun* 2006; **74**: 6037.
61 World Health Organization. Buruli ulcer: diagnosis of *Mycobacterium ulcerans* disease: a manual for health care providers. In: Portaels F, Johnson P, Meyers WM, published in Apr. 2001. (http://whqlibdoc.who.int/hq/2001/WHO_CDS_CPE_GBUI_2001.4.pdf).
62 List of Prokaryotic names with Standing in Nomenclature (http://www.bacterio.cict.fr/, last accessed 19 October 2012).

Infection, Genetics and Evolution 19 (2013) 200–204

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

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

M. Kai [a,*], N. Nakata [a], M. Matsuoka [a], T. Sekizuka [b], M. Kuroda [b], M. Makino [a]

[a] Department of Mycobacteriology, Leprosy Research Center, National Institute of Infectious Diseases, 4-2-1 Aoba-cho, Higashimurayama, Tokyo 189-0002, Japan
[b] Pathogen Genomics Center, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

ARTICLE INFO

Article history:
Received 1 May 2013
Received in revised form 12 July 2013
Accepted 12 July 2013
Available online 24 July 2013

Keywords:
Mycobacterium leprae
Genome
SNPs
Genotyping
Japan

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.

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 (M. Kai), n-nakata@nih.go.jpz (N. Nakata), Matsuoka@nih.go.jp (M. Matsuoka), sekizuka@nih.go.jp (T. Sekizuka), makokuro@nih.go.jp (M. Kuroda), mmaki@nih.go.jp (M. Makino).

1567-1348/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.meegid.2013.07.014

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 QIAamp 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 GAIIx 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

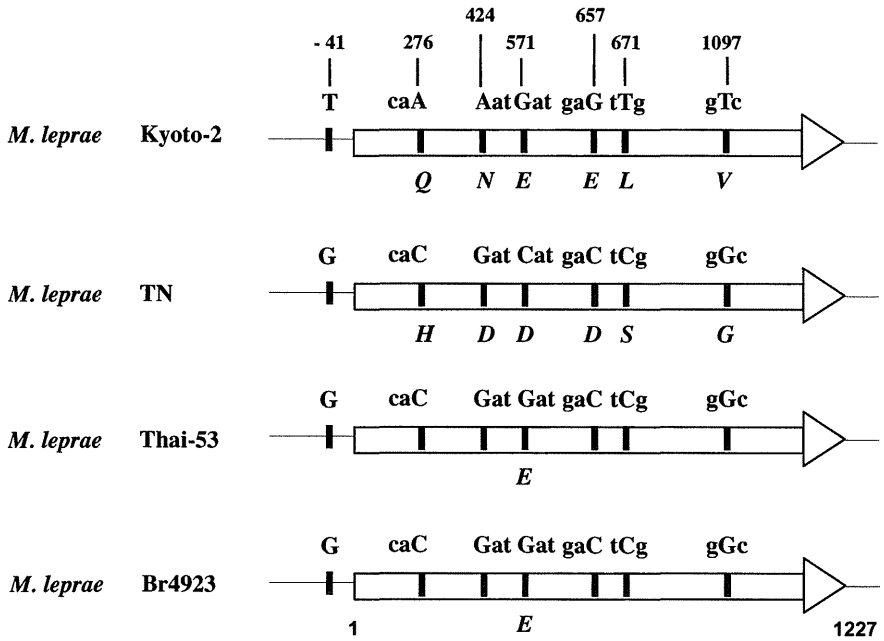

Fig. 1. Comparison of nucleotide sequences of *M. leprae* strains in gene ML0411 and the upstream region. ML0411 gene is indicated with arrows. The letters above arrows show nucleotide bases and set of the three bases indicate codons. SNPs sites of nucleotide bases are indicated with capital letters. 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								Number of 6bp repeat In *rpoT*	SNPs type (I_IV)	SNPs sub-type (A_P)	Distribution*
Nucleotide position	-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	
Kyoto-2	**T**	**A**	**A**	**G**	**G**	**T**	**T**	**4**	**III**	**K**	
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.

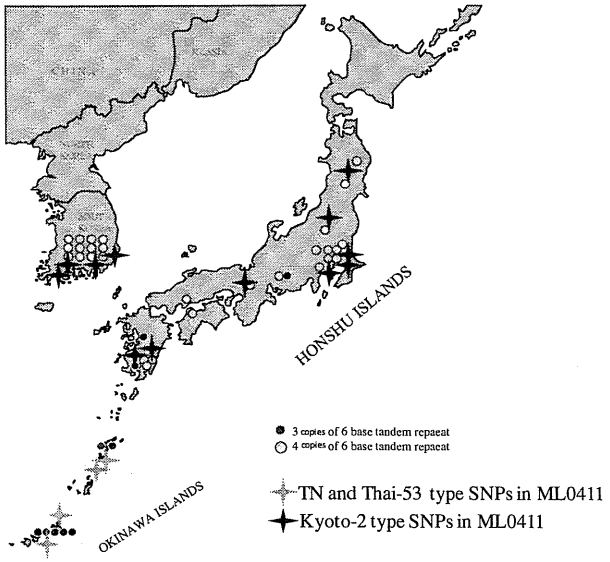

Fig. 2. Geographical distribution of *M. leprae* isolates derived from Japan and Korea having different genotypes.

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, CGAGCCACCAATACAGCATCT 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.

Acknowledgments

We thank Drs. G.T. Chae, Institute of Hansen's Disease, Department of Pathology, College of Medicine, The Catholic University of Korea, Seoul, Korea; P. Saunderson, A.A. Maghanoy, and M.F. Balagon, Leonard Wood Memorial, Cebu, The Philippines for supplying DNA samples. Also, we thank Ms. K. Matsubara for her helpful assistance with sequencing. This research was partially supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by a Research on Emerging and Re-Emerging Infectious Diseases grant from the Ministry of Health, Labor and Welfare of Japan.

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.

References

Bentley, D.R., Balasubramanian, S., Swerdlow, H.P., Smith, G.P., Milton, J., Brown, C.G., Hall, K.P., Evers, D.J., Barnes, C.L., Bignell, H.R., Boutell, J.M., Bryant, J., Carter, R.J., Keira Cheetham, R., Cox, A.J., Ellis, D.J., Flatbush, M.R., Gormley, N.A., Humphray, S.J., Irving, L.J., Karbelashvili, M.S., Kirk, S.M., Li, H., Liu, X., Maisinger, K.S., Murray, L.J., Obradovic, B., Ost, T., Parkinson, M.L., Pratt, M.R., Rasolonjatovo, I.M., Reed, M.T., Rigatti, R., Rodighiero, C., Ross, M.T., Sabot, A., Sankar, S.V., Scally, A., Schroth, G.P., Smith, M.E., Smith, V.P., Spiridou, A., Torrance, P.E., Tzonev, S.S., Vermaas, E.H., Walter, K., Wu, X., Zhang, L., Alam, M.D., Anastasi, C., Aniebo, I.C., Bailey, D.M., Bancarz, I.R., Banerjee, S., Barbour, S.G., Baybayan, P.A., Benoit, V.A., Benson, K.F., Bevis, C., Black, P.J., Boodhun, A., Brennan, J.S., Bridgham, J.A., Brown, R.C., Brown, A.A., Buermann, D.H., Bundu, A.A., Burrows, J.C., Carter, N.P., Castillo, N., Chiara, E.C.M., Chang, S., Neil Cooley, R., Crake, N.R., Dada, O.O., Diakoumakos, K.D., Dominguez-Fernandez, B., Earnshaw, D.J., Egbujor, U.C., Elmore, D.W., Etchin, S.S., Ewan, M.R., Fedurco, M., Fraser, L.J., Fuentes Fajardo, K.V., Scott Furey, W., George, D., Gietzen, K.J., Goddard, C.P., Golda, G.S., Granieri, P.A., Green, D.E., Gustafson, D.L., Hansen, N.F., Harnish, K., Haudenschild, C.D., Heyer, N.I., Hims, M.M., Ho, J.T., Horgan, A.M., Hoschler, K., Hurwitz, S., Ivanov, D.V., Johnson, M.Q., James, T., Huw Jones, T.A., Kang, G.D., Kerelska, T.H., Kersey, A.D., Khrebtukova, I., Kindwall, A.P., Kingsbury, Z., Kokko-Gonzales, P.I., Kumar, A., Laurent, M.A., Lawley, C.T., Lee, S.E., Lee, X., Liao, A.K., Loch, J.A., Lok, M., Luo, S., Mammen, R.M., Martin, J.W., McCauley, P.G., McNitt, P., Mehta, P., Moon, K.W., Mullens, J.W., Newington, T., Ning, Z., Ling Ng, B., Novo, S.M., O'Neill, M.J., Osborne, M.A., Osnowski, A., Ostadan, O., Paraschos, L.L., Pickering, L., Pike, A.C., Pike, A.C., Chris Pinkard, D., Pliskin, D.P., Podhasky, J., Quijano, V.J., Raczy, C., Rae, V.H., Rawlings, S.R., Chiva Rodriguez, A., Roe, P.M., Rogers, J., Rogert Bacigalupo, M.C., Romanov, N., Romieu, A., Roth, R.K., Rourke, N.J., Ruediger, S.T., Rusman, E., Sanches-Kuiper, R.M., Schenker, M.R., Seoane, J.M., Shaw, R.J., Shiver, M.K., Short, S.W., Sizto, N.L., Sluis, J.P., Smith, M.A., Ernest Sohna Sohna, J., Spence, E.J., Stevens, K., Sutton, N., Szajkowski, L., Tregidgo, C.L., Turcatti, G., Vandevondele, S., Verhovsky, Y., Virk,

S.M., Wakelin, S., Walcott, G.C., Wang, J., Worsley, G.J., Yan, J., Yau, L., Zuerlein, M., Rogers, J., Mullikin, J.C., Hurles, M.E., McCooke, N.J., West, J.S., Oaks, F.L., Lundberg, P.L., Klenerman, D., Durbin, R., Smith, A.J., . Accurate whole human genome sequencing using reversible terminator chemistry. Nature 456, 53–59.

Clark-Curtiss, J.E., Docherty, M.A., 1989. A species-specific repetitive sequence in *Mycobacterium leprae* DNA. J. Infect. Dis. 159, 7–15.

Cole, S.T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S.V., Eiglmeier, K., Gas, S., Barry 3rd, C.E., Tekaia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Krogh, A., McLean, J., Moule, S., Murphy, L., Oliver, K., Osborne, J., Quail, M.A., Rajandream, M.A., Rogers, J., Rutter, S., Seeger, K., Skelton, J., Squares, R., Squares, S., Sulston, J.E., Taylor, K., Whitehead, S., Barrell, B.G., 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature 393, 537–544.

Cole, S.T., Eiglmeier, K., Parkhill, J., James, K.D., Thomson, N.R., Wheeler, P.R., Honore, N., Garnier, T., Churcher, C., Harris, D., Mungall, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R.M., Devlin, K., Duthoy, S., Feltwell, T., Fraser, A., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Lacroix, C., Maclean, J., Moule, S., Murphy, L., Oliver, K., Quail, M.A., Rajandream, M.A., Rutherford, K.M., Rutter, S., Seeger, K., Simon, S., Simmonds, M., Skelton, J., Squares, R., Squares, S., Stevens, K., Taylor, K., Whitehead, S., Woodward, J.R., Barrell, B.G., 2001. Massive gene decay in the leprosy bacillus. Nature 409, 1007–1011.

Groathouse, N.A., Rivoire, B., Kim, H., Lee, H., Cho, S.N., Brennan, P.J., Vissa, V.D., 2004. Multiple polymorphic loci for molecular typing of strains of *Mycobacterium leprae*. J. Clin. Microbiol. 42, 1666–1672.

Han, X.Y., Seo, Y.H., Sizer, K.C., Schoberle, T., May, G.S., Spencer, J.S., Li, W., Nair, R.G., 2008. A new Mycobacterium species causing diffuse lepromatous leprosy. Am. J. Clin. Pathol. 130, 856–864.

Irgens, L.M., 1984. The discovery of *Mycobacterium leprae*. A medical achievement in the light of evolving scientific methods. Am. J. Dermatopathol. 6, 337–343.

Kohsaka, K., Mori, T., Ito, T., 1976. Lepromatoid lesion developed in the nude mice inoculated with *Mycobacterium leprae*. La Lepro 45, 177–187.

Lavania, M., Katoch, K., Singh, H., Das, R., Gupta, A.K., Sharma, R., Chauhan, D.S., Sharma, V.D., Sachan, P., Sachan, S., Katoch, V.M., 2007. Predominance of three copies of tandem repeats in *rpoT* gene of *Mycobacterium leprae* from Northern India. Infect. Genet. Evol. 7, 627–631.

Matsuoka, M., 2010. The history of *Mycobacterium leprae* Thai-53 strain. Lepr. Rev. 81, 137.

Matsuoka, M., Maeda, S., Kai, M., Nakata, N., Chae, G.T., Gillis, T.P., Kobayashi, K., Izumi, S., Kashiwabara, Y., 2000. *Mycobacterium leprae* typing by genomic diversity and global distribution of genotypes. Int. J. Lepr. Other Mycobacterial Dis.: Official Organ Int. Lepr. Assoc. 68, 121–128.

Matsuoka, M., Zhang, L., Budiawan, T., Saeki, K., Izumi, S., 2004. Genotyping of *Mycobacterium leprae* on the basis of the polymorphism of TTC repeats for analysis of leprosy transmission. J. Clin. Microbiol. 42, 741–745.

Matsuoka, M., Roa, R.I., Budiawan, T., Kyaw, K., Chae, G.T., 2006. Genotypic analysis of *Mycobacterium leprae* isolates from Japan and other Asian countries reveals a global transmission pattern of leprosy. FEMS Microbiol. Lett. 261, 150–154.

Monot, M., Honore, N., Garnier, T., Araoz, R., Coppee, J.Y., Lacroix, C., Sow, S., Spencer, J.S., Truman, R.W., Williams, D.L., Gelber, R., Virmond, M., Flageul, B., Cho, S.N., Ji, B., Paniz-Mondolfi, A., Convit, J., Young, S., Fine, P.E., Rasolofo, V., Brennan, P.J., Cole, S.T., 2005. On the origin of leprosy. Science 308, 1040–1042.

Monot, M., Honore, N., Garnier, T., Zidane, N., Sherafi, D., Paniz-Mondolfi, A., Matsuoka, M., Taylor, G.M., Donoghue, H.D., Bouwman, A., Mays, S., Watson, C., Lockwood, D., Khamesipour, A., Dowlati, Y., Jianping, S., Rea, T.H., Vera-Cabrera, L., Stefani, M.M., Banu, S., Macdonald, M., Sapkota, B.R., Spencer, J.S., Thomas, J., Harshman, K., Singh, P., Busso, P., Gattiker, A., Rougemont, J., Brennan, P.J., Cole, S.T., 2009. Comparative genomic and phylogeographic analysis of *Mycobacterium leprae*. Nat. Genet. 41, 1282–1289.

Phetsuksiri, B., Srisungngam, S., Rudeeaneksin, J., Bunchoo, S., Lukebua, A., Wongtrungkapun, R., Paitoon, S., Sakamuri, R.M., Brennan, P.J., Vissa, V., 2012. SNP genotypes of *Mycobacterium leprae* isolates in Thailand and their combination with rpoT and TTC genotyping for analysis of leprosy distribution and transmission. Jpn J. Infect. Dis. 65, 52–56.

Sakamuri, R.M., Kimura, M., Li, W., Kim, H.C., Lee, H., Kiran, M.D., Black, W.C.t., Balagon, M., Gelber, R., Cho, S.N., Brennan, P.J., Vissa, V., 2009. Population-based molecular epidemiology of leprosy in Cebu, Philippines. J. Clin. Microbiol. 47, 2844–2854.

Vera-Cabrera, L., Escalante-Fuentes, W.G., Gomez-Flores, M., Ocampo-Candiani, J., Busso, P., Singh, P., Cole, S.T., 2011. Case of diffuse lepromatous leprosy associated with "*Mycobacterium lepromatosis*". J. Clin. Microbiol. 49, 4366–4368.

Watson, C.L., Lockwood, D.N., 2009. Single nucleotide polymorphism analysis of European archaeological *M. leprae* DNA. PLoS One 4, e7547.

Who, S.G., 1982. Chemotherapy of leprosy for control programmes. WHO Tech. Rep. Ser. 675, 1–33.

WHO, 2012. Global leprosy situation, 2012. Wkly Epidemiol. Rec. 87, 12.

Williams, D.L., Gillis, T.P., Portaels, F., 1990. Geographically distinct isolates of *Mycobacterium leprae* exhibit no genotypic diversity by restriction fragment length polymorphism analysis. Mol. Microbiol. 4, 1653–1659.

Zhang, L., Budiawan, T., Matsuoka, M., 2005. Diversity of potential short tandem repeats in *Mycobacterium leprae* and application for molecular typing. J. Clin. Microbiol. 43, 5221–5229.

Jpn. J. Infect. Dis., 66, 83-88, 2013

Invited Review

Buruli Ulcer and Mycolactone-Producing Mycobacteria

Kazue Nakanaga[1]*, Rie Roselyne Yotsu[2], Yoshihiko Hoshino[1], Koichi Suzuki[1], Masahiko Makino[1], and Norihisa Ishii[1]

[1]*Leprosy Research Center, National Institute of Infectious Diseases, Tokyo 189-0002; and* [2]*Department of Dermatology, National Center for Global Health and Medicine, Tokyo 162-8655, Japan*

(Received December 7, 2012)

CONTENTS:

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.

*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

This article is an Invited Review based on a lecture presented at the 22nd Symposium of the National Institute of Infectious Diseases, Tokyo, May 21, 2012.

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).

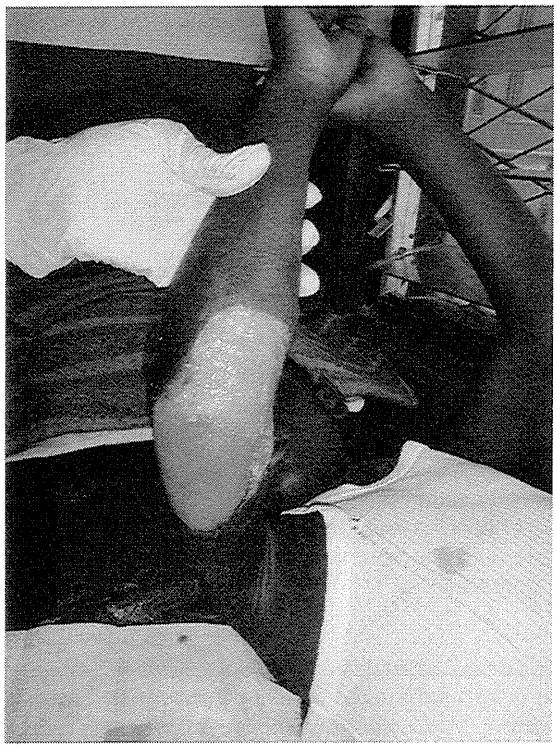

Fig. 1. A Buruli ulcer case in Amasaman Hospital (Accra, Ghana); a large ulcer on the left elbow extensor of an 8-year-old boy. The photograph was taken by Nakanaga, K. and Yotsu, R. R. at the Amasaman Hospital with the approval of the patient's parent, the director of the hospital, and the program manager of the National Buruli Ulcer Control Programme (Dr. Edwin Ampandu), 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-

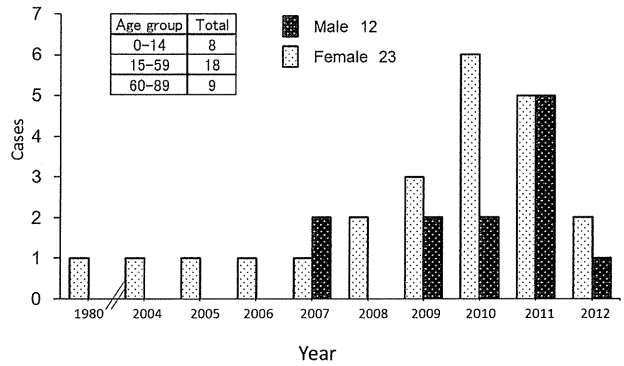

Fig. 2. Buruli ulcer cases in Japan by year diagnosed as of November 2012 (28,29).

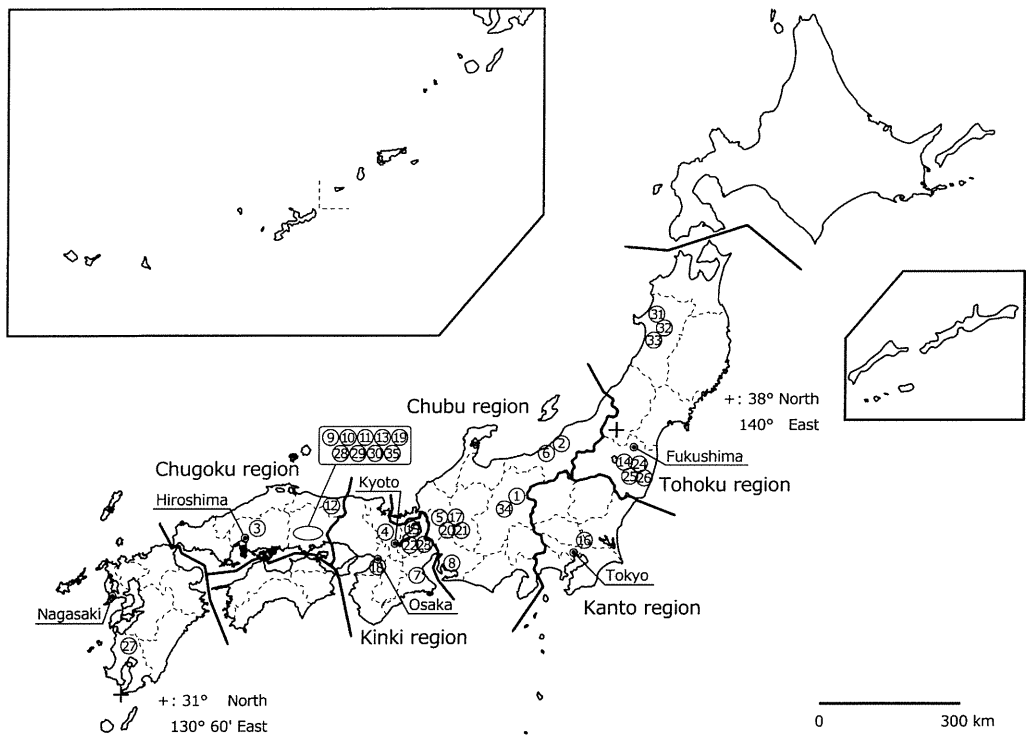

Fig. 3. Distribution of Buruli ulcer cases in Japan: a total of 35 cases as of November 2012 (28,29).

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

Known isolate	"*Mycobacterium ulcerans* subsp. *shinshuense*"
International traveling	None
Mode of transmission	Unknown, not clear with aquatic environment[1)]
Regional bias	Honshu island (northern limit, Akita Prefecture; southern lilmit, 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)

[1)]: Three cases in one family member often contact with tiny stream on their property.

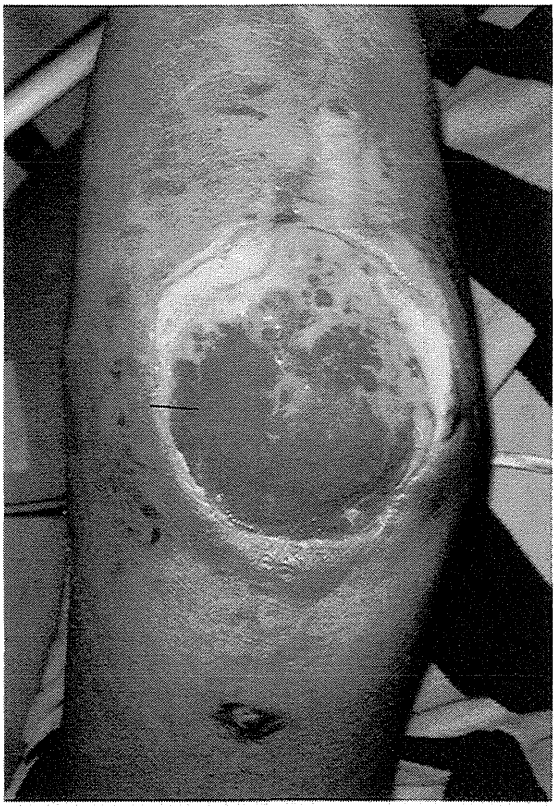

Fig. 4. A Buruli ulcer case in the National Hospital Organization (NHO) Higashihiroshima Medical Center (Hiroshima, Japan); an ulcer on the right elbow extensor of a 20-year-old woman (19,20). The photograph was provided by the attending doctor (Dr. Hideaki Imada) in the NHO Higashihiroshima Medical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

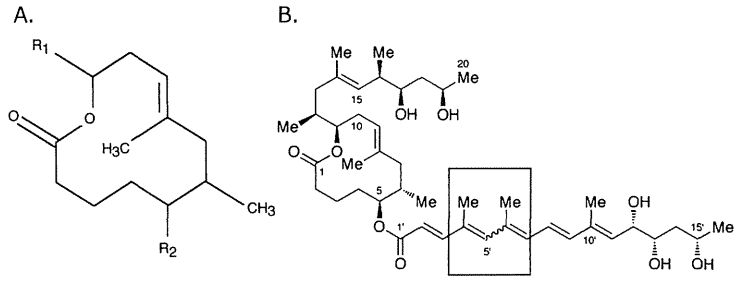

Fig. 5. Basic structure of mycolactone and complete structure of mycolactone A/B. Boxed site indicates isomers A (cis) and B (trans) (33,36).

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-Δ4′,5′- and E-Δ4′,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 β-chemokines macrophage inflammatory protein (MIP)-1α, MIP-1β, regulated on activation, normal T cell expressed and secreted, as well as monocyte chemoattractant protein 1 and interferon γ-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 IS*2404* and IS*2606* 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 IS*2404*, IS*2606*, 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 yellowtail 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.

Acknowledgments This work was supported in part by a Grant-in-Aid for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labour and Welfare of Japan for Y.H., M.M. and N.I.; a Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Culture, Sports, Science and Technology of Japan for Y.H.; and a Grant-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science for K.N.

Conflict of interest None to declare.

REFERENCES

1. Asiedu, K., Scherpbier, R., Raviglione, M., et al. (2000): Buruli ulcer: *Mycobacterium ulcerans* infection. *In* K. Asiedu, R. Scherpbier, and M. Raviglione (ed.). doc. No. WHO/CDS/CPE/GBUI/2000.1. World Health Organization, Geneva.
2. Pszolla, N., Sarkar, M.R., Strecker, W., et al. (2003): Buruli ulcer: a systemic disease. Clin. Infect. Dis., 37, e78-82.
3. Sizaire, V., Nackers, F., Comte, E., et al. (2006): *Mycobacterium ulcerans* infection: control, diagnosis, and treatment. Lancet Infect. Dis., 6, 288-296.
4. Portaels, F., Silva, M.T. and Meyers, W.M. (2009): Buruli ulcer. Clin. Dermatol., 27, 291-305.
5. Walsh, D.S., Portaels, F. and Meyers, W.M. (2011): Buruli ulcer: advances in understanding *Mycobacterium ulcerans* infection. Dermatol. Clin., 29, 1-8.
6. World Health Organization (2012): Buruli ulcer endemic countries (internet). Online at <http://www.who.int/buruli/country/en/>. Accessed December 5, 2012.
7. Mve-Obiang, A., Lee, R.E., Umstot, E.S., et al. (2005): A newly discovered mycobacterial pathogen isolated from laboratory colonies of *Xenopus* species with lethal infections produces a novel form of mycolactone, the *Mycobacterium ulcerans* macrolide toxin. Infect. Immun., 73, 3307-3312.
8. Ranger, B.S., Mahrous, E.A., Mosi, L., et al. (2006): Globally distributed mycobacterial fish pathogens produce a novel plasmid-encoded toxic macrolide, mycolactone F. Infect. Immun., 74, 6037-6045.
9. Sakiguchi, K., Iimai H., Hirayama K., et al. (2011): *Mycobacterium ulcerans* infection in an indian flap-shelled turtle (*Lissemys punctata punctata*). J. Vet. Med. Sci., 73, 1217-1220.
10. Clancey, J.K., Dodge, O.G., Lunn, H.F., et al. (1961): Mycobacterial skin ulcers in Uganda. Lancet, 278, 951-954.
11. The Uganda Buruli Group (1971): Epidemiology of *Mycobacterium ulcerans* infection (Buruli ulcer) at Kinyara, Uganda. Trans. R. Soc. Trop. Med. Hyg., 65,763-775.
12. Alsop, D.G. (1972): The Bairnsdale ulcer. Aust. NZ. J. Surg., 41, 317-319.
13. MacCallum, P., Tolhurst, J.C., Buckle, G., et al. (1948): A new mycobacterial infection in man. I. Clinical aspects. II. Experimental investigations in laboratory animals. III. Pathology of the experimental lesions in the rat. IV. Cultivation of the new mycobacterium. J. Pathol. Bacteriol., 60, 92-122.
14. Fenner, F. (1951): The significance of the incubation period in infectious diseases. Med. J. Austr., 2, 813-818.
15. Boyd, S.C., Athan, E., Friedman, N.D. et al. (2012): Epidemiology, clinical features and diagnosis of *Mycobacterium ulcerans* in an Australian population. Med. J. Aust., 196, 341-344.
16. Mikoshiba, H., Shindo, Y., Matsumoto, H., et al. (1982): A case of atypical mycobacteriosis due to *Mycobacterium ulcerans*-like organism. Nihon Hifukagakkaizassi, 92, 557-565 (in Japanese).
17. Tsukamura, M., Kaneda, K., Imaeda, T., et al. (1989): A taxonomic study on a mycobacterium which caused skin ulcer in a Japanese girl and resembled *Mycobacterium ulcerans*. Kekkaku, 64, 691-697 (in Japanese).
18. Kazumi, Y., Ohtomo, K., Takahashiet, M., et al. (2004): *Mycobacterium shinshuense* isolated from cutaneous ulcer lesion of right lower extremity in a 37-year-old woman. Kekkaku, 79, 437-441 (in Japanese).
19. Nakanaga, K., Ishii, N., Suzuki, K., et al. (2007): "*Mycobacterium ulcerans* subsp. *shinshuense*" isolated from a skin ulcer lesion: identification based on 16S rRNA gene sequencing. J. Clin. Microbiol., 45, 3840-3843.
20. Imada, H., Kishi, K., Okabe, T., et al. (2008): Cutaneous ulcer of a right olecranon due to *Mycobacterium shinshuense*; a case report. Seikeigeka, 59, 1440-1445 (in Japanese).
21. Suzuki, S., Arakawa, C., Shibuya, S., et al. (2008): Skin ulcer caused by '*Mycobacterium ulcerans* subsp. *shinshuense*' infection. Hifubyou Shinryo, 30, 145-148 (in Japanese).
22. Kondo, M., Kurokawa, I., Ito, Y., et al. (2009): Leg ulcer caused by *Mycobacterium ulcerans* ssp. *shinshuense* infection. Int. J. Dermatol., 48, 1330-1333.
23. Funakoshi, T., Kazumi, Y., Okada, R., et al. (2009): Intractable ulcer caused by *Mycobacterium shinshuense*: successful identification of mycobacterium strain by 16S ribosomal RNA 3'-end sequencing. Clin. Exp. Dermatol., 34, e712-715.
24. Watanabe, T., Ohkusu, K., Nakanaga, K., et al. (2010): Buruli ulcer caused by "*Mycobacterium ulcerans* subsp. *shinshuense*." Eur. J. Dermatol., 20, 809-810.
25. Matsumura, Y., Taki, R., Miyachi, Y., et al. (2012): Case of Buruli ulcer due to *Mycobacterium ulcerans* ssp. *shinshuense* with distal cutaneous involvement and synovitis. J. Dermatol., 39, 80-83.
26. Onoe, H., Yotsu, R.R., Nakanaga, K., et al. (2012): Buruli ulcer accompanied by pain in a Japanese patient. J. Dermatol., 39, 869-870.
27. Faber, W.R., Arias-Bouda, L.M., Zeegelaar, J.E., et al. (2000): First reported case of *Mycobacterium ulcerans* infection in a patient from China. Trans. R. Soc. Trop. Med. Hyg., 94, 277-279.
28. Nakanaga, K., Hoshino, Y., Yotsu, R.R., et al. (2011): Nineteen cases of Buruli ulcer diagnosed in Japan from 1980 to 2010. J. Clin. Microbiol., 49, 3829-3836.
29. Yotsu, R.R., Nakanaga, K., Hoshino, Y., et al. (2012): Buruli ulcer and current situation in Japan: a new emerging cutaneous *Mycobacterium* infection. J. Dermatol., 39, 587-593.
30. Read, J.K., Heggie, C.M., Meyers, W.M., et al. (1974): Cytotoxic activity of *Mycobacterium ulcerans*. Infect. Immun., 9, 1114-1122.
31. Hockmeyer, W.T., Krieg, R.E., Reich, M., et al. (1978): Further characterization of *Mycobacterium ulcerans* toxin. Infect. Immun., 21, 124-128.
32. Pimsler, M., Sponsler, T.A., Meyers, W.M. et al. (1988): Immunosuppressive properties of the soluble toxin from *Mycobacterium ulcerans*. J. Infect. Dis., 157, 577-580.
33. George, K.M., Chatterjee, D., Gunawardana, G., et al. (1999): Mycolactone: a polyketide toxin from *Mycobacterium ulcerans* required for virulence. Science, 283, 854-857.
34. George, K.M., Pascopella, L., Welty, D.M., et al. (2000): *Mycobacterium ulcerans* toxin, mycolactone, causes apoptosis in guinea pig ulcers and tissue culture cells. Infect. Immun., 68, 877-883.
35. Mve-Obiang, A., Lee, R.E., Portaels, F., et al. (2003): Heterogeneity of mycolactones produced by clinical isolates of *Mycobacterium ulcerans*: implications for virulence. Infect. Immun., 71, 774-783.
36. Kishi, Y. (2011): Chemistry of mycolactones, the causative toxins of Buruli ulcer. Proc. Natl. Acad. Sci. USA, 108, 6703-6708.
37. Stinear, T.P., Jenkin, G.A., Johnson, P.D., et al. (2000): Comparative genetic analysis of *Mycobacterium ulcerans* and *Mycobacterium marinum* reveals evidence of recent divergence. J. Bacteriol., 182, 6322-6330.
38. Jenkin, G.A., Stinear, T.P., Johnson, P.D., et al. (2003): Subtractive hybridization reveals a type I polyketide synthase locus specific to *Mycobacterium ulcerans*. J. Bacteriol., 185, 6870-6882.
39. Stinear, T.P., Mve-Obiang, A., Small, P.L., et al. (2004): Giant plasmid-encoded polyketide synthases produce the macrolide toxin of *Mycobacterium ulcerans*. Proc. Natl. Acad. Sci. USA, 101, 1345-1349.
40. Stinear, T.P., Pryor, M.J., Porter, J.L., et al. (2005): Functional analysis and annotation of the virulence plasmid pMUM001 from *Mycobacterium ulcerans*. Microbiology, 151, 683-692.
41. Goto, M., Nakanaga, K., Aung, T., et al. (2006): Nerve damage in *Mycobacterium ulcerans*-infected mice. Probable cause of painlessness in Buruli ulcer. Am. J. Pathol., 168, 805-811.
42. En, J., Goto, M., Nakanaga, K., et al. (2008): Mycolactone is responsible for the painlessness of *Mycobacterium ulcerans* infection (Buruli ulcer) in a murine study. Infect. Immun., 76, 2002-2007.
43. Coutanceau, E., Decalf, J., Martino, A. et al. (2007): Selective suppression of dendritic cell functions by *Mycobacterium ulce-*

rans toxin mycolactone. J. Exp. Med., 204, 1395–1403.
44. Trott, K.A., Stacy, B.A., Lifland, B.D., et al. (2004): Characterization of a *Mycobacterium ulcerans*-like infection in a colony of African tropical clawed frogs (*Xenopus tropicalis*). Comp. Med., 54, 309–317.
45. Chai, N., Bronchain, O., Panteix, G., et al. (2012): Propagation method of saving valuable strains from a *Mycobacterium liflandii* infection in Western clawed frogs (*Silurana tropicalis*). J. Zoo. Wildl. Med., 43, 15–19.
46. Nakanaga, K., Hoshino, Y., Hattori, Y., et al. (2012): *Mycobacterium pseudoshottsii* isolated from 24 farmed fishes in western Japan. J. Vet. Med. Sci., 74, 275–278.
47. Yip, M.J., Porter, J.L., Fyfe, J.A.M., et al. (2007): Evolution of *Mycobacterium ulcerans* and Other mycolactone-producing mycobacteria from a common *Mycobacterium marinum* progenitor. J. Bacteriol., 189, 2021–2029.
48. Pidot, S.J., Hong, H., Seemann, T., et al. (2008): Deciphering the genetic basis for polyketide variation among mycobacteria producing mycolactones. BMC Genomics, 9, 462.
49. Pidot, S.J., Asiedu, K., Kaser, M., et al. (2010): *Mycobacterium ulcerans* and other mycolactone-producing mycobacteria should be considered a single species. PLoS Negl. Trop. Dis., 27, 4(7), e663.

Basophils Infiltrate the Skin Lesions in Lepromatous Leprosy

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

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

Accepted November 20, 2011.

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 FcεRI 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 $2D7^+$ 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.

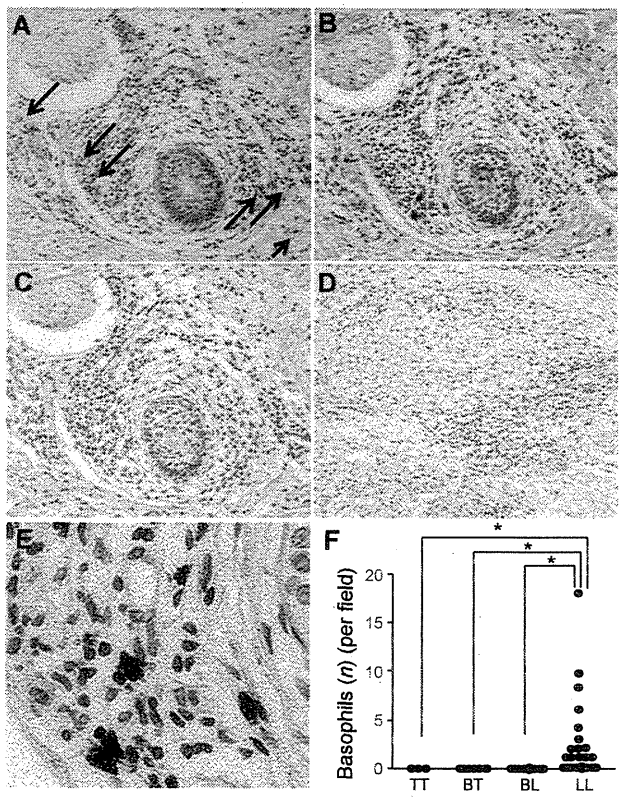

Fig. 1. Immunohistochemistry of basophils in leprosy skin lesions. Positive cells for (A) BB1 and (B) 2D7, but not for (C) isotype-matched control antibodies, were observed in the skin lesions of LL, but (D) not in the skin lesion of TT (E) High-power field of B. (F) The number of 2D7 cells was calculated in five high-power fields (×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 ×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 legions 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.

REFERENCES

1. Stefani MM, Guerra JG, Sousa AL, Costa MB, Oliveira ML, Martelli CT, et al. Potential plasma markers of Type 1 and Type 2 leprosy reactions: a preliminary report. BMC Infect Dis 2009; 9: 75.
2. Yoshimoto T, Yasuda K, Tanaka H, Nakahira M, Imai Y, Fujimori Y, et al. Basophils contribute to T(H)2-IgE responses in vivo via IL-4 production and presentation of peptide-MHC class II complexes to CD4+ T cells. Nat Immunol 2009; 10: 706–712.
3. Sokol CL, Chu NQ, Yu S, Nish SA, Laufer TM, Medzhitov R. Basophils function as antigen-presenting cells for an allergen-induced T helper type 2 response. Nat Immunol 2009; 10: 713–720.
4. Ito Y, Satoh T, Takayama K, Miyagishi C, Walls AF, Yokozeki H. Basophil recruitment and activation in inflammatory skin diseases. Allergy 2011; 66: 1107–1113.
5. Otsuka A, Walls A, Miyachi Y, Kabashima K. Comparison of basophil infiltration into the skin between eosinophilic pustular folliculitis and neutrophilic folliculitis. J Eur Acad Dermatol Venereol 2012; 26: 527–529.
6. Chatterjee BR. Leprosy immunopathogenesis and vaccine development. Indian J Lepr 1992; 64: 359–374.

JCM
Journals.ASM.org

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

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

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

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 IS*6110* 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 tuberculosis (TB) control program, several methods to distinguish this clade have been developed. First, van Soolingen et al. (1) identified this clade by its specific IS*6110* 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 IS*6110* 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 IS*6110* 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-

Received 23 December 2012 Returned for modification 11 February 2013
Accepted 4 April 2013
Published ahead of print 17 April 2013
Address correspondence to Takashi Matsuba, matsubat@med.tottori-u.ac.jp, or Yasuhiko Suzuki, suzuki@czc.hokudai.ac.jp.

doi:10.1128/JCM.03404-12

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 × *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 orygis* 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 μM 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.tbdb.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 μM (each) two or three primers (Table 1), 0.5 M betaine, 1 μl 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 μl, contained 1× PCR buffer (1.5 mM Mg; TaKaRa Bio, Inc., Shiga, Japan), 0.5 μl dNTP solution mix (10 mM each dNTP; New England BioLabs, Inc., Ipswich, MA), 0.5 μl each of Fw and R1 primers, 0.2 μl R2 primer (primer solutions in 10 μM; Table 1), 1.5 μl of 5 M betaine, 0.45 μl of 25 mM $MgCl_2$ (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/μl 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 evolutional 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	CCGGGAACTAGGAATGGTAA	Sequencing	This study
	og0002	AGCAACCTCGCAATCTGAC	Sequencing	This study
	ON-1002 (Fw)	GTCACTGAACGTGGCCGGCTC	Multiplex PCR for Beijing type identification	This study
	ON-1258 (R1)[a]	TCGGTCACCGTTTTTGTAGGTGACCGTC	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[b]	This study
	RD105-R	AGGCCGCATAGTCACGGTCG	Multiplex PCR for east Asian type determination[b]	This study
	RD105-M (+304~323)	TCCTGGGTGCCGAACAAGTG	Multiplex PCR for east Asian type determination[b]	This study
	RD105EA-F (−80~−60)	TCGGACCCGATGGCTTCGGTG	PCR for east Asian type determination[c]	This study
	RD105EA-R (61~42)	TGATCACGGTTCGCCCGCAG	PCR for east Asian type determination[c]	This study
RD207	RD207-1F (Warren)	TTCAACCATCGCCGCCTCTAC	PCR for Beijing type identification (set 1)	22
	RD207-1R (Warren)	CACCCTCTACTCTGCGCTTTG	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	CGTTCAACCCCAAACAGGTA	PCR for ancestral *M. tuberculosis* determination	25
	TbD1R	AATCGAACTCGTGGAACACC	PCR for ancestral *M. tuberculosis* determination	25
797736[d]	Beijing ST-1F	GACGGCCGAATCTGACACTG	MLST for Beijing lineage	This study
	Beijing ST-1R	CCATTCCGGGTGGTCACTG	MLST for Beijing lineage	This study
909164[d]	Beijing ST-2F	CGTCGAGCTCCCACTTCTTG	MLST for Beijing lineage	This study
	Beijing ST-2R	TCGTCGAAGTGGACGAGGAC	MLST for Beijing lineage	This study
1477596[d]	Beijing ST-3F	GTCGACAGCGCCAGAAAATG	MLST for Beijing lineage	This study
	Beijing ST-3R	GCTCCTATGCCACCCAGCAC	MLST for Beijing lineage	This study
1692067[d]	Beijing ST-5F	GATTGGCAACTGGCAACAGG	MLST for Beijing lineage	This study
	Beijing ST-5R	TGGCCGTTTCAGATAGCACAC	MLST for Beijing lineage	This study
1892015[d]	Beijing ST-6F	GCTGCACATCATGGGTTGG	MLST for Beijing lineage	This study
	Beijing ST-6R	GTATCGAGGCCGACGAAAGG	MLST for Beijing lineage	This study
2376133[d]	Beijing ST-7F	TCTTGCGACCCGATGTGAAC	MLST for Beijing lineage	This study
	Beijing ST-7R	GAGCGCAACATGGGTGAGTC	MLST for Beijing lineage	This study
2532614[d]	Beijing ST-8F	CCCTTTTCTGCTCGGACACG	MLST for Beijing lineage	This study
	Beijing ST-8R	GATCGACCTTCGTGCACTGG	MLST for Beijing lineage	This study
2825579[d]	Beijing ST-9F	CCTTGGAGCGCAACAAGATG	MLST for Beijing lineage	This study
	Beijing ST-9R	CTGGCCGGACGATTTTGAAG	MLST for Beijing lineage	This study
4137829[d]	Beijing ST-10F	CGTCGCTGCAATTGTCTGG	MLST for Beijing lineage	This study
	Beijing ST-10R	GGACGCAGTCGCAACAGTTC	MLST for Beijing lineage	This study

[a] Beijing-type specific mutation-detection primer. Underlined 2-base sequences at the 5′ end are not complementary sequences.
[b] This assay was used for Beijing genotype strains.
[c] This assay was used for non-Beijing genotype strains.
[d] 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.

TABLE 2 *Rv0679c* multiplex PCR results compared with other typing results in 619 *M. tuberculosis* clinical isolates

Isolate origin	Spoligotype family[a]	RD207, RD105, or other typing methods[b]	Sequence type[c]	*Rv0679c* M-PCR type[d]	No. of isolates
Beijing or Beijing-like					393
Japan	Beijing	ND	26	Beijing	10
	Beijing	ND	3	Beijing	24
	Beijing	ND	STK	Beijing	13
	Beijing-like	RD207$^+$	STK	Beijing	1
	Beijing	ND	25	Beijing	3
	Beijing	ND	19	Beijing	9
	Beijing	ND	10	Beijing	12
	Beijing	ND	22	Beijing	4
	Beijing	ND	ND	Beijing	23
Bangladesh	Beijing	ND	26	Beijing	3
	Beijing	ND	10	Beijing	12
	Beijing	ND	22	Beijing	2
	Beijing	ND	8	Beijing	1
	Beijing	ND	ND	Beijing	29
	Beijing-like	RD105$^+$, RD207$^+$	ND	Beijing	1
Nepal	Beijing	ND	ND	Beijing	64
Myanmar	Beijing	ND	ND	Beijing	141
	Beijing-like	RD105$^+$, RD207$^+$	ND	Beijing	1
China (Heilongjiang)	Beijing	ND	ND	Beijing	40
Non-Beijing or undesignated/new[a]					216
Japan	Undesignated/new[e]	RD105$^+$, RD207$^-$	ND	Non-Beijing	29
	Others[f]	ND	ND	Non-Beijing	16
Bangladesh	—[g]	ND	ND	Non-Beijing	73
Nepal	—[h]	ND	ND	Non-Beijing	45
Myanmar	—[i]	ND	ND	Non-Beijing	51
China (Heilongjiang)	Undesignated/new	ND	ND	Non-Beijing	2
Mixed clone samples					6
Bangladesh	Undesignated/new	Mixed peak in sequence[j] RD105$^+$, RD207$^+$	ND	Beijing	1
Myanmar	Undesignated/new	RD105$^+$, RD207$^+$	ND	Beijing	2
	EAI2_NTB	RD105$^+$	ND	Beijing	1
	EAI5	RD105$^+$	ND	Beijing	1
China (Heilongjiang)	Undesignated/new	RD105$^+$	ND	Beijing	1
New spoligotype lacking spacers 1–34[k]					4
Japan	New	RD105$^+$, RD207$^+$[k]	ND	Beijing	1
Nepal	New	RD105$^-$, TbD1$^+$[k]	ND	Non-Beijing	1
Myanmar	New	RD105$^+$, RD207$^+$	ND	Beijing	1
China (Heilongjiang)	New	RD105$^+$, RD207$^+$	ND	Beijing	1

[a] Spoligotype labeling is according to SpolDB4 (3).
[b] A positive superscript indicates that a deletion was detected; a minus superscript indicates that the RD was not deleted or the region was intact. ND, not determined.
[c] Sequence type is according to reference 26.
[d] M-PCR, multiplex PCR.
[e] East Asian lineage.
[f] Including the clades LAM1, LAM9, T1, T2, T3, T3-Osaka, and new (other than the east Asian lineage).
[g] Including the clades EAI1_SOM, EAI2-MANILA, EAI3_IND, EAI5, EAI6_BGD1, EAI7_BGD2, EAI unidentified, CAS, CAS1-DHLHI, CAS2, LAM9, T1, T4, H1, H3, X1, X2, and undesignated/new.
[h] Including the clades EAI3_IND, EAI5, CAS, CAS1-DHLHI, LAM1, LAM5, T1, T2, T3, H3, S, and undesignated/new.
[i] Including the clades EAI2_MANILA, EAI2_NTB, EAI5, EAI6_BGD1, EAI7_BGD2, CAS1-DHLHI, LAM9, T1, T3, X2, S, and undesignated/new.
[j] Overlapped peak of C and G was observed at nucleic acid position 426.
[k] Details are described in Table 3.

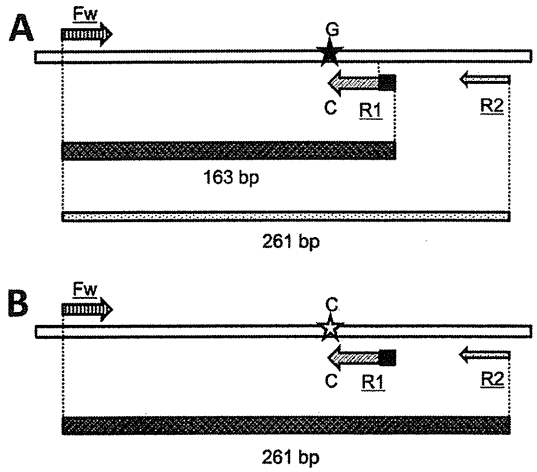

FIG 1 PCR primers and products of *Rv0679c*-targeting multiplex PCR for Beijing lineage discrimination. (A) In the Beijing sample, the 163-bp product is amplified more dominantly than is the 261-bp product. (B) In the non-Beijing sample, 163-bp product is not amplified because of the mismatch of the 3′ end of R1. Fw, forward primer; R1, reverse primer 1 (Beijing lineage specific); R2, reverse primer 2. Two-base noncomplement nucleotides at the 5′ end are shown by black squares.

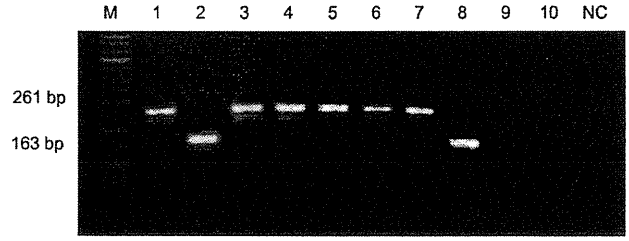

FIG 2 Electrophoresis results of the multiplex PCR products. Lane M, 50-bp ladder DNA size marker; lane 1, *M. bovis* BCG Tokyo 172 (non-Beijing lineage control) strain; lane 2, *M. tuberculosis* OM-9 strain (Beijing lineage control); lane 3, *M. tuberculosis* H37Rv; lane 4, *M. africanum* ATCC 25420; lanes 5–8, *M. tuberculosis* clinical isolates (lane 5, non-Beijing east Asian; lane 6, EAI; lane 7, LAM9; lane 8, Beijing); lane 9, *M. avium* strain JATA51-1; lane 10, *M. kansasii* JATA21-1; lane NC, negative control.

In strain C, the C185T SNP was observed, and in T17, a cytosine was inserted at position 92. In *M. canettii* CIPT 140010059, two SNPs and a codon insertion, ACC at position 154, were observed.

Beijing lineage identification by multiplex PCR. Multiplex PCR was developed targeting the Beijing-specific SNP on *Rv0679c*, employing a primer with the mutated nucleic acid at the 3′ end of the sequence (primer R1; Fig. 1 and Table 1); the optimal reaction conditions were determined as described in Materials and Methods. With this system, a bright band of 163 bp was observed as an amplified product of the primers Fw and R1 in the Beijing genotype samples (Fig. 1A and 2). An additional band of 261 bp, which is the product of primers Fw and R2, can be seen depending on the conditions, although it is always significantly thinner than the 163-bp band because of the low R2-primer concentration (see Materials and Methods). In contrast, only the 261-bp band is observed in a non-Beijing genotype sample (Fig. 1B and 2). Since the sequences of the primers are specific to the MTC, no amplification occurs in the absence of MTC genomic DNA (Fig. 2, data for *M. avium* and *M. kansasii*). A total of 619 clinical isolates obtained in the five Asian countries of Japan, Bangladesh, Nepal, Myanmar, and China were subjected to this Beijing lineage-identifying multiplex PCR, and the results were compared with their spoligotypes. All the isolates determined as having a Beijing or Beijing-like genotype by the SpolDB4 (n = 393) were determined to be in the Beijing lineage by the multiplex PCR (Table 2). On the other hand, no samples that included only non-Beijing genotype DNA (n = 216) were identified as being in the Beijing lineage. Twenty-nine non-Beijing east Asian lineage strains, which were suggested by a characteristic spoligotype having spacer 34 and were defined by RD105 detection, were determined to be non-Beijing by the multiplex PCR. Six isolates that showed a discrepancy between their spoligotype and the multiplex PCR result were further determined by RD207 or RD105 detection PCR and were revealed to be a mixture of Beijing and other subtype strains (mixed clone samples, Table 2). Four samples from different countries had confusing spoligotypes that lacked spacers 1 to 34 and additionally lacked some of the spacers from 35 to 43. These samples could also be identified correctly (Tables 2 and 3). The minimum detection limits were 100 and 1,000 cells per reaction in the Beijing genotype and BCG strains, respectively (data not shown).

DISCUSSION

In this study, we demonstrated that the SNP of C to G at position 426 in the *Rv0679c* gene is specific to the Beijing genotype strains. We developed a new multiplex PCR using this SNP to identify Beijing lineage isolates. This PCR assay successfully distinguished Beijing genotype strains from others, including the non-Beijing east Asian strains, with 100% accuracy. The Beijing lineage genotype is usually identified by spoligotyping, specific patterns of IS*6110* RFLP, or the detection of RD207, which is led by an insertion of IS*6110* in the DR region. However, spoligotyping is well known to show gene conversions, and strains having no genetic relationship sometimes show the same spoligotype (3, 26). Fenner et al. (35) reported pseudo-Beijing strains that had a typical Beijing spoligotype even though they actually belonged to the CAS family. This type of confusion seems to occur especially in areas that have a higher prevalence of principal genetic group 1 (PGG1) lineages, including the EAI, CAS, and east Asian lineages, since PGG1 strains usually possess spacers 35 and 36, which are lacking in PGG2 and PGG3 strains (3, 36). In other areas, mixed infections of more than two strains sometimes disrupt correct spoligotyping by showing mixed spacer patterns. The Manu1-SIT100 and Manu2-SIT54 types, which lack the spacers 34 or 33 and 34, respectively, are known to be producible by the mixture of Beijing family and T1 strains (3, 37). In this study, we found that some samples showed discrepant results between *Rv0679c* multiplex PCR and spoligotyping that determined a strain to be of the Beijing genotype by multiplex PCR, despite having another spoligotype. Using RD105 and RD207 detection methods, all of these samples were confirmed to be a mixture of Beijing and another strain. This type of mixed culture is sometimes observed in countries with a higher TB burden, where a coinfection of more than two strains is not rare (22). Some of the spoligopatterns of those samples showed faint positive spacers, suggesting the mixed presence of other strains. Even clear and correct spoligotypes can sometimes lead to misjudgments. In the current study, some samples showed only one to several spacers to be positive in the Beijing spacer area,

TABLE 3 Typing result comparison in clinical isolates having confusing spoligotype patterns

Sample (identification)	Spoligotype pattern	Spoligotype family[a]	*Rv0679c* M-PCR type	Detection type				Final typing result
				RD207 set 1[b]	RD207 set 2[b]	RD105	TbD1	
Japan (O-05-44)		New	Beijing	−	+[c]	+	−	Beijing
Nepal (no. 51)		New	Non-Beijing	−	−	−	+	Ancestral[d]
Myanmar (no. 95)		New	Beijing	+	+	+	ND	Beijing
China (2460)		New[e]	Beijing	+	+	+	ND	Beijing

[a] These patterns were not found in the SpolDB4 list.
[b] PCR sets 1 and 2 in reference 22.
[c] A faint correctly sized band and an additional band of a different size were observed.
[d] Ancestral type of *M. tuberculosis* strain possessing TbD1 region (25).
[e] The spoligotype pattern of this sample has been reported in reference 13.

namely, from spacers 35 to 43. Most were identified as being of the Beijing genotype by multiplex PCR, while one was judged to be a non-Beijing strain. All Beijing genotype-positive results were confirmed by RD105 and RD207 PCRs, and the non-Beijing isolate was revealed as an "ancestral type," which involves EAI but not the Beijing lineage, by TbD1 detection (Table 3) (25). These examples support the high specificity and applicability of this SNP-targeting PCR. The disadvantages of IS*6110* RFLP and RD207 detection have already been described above. RD207-detection PCR did not work as expected in the sample that lost spacers 1 to 42 (Japan O-05-44; Table 3), suggesting that some additional reconstruction had occurred at the IS*6110* insertion site of the DR region. SNPs in MTC genomes can provide robust lineage information, whereas repetitive elements, such as direct repeats in the DR region, the mycobacterial interspersed repetitive unit (MIRU) tandem repeats (38), or IS*6110*, are prone to alteration. One hundred percent concordance of the PCR results with the genetically confirmed Beijing type is not surprising because of the rigidity of the SNPs in the MTC (25, 26). Of the 393 Beijing family isolates, 94 were subtyped by MLST and consisted of 8 STs covering a wide range of the Beijing family, from ancient to modern types (Table 2). This suggested that a specific mutation in *Rv0679c* seemed to have occurred in the Beijing lineage at the same time as the RD207 deletion event.

Rv0679c is an MTC-specific gene, as shown by Cifuentes et al. (27), and no significantly similar sequence was detected by an NCBI BLASTn search in the GenBank database. Thus, this multiplex PCR assay can be used for the identification of the MTC, as well as for the differentiation of Beijing and non-Beijing lineages (Fig. 2). The Beijing mutation detection primer (R1; Fig. 1 and Table 1) was designed to have two additional noncomplement bases at the 5′ end to block the second amplification by the PCR product that produces the 261-bp fragment with an outer R2 primer. Additionally, the higher concentration and melting temperature of the R1 primer compared to those of the outer R2 primer increase the Fw-R1 product more than the Fw-R2 product. With these techniques, the Beijing band (163 bp) can be shown to be significantly brighter than the non-Beijing band (261 bp) when the sample is derived from Beijing lineage *M. tuberculosis* strains (Fig. 2). The relatively higher annealing temperature of 66°C gave good contrast of those two bands and prevented nonspecific amplifications. Modified *Taq* or other polymerases that have 3′-to-5′ exonuclease activity should be avoided, since those enzymes can trim the mutated nucleotide at the R1 primer end. It is recommended to check the PCR conditions using positive controls for Beijing and non-Beijing types (i.e., BCG) every time (Fig. 2). The detection limit of 100 to 1,000 copies per reaction might be relatively high; however, it can be improved by about 10 times by increasing the PCR cycle number to 40, although the necessity of identifying the MTC lineage in direct clinical specimens seems to be low.

In papers featuring SNPs as epidemiological markers, synonymous mutations are usually selected to avoid the effect of evolutional pressure (26). However, both SNPs for the differentiation of PGG1, PGG2, and PGG3 were nonsynonymous mutations in *katG* and *gyrA* (36), and so far, they have provided robust differentiation results. In the MTC, nonsynonymous mutations on functional genes can be observed in a relatively higher frequency than in other bacteria because of extremely reduced purifying selection pressure (39). Thus, nonsynonymous mutations can be preserved unless they are significantly disadvantageous. Indeed, 100% of the Beijing family strains in the current study could be identified with this nonsynonymous mutation, suggesting that it at least has no adverse effect on those strains. The function of the Rv0679c protein is still unclear, although its expression on the cell surface has been confirmed (27, 28). Cifuentes et al. (27) reported that the surface-localized Rv0679c protein contributed to the *M. tuberculosis* invasion of host cells and proposed the protein as a vaccine candidate. The substituted amino acid at position 142 was located in the C-terminus region of the protein, which was included in the "high-activity binding peptide" to target cells (27). Thus, this highly conserved nonsynonymous SNP, which results in an amino acid substitution with different characteristics (Asn → Lys), might have some biological meaning in explaining Beijing lineage pathogenicity. Since BCG vaccine strains, as well as other non-Beijing strains, have Rv0679c-Asn142, this substitution might affect the antigenicity of the Beijing bacterial surface and might contribute to the possible evasion of BCG-derived immunity. Further investigation of the association of the Rv0679c Asn142Lys substitution with Beijing strain outer membrane characteristics and antigenicity is ongoing.

In conclusion, a simple, robust, and low-cost multiplex PCR assay for the detection of Beijing lineage *M. tuberculosis* strains was successfully developed using a Beijing-specific SNP on *Rv0679c*. This PCR assay can be used in local laboratories to monitor the prevalence of the Beijing genotype, and this is strongly recommended to control this possibly highly pathogenic and drug resistance-prone sublineage.