Genotypic Analysis of *Mycobacterium tuberculosis* in Bangladesh and Prevalence of the Beijing Strain

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Genotypic analysis was performed on 48 *Mycobacterium tuberculosis* complex strains collected from a hospital in Dhaka city. Deletion analysis showed that the isolates were all *M. tuberculosis;* 13 of them were found to be of the "ancestral" type, while 35 were of the "modern" type, indicating that both endemic (ancestral type) and epidemic (modern type) strains cause tuberculosis in Bangladesh. Genotyping based on the spoligotype and variable-number tandem repeats (VNTR) of mycobacterial interspersed repetitive units (MIRU) was also done. A total of 34 strains (71%) were grouped by spoligotyping into nine different clusters; the largest comprised 15 isolates of the Beijing genotype, whereas the remaining eight clusters consisted of two to five isolates. MIRU-VNTR typing detected 32 different patterns among 44 tested strains, and the 15 Beijing strains were further discriminated by MIRU-VNTR typing (7 distinct patterns for the 15 isolates). These results indicate that MIRU-VNTR typing, along with spoligotyping and deletion analysis, can be used effectively for molecular epidemiological studies to determine ongoing transmission clusters; to our knowledge, this is the first report about the type of strains prevailing in Bangladesh.

Despite global efforts to combat tuberculosis (TB), the disease remains a major public health problem worldwide, especially in developing countries such as Bangladesh. Key factors in the control of TB are rapid detection and adequate therapy to arrest further transmission. Outbreaks of infectious disease often result from exposure to a common source of the etiologic agent (9). Generally, the etiologic agent causing an outbreak of infection is derived from a single cell whose progeny are genetically identical or closely related to the source organism. In epidemiological terms, the organisms involved in the outbreak are clonally related. DNA fingerprinting techniques now exist which identify specific strains of *Mycobacterium tuberculosis* (22). These techniques, along with conventional approaches, have become powerful tools in TB epidemiology. Unfortunately, epidemiological data for Bangladesh are scarce. Hence, monitoring the control of TB by epidemiological investigation is of utmost importance.

Large-scale genotyping of *M. tuberculosis* using IS6110 restriction fragment length polymorphism is labor-intensive and requires culturing of the slow-growing mycobacteria, and the results are sometimes difficult to compare among laboratories. After the completion of the genome sequence of *M. tuberculosis* H37Rv, comparative-genomics approaches greatly enhanced our understanding of the mechanisms of insertion and deletion of DNA and the resulting distribution of variable regions around the genomes of tubercle bacilli (4, 6, 7). Based on this knowledge, a deletion analysis system has been developed to differentiate the members of the *M. tuberculosis* complex (4, 23). There are 20 variable regions, of which 14 regions of difference (RD1 to RD14) were found to be absent from bacillus Calmette-Guérin (BCG) Pasteur relative to *M. tuberculosis* H37Rv (2, 7, 10). Six regions, H37Rv-related deletions (RvD1 to RvD5 and *M. tuberculosis* specific deletion 1 (TbD1), are absent from the *M. tuberculosis* H37Rv genome relative to other members of the *M. tuberculosis* complex. Based on the presence or absence of the TbD1 region, *M. tuberculosis* strains can be divided into "ancestral" and "modern" types. The Beijing, Haarlem, and African strains responsible for major epidemics are modern types (4, 16).

Spoligotyping is a PCR-based method which allows simultaneous detection and strain differentiation of *M. tuberculosis* present in clinical specimens without the need for culture (13). The method is based on strain-dependent hybridization patterns of in vitro-amplified DNA with multiple spacer oligonucleotides. This region contains multiple short 36-bp direct repeats (DRs) and nonrepetitive spacers, which are 35 to 41 bp in length, interspersed between the DRs. Spoligotyping is a rapid method that allows large numbers of isolates to be handled in a short time. The DRs are extremely well conserved among *M. tuberculosis* complex strains, making spoligotyping a specific method for the detection of *M. tuberculosis* complex members (13).

Recently, a typing method based on variable-number tandem repeats (VNTR) of genetic elements named mycobacterial interspersed repetitive units (MIRU) in 12 human minisatellite-like regions of the *M. tuberculosis* genome has been developed (20, 28). MIRUs are composed of 40- to 100-bp repetitive DNA sequences, dispersed in 41 intergenic regions of the *M. tuberculosis* complex genome (18, 29, 30). Twelve of these sites display polymorphisms in MIRU copy number among nonrelated *M. tuberculosis* isolates. Typing using MIRUs

TABLE 1. Patient characteristics

Strain	Age (yr)	Sex[a]	Treatment history	BCG scar	Sensitivity[b] to:				Origin
					INH	RMP	EMB	SM	
TB1	28	M	Previously treated	Present	ND	ND	ND	ND	Dhaka
TB2	30	M	Previously treated	Present	S	S	S	S	Dhaka
TB6	18	M	New case	Absent	S	S	S	S	Dhaka
TB8	25	M	New case	Present	ND	ND	ND	ND	Dhaka
TB9	60	M	New case	Absent	S	S	S	S	Dhaka
TB10	50	M	Previously treated	Present	ND	ND	ND	ND	Patuakhali
TB11	30	M	New case	Present	ND	ND	ND	ND	Dhaka
TB12	56	M	New case	Present	S	S	S	S	Natore
TB14	52	M	Previously treated	Present	R	R	R	R	Rangpur
TB16	20	F	New case	Present	ND	ND	ND	ND	Gazipur
TB19	50	F	New case	Present	S	S	S	S	Dhaka
TB20	16	M	New case	Absent	ND	ND	ND	ND	Narayanganj
TB21	22	M	New case	Absent	S	S	S	S	Narayanganj
TB23	35	M	New case	Present	S	S	S	S	Kishoreganj
TB24	20	F	New case	Absent	ND	ND	ND	ND	Dhaka
TB27	26	M	New case	Present	S	S	S	S	Dhaka
TB29	22	F	New case	Present	S	S	S	S	Dhaka
TB30	20	F	New case	Absent	S	S	S	S	Dhaka
TB32	35	F	New case	Present	ND	ND	ND	ND	Dhaka
MA1	30	M	Previously treated	Not known	S	S	S	S	Dhaka
MA2	32	F	Previously treated	Absent	S	S	S	S	Dhaka
MA5	42	M	Previously treated	Absent	S	S	S	S	Dhaka
MA6	32	M	Previously treated	Absent	R	R	S	S	Shariatpur
MA7	50	M	Previously treated	Absent	R	R	S	S	Noakhali
MA9	33	M	Previously treated	Absent	R	R	S	S	Munshiganj
MA11	27	M	Previously treated	Present	S	S	S	S	Comilla
MA12	38	F	Previously treated	Absent	R	S	S	R	Dhaka
MA13	33	F	Previously treated	Absent	S	S	S	S	Dhaka
MA16	35	M	Previously treated	Absent	R	R	R	R	Dhaka
MA17	46	M	Previously treated	Not known	R	S	R	S	Narayanganj
MA19	25	M	Previously treated	Present	R	S	S	S	Narayanganj
MA20	40	M	Previously treated	Absent	R	R	R	R	Kishoreganj
MA25	55	M	Previously treated	Present	S	R	S	R	Dhaka
MA27	50	M	Previously treated	Present	S	R	S	S	Shariatpur
MA28	40	M	Previously treated	Absent	R	R	S	R	Narsingdi
MA29	30	M	Previously treated	Present	R	S	S	S	Kishoreganj
MA31	31	M	Previously treated	Absent	R	R	R	R	Narayanganj
MA32	28	M	Previously treated	Absent	S	S	S	S	Dhaka
MA35	23	M	Previously treated	Present	S	S	S	R	Dhaka
MA36	50	M	Previously treated	Not known	S	S	S	S	Dhaka
MA37	45	M	Previously treated	Present	S	S	S	S	Habiganj
MA38	39	M	Previously treated	Absent	ND	ND	ND	ND	Comilla
MA39	22	F	Previously treated	Present	R	R	S	R	Gazipur
MA41	45	M	Previously treated	Absent	R	S	S	S	Dhaka
MA42	22	M	Previously treated	Present	R	S	R	R	Shariatpur
MA45	17	F	Previously treated	Not known	S	S	S	S	Dhaka
MA46	40	M	Previously treated	Not known	S	S	S	S	Dhaka
MA48	20	F	Previously treated	Not known	R	S	S	S	Dhaka

[a] M, male; F, female.
[b] INH, isoniazid; RMP, rifampin; EMB, ethambutol; SM, streptomycin; R, resistant; S, sensitive; ND, not done.

is a PCR-based method where strains can be typed by a numerical code corresponding to the numbers of MIRUs in the different loci. Here, we apply a combination of all three different genotyping methods to a random collection of isolates of *M. tuberculosis* from Bangladesh.

MATERIALS AND METHODS

Mycobacterial strains and genomic DNA. A total of 48 cases of TB were investigated (Table 1). Samples were collected randomly from patients attending the Institute of Diseases of Chest and Hospital (IDCH) between 1999 and 2000. IDCH is a national facility for the diagnosis and treatment of TB in Dhaka city. Treatment is free, and the hospital serves a large segment of the city's population. It also handles a substantial number of patients with complications referred from other hospitals and Thana Health Complexes in and outside Dhaka city.

Twenty-six of the patients were from different parts of Dhaka city (Table 1). Others were from different regions of Bangladesh outside Dhaka (Table 1). Most of them (>70%) were from a low socioeconomic condition. Thirty-six were male, and 12 were female. Their ages ranged from 16 to 60 years (the mean age was 34 years). Twenty-two patients had a scar present, indicating that they were vaccinated with BCG during their childhood. Twenty patients had no scar, and the result was not recorded for the other six. All of the patients were sputum microscopy positive and culture positive. Thirty-three samples were from previously diagnosed patients who had been treated with antitubercular drugs for certain periods. Fifteen of the isolates were primary isolates. Drug sensitivity testing was performed on 39 samples.

Genomic DNA was obtained by resuspending mycobacterial colonies in 100 to 200 µl of distilled H_2O and incubating them at 85°C for 30 min. After centrifugation of the suspension, the supernatant containing the DNA was removed and stored at −20°C until further use.

RD PCR analysis. RD PCR analysis was done using the methods described by Brosch et al. (4). Sequences inside or flanking RD and RvD regions were

TABLE 2. Deletion analysis and spoligotyping of Bangladeshi strain

Strain	Presence of region[a]:																				
	RD 1	RD 2	RD 3	RD 4	RD 5B	RD 6	RD 7	RD 8	RD 9	RD 10	RD 11	RD 12	RD 13	RD 14	15-Be	RvD1	RvD2	RvD3	RvD4	RvD5	TbD 1
Block 1																					
TB1	1	1	0	1	1	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1
TB32	1	1	1	1	0	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1
MA25	1	1	0	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1
MA31	1	1	0	1	1	1	1	1	1	1	2	1	1	1	0	1	1	1	1	1	1
TB16	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1
TB29	1	1	0	1	0	1	1	1	1	1	0	1	1	1	0	1	1	1	1	1	1
TB23	1	1	1	1	0	1	1	1	1	1	0	1	1	1	0	1	1	1	1	1	1
TB21	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	1	1	1	1
TB27	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	1	1	1	1
TB30	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	1	1	1	1
TB19	1	1	1	1	0	1	1	1	1	1	0	1	1	1	0	1	1	1	1	1	1
TB2	1	1	1	1	0	1	1	1	1	1	0	1	1	1	0	1	1	0	1	1	1
TB20	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1
Block 2																					
TB8	1	1	0	1	1	1	1	1	1	1	0	1	1	1	ND	1	0	1	1	1	0
MA9	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	0	1	1	0
MA36	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	0	1	1	0
TB24	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0
MA5	1	1	1	1	1	1	1	1	1	1	2	1	1	1	0	1	0	1	1	1	0
MA29	1	1	0	1	1	1	1	1	1	1	1	1	1	1	ND	1	0	0	1	1	0
MA39	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	0	0
MA41	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	0	1	1	0
Block 3																					
MA6	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0
MA11	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0
MA19	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0
MA35	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0
MA42	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0
MA45	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0
MA46	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0
MA17	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0
MA27	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0
MA32	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0
MA37	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0
TB6	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0
MA1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0
MA16	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0
MA28	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0
Block 4																					
TB9	1	1	0	1	1	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0
TB10	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0
MA2	1	1	0	1	1	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0
MA7	1	1	0	1	1	0	1	1	1	1	1	1	1	1	0	1	0	0	0	1	0
MA12	1	1	0	1	1	0	1	1	1	1	1	1	1	1	0	1	0	0	0	1	0
MA13	1	1	0	1	1	0	1	1	1	1	1	1	1	1	0	1	0	0	0	1	0
MA20	1	1	0	1	1	0	1	1	1	1	2	1	1	1	0	1	0	0	1	1	0
TB14	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0
TB12	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0
MA38	1	1	0	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0
MA48	1	1	0	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0
TB11	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0

[a] 0, region not present; 1, region present; 2, both internal and flanking primers give product. IS-Be = IS *6110* Beijing strains; ND, not determined.

[b] ., spacer not present; X, spacer present.

obtained from the websites http://genolist.pasteur.fr/TuberculList/ and http://www.sanger.ac.uk/Projects/M bovis/. Primers that would amplify ~500-bp fragments were designed by using the Primer3 website http://www-genome.wi.mit.edu/cgi-bin/primer3-www.cgi. A detailed list of all primer sequences used in this study is available at http://www.pnas.org/cgi/data/052548299/DC1/1. PCR amplifications with mixtures containing, per reaction, 1.25 μl of 10× PCR buffer [600 mM Tris HCL (pH 8.8), 20 mM MgCl₂, 170 mM (NH₄)₂SO₄, 100 mM β-mercaptoethanol], 1.25 μl of 20 mM nucleotide mix, 50 nM each primer, 1 to 10 ng of template DNA, 10% dimethyl sulfoxide, 0.2 U of *Taq* polymerase (Gibco-BRL), and sterile distilled water to 12.5 μl were performed on a PTC-100 amplifier (MJ Inc.) with an initial denaturation step of 90 s at 95°C followed by 35 cycles of 30 s at 95°C, 1 min at 58°C, and 4 min at 72°C. Sequence analysis of the *katG* codon 463 polymorphism was done as described previously (4).

Spoligotyping. Spoligotyping was performed as previously described by Kamerbeek et al. (13) with minor modifications. The DR region was amplified by PCR with oligonucleotide primers derived from the DR sequence. Mycobacterial genomic DNA was extracted from cultured cells as described previously (14, 15). Twenty-five microliters of the following reaction mixture were used for the PCR: 12.5 μl of HotStarTaq Master Mix (Qiagen; this solution provides a final concentration of 1.5 mM MgCl₂ and 200 μM each deoxynucleoside triphosphate), 2 μl of each primer (20 pmol each), 5 μl of DNA solution (ca. 10 ng), and 3.5 μl of distilled water). The mixture was heated for 15 min at 96°C and subjected to 30 cycles of 1 min at 96°C, 1 min at 55°C, and 30 s at 72°C. The amplified product was hybridized to a set of 43 immobilized oligonucleotides, each corresponding to one of the unique spacer DNA sequences within the DR locus. After hybridization, the membrane was washed twice for 10 min in 2× SSPE (1× SSPE is 0.18

TABLE 2—*Continued*

Presence of spacer[b]:

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43
X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	.	.	.	.	X	.	X	X	X	X	X	.	X	X	X
X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	.	.	.	.	X	.	X	X	X	X	X	.	X	X	X
X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	.	.	.	.	X	.	X	X	X	X	X	.	.	.	.
X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	.	.	.	.	X	.	X	X	X	X	.	.	.	.	.
X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	.	.	.	.	X	.	X	X	X	X	X	X	X	X	X
X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	.	X	X	X	X	X	X	X	.	.	.	.	X	.	.	.	.	.	.	.	.	.	.
X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	.	X	X	X	X	X	X	X	.	.	.	.	X	.	X	X	X	X	X	X	X	X	X
X	.	.	X	.	X	X	X	X	.	.	.	.	.	.	.	X	X	X	X	X	X	X	X	X	X	X	X	X	.	.	.	.	X	.	X	X	.	.	X	X	X	X	X
X	.	.	X	X	X	X	X	X	.	.	.	.	.	.	X	X	X	X	X	X	X	X	X	X	X	X	X	X	.	.	.	.	X	.	X	X	.	.	.	X	X	X	X
X	.	.	X	X	X	X	X	X	X	.	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	.	.	.	.	X	.	X	X	.	.	.	X	X	X	X
X	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	X	X	.	.	.	.	X	.	X	X	.	X	X	X	X	X
X	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	X	X	X	X
X	X	X	X	X	X	X	X	X	.	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	.	.	.	.	.	.	X	X	X	X	X	.	X	X	X
X	X	X	.	.	.	X	X	X	X	X	X	X	X	X	X	X	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	X	X	X	X	X	X	X	X	X
X	X	X	.	.	.	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	.	X	.	.	.	.	.	.	.	.	.	X	X	X	X	X	X	X	X	X
X	X	X	.	.	.	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	.	X	.	.	.	.	.	.	.	.	.	.	.	X	X	X	X	X	X	X	X	X
X	X	X	.	.	.	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.	X	X	X	X	X	X	X	X	X
X	X	X	.	.	.	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.	X	X	X	X	X	X	X	X	X
X	X	X	.	.	.	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.	X	X	X	X	X	X	X	X	X
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	X	X	X	X	X	X	X	X	X
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	X	X	X	X	X	X	X	X	X
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M NaCl, 10 mM NaH$_2$PO$_4$, and 1 mM EDTA [pH 7.7])–0.5% sodium dodecyl sulfate at 60°C and then incubated in 1:4,000-diluted streptavidin-peroxidase conjugate (Boehringer) for 45 to 60 min at 42°C. The membrane was washed twice for 10 min in 2× SSPE–0.5% sodium dodecyl sulfate at 42°C and rinsed with 2× SSPE for 5 min at room temperature. Hybridizing DNA was detected by the enhanced chemiluminescence method (Amersham) (32, 33) and by exposure to X ray film (Hyperfilm ECL; Amersham) as specified by the manufacturer.

PCR and MIRU analysis. PCRs were carried out by using the PCR reagent system (Gibco-BRL). Five microliters from fivefold-diluted DNA solutions was added to a final volume of 50 μl containing 0.2 μl of DNA polymerase (1 U), 0.2 mM each dATP, dCTP, dGTP, and dTTP, 5 μl of PCR buffer, 0.4 μM (2 μM for locus 7) primers and 1 to 3.5 mM MgCl$_2$. The primers and MgCl$_2$ concentrations used were as described by Mazars et al. (20). The PCR fragments were analyzed by agarose gel electrophoresis with 1.5% agarose. The sizes of the amplicons were estimated by comparison with 50- and 100-bp ladders. The MIRU copy number per locus was calculated by using the conventions described by Supply et al. (30).

RESULTS

Deletion analysis. Forty-eight strains isolated from pulmonary TB patients were investigated by PCR-based deletion analysis using the 20 variable regions. Most of the RD regions were present in all the strains tested. RD1, RD2, RD4, RD7, RD8, RD9, RD10, RD12, RD13, RD14 and RvD1 were present in all strains (Table 2). The presence of these 10 RD regions, which are strictly conserved in *M. tuberculosis*, indicates that all the strains tested belonged to the species *M.*

TABLE 3. MIRU-VNTR analysis of *M. tuberculosis* isolates

| Strain | Copy no. at MIRU locus: | | | | | | | | | | | Cluster | |
	2	4	10	16	23	24	26	27	31	39	40	Spoligotype	MIRU-VNTRs
Block 1													
TB1	2	3	3	0	6	2	2	0	5	1	3	ST1	
TB32	2	5	4	3	7	2	2	3	5	1	3		
MA25	2	5	6	2	6	2	2	3	3	1	3	ST2	MI1
MA31	2	5	6	2	6	2	2	3	3	1	3		
TB16	2	2	4	3	5	2	2	3	5	2	3		
TB29	2	5	3	3	4	2	2	3	5	3	3		
TB23	2	5	3	4	6	2	3	5	3	3	2		
TB21	2	5	4	4	6	2	2	3	5	3	3		
TB27	2	5	3	1	6	2	2	3	5	3	4	ST3	
TB30	2	5	3	1	5	2	2	3	5	3	4		
TB19	2	4	4	4	7	2	2	1	5	2	3		
TB2	2	2	0	0	5	2	2	1	5	3	3		
TB20	2	5	4	3	6	2	2	3	5	1	3		
Block 2													
TB8	2	2	6	3	5	1	6	3	5	3	3		
MA9	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ST4	
MA36	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND		
TB24	2	2	6	4	5	1	7	3	5	2	3	ST5	
MA5	2	2	5	4	5	1	8	3	5	3	3		
MA29	2	4	4	4	5	1	8	3	6	3	3		
MA39	2	2	6	4	5	1	6	3	4	2	3		
MA41	2	2	6	4	5	1	7	3	4	2	3		
Block 3													
MA6	2	2	3	3	5	1	5	3	5	3	3	ST6	MI2
MA11	2	2	3	3	5	1	5	3	5	3	3		
MA19	2	2	3	3	5	1	5	3	5	3	3		
MA35	2	2	3	3	5	1	5	3	5	3	3		
MA42	2	2	3	3	5	1	5	3	5	3	3		
MA45	2	2	3	3	5	1	5	3	5	3	3		
MA46	2	2	3	3	5	1	5	3	5	3	3		
MA17	2	2	3	3	5	1	7	3	4	3	3		MI3
MA27	2	2	3	3	5	1	7	3	4	3	3		
MA32	2	2	3	3	5	1	6	3	4	3	3		MI4
MA37	2	2	3	3	5	1	6	3	4	3	3		
TB6	2	4	3	3	5	1	5	3	5	3	3		
MA1	2	2	2	3	5	1	5	3	5	3	3		
MA16	2	2	3	3	5	1	5	3	5	2	3		
MA28	2	2	3	3	5	1	2	3	5	3	3		

tuberculosis (4). The RD3 region was absent in 29 strains, and the product amplified using the second set of internal primers was smaller in 2 strains. RD11, was also absent from 8 of 48 strains tested. The RD3 and RD11 regions correspond to prophages phiRv1 and phiRv2 of *M. tuberculosis* H37Rv (7). RD5, which contains the genes *plcA* to *plcC* that encode proteins with phospholipase C activity, was absent from five strains. Such genes have been identified as hot spots for inser-

TABLE 3—*Continued*

| Strain | Copy no. at MIRU locus: | | | | | | | | | | | Cluster | |
	2	4	10	16	23	24	26	27	31	39	40	Spoligotype	MIRU-VNTRs
Block 4													
TB9	2	2	5	3	5	1	5	3	2	2	3	ST7	MI5
TB10	2	2	5	3	5	1	5	3	2	2	3		
MA2	2	2	4	3	6	1	6	3	3	2	1	ST8	MI6
MA7	2	2	4	3	6	1	6	3	3	2	1		
MA12	2	2	4	2	6	1	6	3	3	2	1	ST9	MI7
MA13	2	2	4	2	6	1	6	3	3	2	1		
MA20	2	2	0	2	6	1	6	3	3	2	1		
MA38	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND		
MA48	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND		
TB14	2	2	4	3	5	1	6	3	3	2	4		
TB12	2	2	5	3	5	1	5	3	3	2	3		
TB11	2	2	3, 4	3	5	1	5	3	3	2	3, 5		

Vertical bars correspond to spoligotype (ST) and MIRU-VNTR (MI) clusters. ND, not determined.

tion of the mobile element IS*6110* (35). Homologous recombination of two copies of IS*6110* oriented in the same direction may cause deletion of the intervening sequences, and this mechanism contributes to the hypervariability of such genomic regions (5). RD6, containing IS*1532* (10), was absent from 10 strains. These four regions were found to be absent from some of the *M. tuberculosis* strains investigated previously (4).

In a large number of the isolates tested (35 isolates), the TbD1 region was deleted, indicating that they are all modern strains of *M. tuberculosis*. However, 13 of 48 strains were found to harbor the TbD1 region (Table 2). As expected, the presence or absence of RvD regions was variable. RvD1 was present in all 48 strains, whereas RvD2 and RvD3 were highly variable in their presence. RvD2 and RvD3 were absent from 27 and 24 strains, respectively, with both RvD2 and RvD3 being deleted from 21 strains. Strains belonging to the Beijing cluster (see below) were shown to lack both RvD2 and RvD3, consistent with previous findings (4). Like RD5, RvD2 also contains a *plc* gene (*plcD*), and it has been shown that deletions in the RvD2 region can be as large as 20 kb in some *M. tuberculosis* strains (12). RvD2 and RvD3 are located very close to each other on the chromosome of *M. tuberculosis* H37Rv at positions 1989 and 1997 kb, respectively. As such, the constant absence of region RvD2 and RvD3 from *M. tuberculosis* strains belonging to the Bejing cluster suggests that these two regions may have been removed by a single deletion of a larger chromosomal region. RvD4 was present in 45 strains and absent from 3 strains. RvD5 was absent from one of the strains tested.

Spoligotyping. All 48 isolates were analyzed by spoligotyping (Table 2). A cluster was defined as two or more isolates from different patients with identical spoligotype patterns, whereas nonclustered patterns were referred to as unique. The largest cluster (ST6) consists of 15 strains, one cluster contains five strains (ST5), and there are seven other clusters (ST1, ST2,

ST3, ST4, ST7, ST8, and ST9) comprising two strains each. Fourteen isolates (29%) exhibited unique (nonclustered) patterns. All strains of the largest cluster comprising 15 isolates contained only 9 of the 43 spacer sequences tested. They showed a spoligotype pattern with hybridization only to the 3'-terminal spacer 35 to 43, which is characteristic of the Beijing genotype. In all strains identified as the Beijing type, RD3, RvD2, and RvD3 regions were found to be absent by deletion analysis. We examined 46 strains for the presence of the Beijing genotype-specific IS*6110* in the *dnaA-dnaN* region (16). This marker was present only in the largest cluster of strains identified as Beijing members by spoligotyping and deletion analysis (Table 2).

The 48 isolates analyzed by deletion analysis and spoligotyping were organized in four blocks according to their degree of relatedness (Table 2). Block 1 comprised the 13 ancestral strains having TbD1 present. *M. bovis* strains usually lack spacer sequences 39 to 43 (16). The spoligotypes of three strains in block 1, MA25 and MA31, lacking spacers 40 to 43, and TB29, lacking spacers 34 to 43, highly resembled those of *M. bovis*, but the presence of region RD9 and some other RD regions that are not contained in the genome of *M. bovis* confirmed that these strains were indeed *M. tuberculosis* strains.

All 13 strains in block 1 belonged to phylogenetic group 1 defined by Sreevatsan et al. (27) based on the *katG* codon 463 (CTG) sequence polymorphism. It is noteworthy that the great majority of these strains (85%) carried spacer 33, which was absent from all other tested *M. tuberculosis* strains, whereas spacers 29 to 32 were missing (Table 2). It seems that this particular combination is a characteristic part of the spoligotypes common in *M. tuberculosis* strains that have region TbD1 still present; therefore they have been named ancestral *M. tuberculosis* strains (4).

In contrast, block 2 contains eight strains from phylogenetic

group 1 with TbD1 deleted. These isolates showed high similarity in their spoligotype pattern for spacers 23 to 43 to those from Beijing strains, which are regrouped in block 3. The major difference between the spoligotypes of strains from block 2 and the Bejing strains in block 3 is that many of the spacers from 1 to 22 are still present in strains from block 2 whereas they are absent from Bejing strains, which characteristically harbor only spacers 35 to 43 (Table 2).

Block 4 contains strains of phylogenetic group 2 or 3 of Sreevatsan et al. (27), showing the $katG^{463}$ (where 463 is the codon number) sequence CGG. Interestingly, all these strains lacked spacers 33 to 36 but had the flanking spacers present. This spoligotype signature is typical of strains from phylogenetic groups 2 and 3 (4, 25). According to data from a large spoligotype collection, strains with this characteristic combination (spacer 32 present, spacers 33 to 36 absent, and spacer 37 present) represent the great majority of *M. tuberculosis* strains isolated throughout the world (26).

Genotyping by MIRU-VNTR. Forty-four of the 48 isolates were also typed using the MIRU-VNTR typing method. This detected 32 different patterns (Table 3), 19 of which were grouped into seven clusters. The largest cluster comprised seven strains (MI2), and six clusters comprised two strains each. Twenty-five strains had unique patterns. The 15 Beijing strains clustered in block 3 by spoligotyping were divided into seven different MIRU genotypes. Eleven of them were grouped into three MIRU-VNTR patterns, and two groups comprised two identical strains. The remaining four had unique MIRU patterns (Table 3). Unexpectedly, most of the strains (38 strains) failed to give a PCR product with primers for the MIRU 20 locus and only 6 strains gave similar-sized bands (data not shown). Since the reason for the failure of amplification at this locus remains unclear, the results of MIRU locus 20 were not included in the present MIRU-VNTR typing analysis. Another unusual finding was that TB11 isolates gave double bands of different sizes for MIRU loci 10 and 40 (Table 3). Although it is possible that the patient was coinfected with two different strains, this result is still surprising, given that the DNA was prepared from a single colony. Both MIRU 10 and 40 are highly polymorphic.

DISCUSSION

The aim of this study was to characterize the strains causing TB in Bangladesh by using three different molecular typing methods: RD-based deletion analysis, spoligotyping, and MIRU-VNTR typing. A total of 48 strains were studied, which were clinical isolates from adult pulmonary TB patients treated in a hospital in Dhaka during different periods. Deletion analysis using RD1 to RD14 revealed that all the samples investigated were *M. tuberculosis*. The use of primers internal to the RD regions, in parallel with primers flanking these regions, allowed us to identify possible false-positive or false-negative PCR amplification and contributed to the reproducibility and accuracy of the results. One very interesting finding of the study was that 13 of the 48 *M. tuberculosis* strains tested had the TbD1 region intact, indicating that a considerable number of such strains transmit TB in Bangladesh. TbD1, the "*M. tuberculosis* specific deletion 1" was originally identified as a 2,153-bp region that was specifically lacking from the *mmpL6*

genes of almost all *M. tuberculosis* strains (4). In contrast, all other members of the *M. tuberculosis* complex have this 2.1-kb region present. However, a few *M. tuberculosis* strains included in the initial analysis contained the TbD1 region, and these strains were named ancestral type *M. tuberculosis* strains because they belong to a lineage of strains that divided from all other *M. tuberculosis* strains before the deletion of TbD1 occurred (4). From an evolutionary standpoint, it seems that this group of strains is quite distant from all other *M. tuberculosis* strains, which is also reflected by the particular spoligotypes of these isolates. As shown in Table 2, most of these strains have the following spoligotype signature, i.e., absence of spacers 29 to 32, presence of spacer 33, and absence of spacer 34. The finding that this section of the spoligotype is conserved in most ancestral *M. tuberculosis* strains allows one to predict the presence or absence of TbD1 by simple analysis of the spoligotype.

By consulting the spoligotype database at the Pasteur Institute of Guadeloupe, which contains more than 3,300 spoligotypes from *M. tuberculosis* strains isolated in different parts of the world (26), it is possible to identify strains that resemble ancestral *M. tuberculosis* strains. However, the number of isolates with such spoligotypes is small. The few ancestral *M. tuberculosis* strains that have previously been analyzed had a very low copy number of IS*6110* and were isolated from East African or Indian patients (16); it has been suggested that such strains may originate from foci of endemic infection (4, 24). Bangladesh has long been a region where TB is endemic. The large number of ancestral-type *M. tuberculosis* strains (13 of 48, represented in block 1 of Table 2) identified in the present study suggests that, indeed, these strains belong to the endemic strain pool, and that they have probably persisted in this region for a considerable time. Unfortunately, it is difficult to calculate with precision the time that has elapsed since their divergence from the putative common ancestor. However, some indication is available now from studies of mummified human remains. Zink et al. recently analyzed mycobacterial DNA sequences from Egyptian mummies that were at least 2,500 years old and were characterized by spinal and rib lesions pathognomonic for TB (36). Interestingly, the spoligotypes of these amplified mycobacterial DNAs showed many characteristics of "modern" *M. tuberculosis* strains of genetic group 2 or 3, similar to the ones depicted in block 4 of Table 2, i.e., absence of spacers 33 to 36 and presence of the flanking spacers 32 and 37. Considering the clonal structure of the *M. tuberculosis* strain population (31), with little or no exchange of genetic material between different lineages of *M. tuberculosis*, this observation suggests that the branches of ancestral and modern *M. tuberculosis* strains, both prevalent in Bangladesh, separated more than 2,500 years ago and represent phylogenetically quite distinct populations of *M. tuberculosis* strains present in the same geographical region.

The modern *M. tuberculosis* strains comprise representatives of major epidemics like the Beijing, Haarlem, and African clusters. The majority of *M. tuberculosis* strains of the Beijing family originated from the province of Beijing in China, and strains of this family were found to dominate in neighboring countries such as Mongolia, South Korea, Thailand, and Vietnam (1, 34). In contrast to the dominance of the Beijing genotype in many Asian countries, a low frequency (3%) of this genotype was reported among the strains from India (21).

Strains of the Beijing family have also been found in Europe, Africa, and the United States. The "W" strain, which caused a large outbreak of multidrug-resistant TB in New York and other U.S. cities, belongs to the Beijing family (16).

In the neighboring countries in Asia, rates of infection with the Beijing family strains are higher than those in the more distant countries, suggesting that the Beijing family may have radiated from the Beijing area to other regions. The factor which was responsible for the selection and dissemination of the Beijing strains is not known, but there is evidence that Beijing strains, like strain HN878, are hypervirulent, as demonstrated by the unusually early death of infected immunocompetent mice (19). Beijing strains are more common in areas where BCG vaccination coverage is extensive. Most countries in Southeast Asia have used BCG vaccination for the past two to six decades. It has been suggested that BCG vaccination may have favored the selection of *M. tuberculosis* strains that resist BCG-induced immunity (11). The high prevalence of Beijing-type *M. tuberculosis* strains in Bangladesh may be linked to a similar phenomenon.

The samples for this study were collected from patients coming to a hospital from different parts of Dhaka city and also from areas outside Dhaka. There was no apparent epidemiological link among the strains shown to be identical by both spoligotyping and MIRU typing. Strains in the same cluster came from patients from different regions of the country. Although it is difficult to draw conclusions about the transmission pattern of TB in a particular area of Bangladesh, these results suggest that a number of cases may be due to recent transmission. Beijing strains were isolated from patients from different regions of Bangladesh, suggesting that these virulent strains are aggressively spreading throughout the country. Almost all of the strains of Beijing type were from patients who were previously treated with antitubercular drugs for certain periods. Many of them were from patients whose treatment had failed. Due to lack of information about the previous episode and the treatment history, it was not possible to confirm whether these were cases of reactivation or reinfection. Of 15 Beijing strains, 8 were found to be resistant to one or more antitubercular drugs, although we cannot differentiate between primary and secondary drug resistance since all patients were previously treated with antitubercular drugs. In earlier work, it has been shown that Beijing strains are often associated with drug-resistant TB (3, 8, 17, 21). Further studies using a larger sample size in a particular area are needed to investigate the incidence of Beijing-type strains in Bangladesh and to determine the transmission dynamics of TB caused by these virulent strains in the community. It is also of interest that drug resistance appears less common among ancestral strains, where only two cases were found among the 13 strains examined (Tables 1 and 2).

Most of the strains (38 strains) gave no PCR product with primers for MIRU locus 20; only 6 strains have two copies of the MIRU 20 locus present (data not shown). These results are very different from those of other studies done elsewhere (20, 28, 30) and should be further investigated. Since the MIRU 20 locus is less polymorphic and more than 90% discrimination can be obtained without its use, we have excluded MIRU 20 from our present analysis. Fifteen strains in the Beijing cluster identified by spoligotyping were further discriminated by MIRU-VNTR analysis. Eleven of them clustered into four groups, each consisting of two to seven strains, and four were found to have unique patterns. All MIRU-VNTR patterns of Beijing family strains were highly similar, differing only in copy numbers for one or two loci, indicating that *M. tuberculosis* isolates grouped into the Beijing family by spoligotyping also have a similar grouping pattern when other genetic markers like MIRU-VNTR typing are used. As shown in Table 2, this study also revealed that most spoligotype patterns of *M. tuberculosis* strains contain characteristic signatures which are specific for certain subpopulations. These characteristic signatures correlate strongly with the results of deletion analysis and the presence of *katG*^463 alleles. The correct identification of clinical isolates and the appropriate estimation of the phylogenetic relatedness among various *M. tuberculosis* strains prevalent in a given geographic area is of utmost importance for epidemiological investigations and represents a prerequisite for the identification of emerging clones. As such, it is evident that MIRU-VNTR analysis has the potential to discriminate among the strains prevailing in Bangladesh and, in combination with spoligotyping and RD analysis, fulfills the above-mentioned requirements for performing effective molecular epidemiological studies.

REFERENCES

1. **Anh, D. D., M. W. Borgdorff, L. N. Van, N. T. Lan, T. van Gorkom, K. Kremer, and D. van Soolingen.** 2000. *Mycobacterium tuberculosis* Beijing genotype emerging in Vietnam. Emerg. Infect. Dis. **6:**302–305.
2. **Behr, M. A., M. A. Wilson, W. P. Gill, H. Salamon, G. K. Schoolnik, S. Rane, and P. M. Small.** 1999. Comparative genomics of BCG vaccines by whole-genome DNA microarray. Science **284:**1520–1523.
3. **Bifani, P. J., B. Mathema, Z. Liu, S. L. Moghazeh, B. Shopsin, B. Tempalski, J. Driscol, R. Frothingham, J. M. Musser, P. Alcabes, and B. N. Kreiswirth.** 1999. Identification of a W variant outbreak of *Mycobacterium tuberculosis* via population-based molecular epidemiology. JAMA **282:**2321–2327.
4. **Brosch, R., S. V. Gordon, M. Marmiesse, P. Brodin, C. Buchrieser, K. Eiglmeier, T. Garnier, C. Gutierrez, G. Hewinson, K. Kremer, L. M. Parsons, A. S. Pym, S. Samper, D. van Soolingen, and S. T. Cole.** 2002. A new evolutionary scenario for the *Mycobacterium tuberculosis* complex. Proc. Natl. Acad. Sci. USA **99:**3684–3689.
5. **Brosch, R., W. J. Philipp, E. Stavropoulos, M. J. Colston, S. T. Cole, and S. V. Gordon.** 1999. Genomic analysis reveals variation between *Mycobacterium tuberculosis* H37Rv and the attenuated *M. tuberculosis* H37Ra strain. Infect. Immun. **67:**5768–5774.
6. **Brosch, R., A. S. Pym, S. V. Gordon, and S. T. Cole.** 2001. The evolution of mycobacterial pathogenicity: clues from comparative genomics. Trends Microbiol **9:**452–458.
7. **Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry III, F. Tekaia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, B. G. Barrell, et al.** 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature **393:**537–544.
8. **Diaz, R., K. Kremer, P. E. de Haas, R. I. Gomez, A. Marrero, J. A. Valdivia, J. D. van Embden, and D. van Soolingen.** 1998. Molecular epidemiology of tuberculosis in Cuba outside of Havana, July 1994–June 1995: utility of spoligotyping versus IS6110 restriction fragment length polymorphism. Int. J. Tuberc. Lung Dis. **2:**743–750.

9. **French, A. L., S. F. Welbel, S. E. Dietrich, L. B. Mosher, P. S. Breall, W. S. Paul, F. E. Kocka, and R. A. Weinstein.** 1998. Use of DNA fingerprinting to assess tuberculosis infection control. Ann. Intern. Med. **129:**856–861.

10. **Gordon, S. V., R. Brosch, A. Billault, T. Garnier, K. Eiglmeier, and S. T. Cole.** 1999. Identification of variable regions in the genomes of tubercle bacilli using bacterial artificial chromosome arrays. Mol. Microbiol. **32:**643–655.

11. **Hermans, P. W., F. Messadi, H. Guebrexabher, D. van Soolingen, P. E. de Haas, H. Heersma, H. de Neeling, A. Ayoub, F. Portaels, D. Frommel, et al.** 1995. Analysis of the population structure of *Mycobacterium tuberculosis* in Ethiopia, Tunisia, and The Netherlands: usefulness of DNA typing for global tuberculosis epidemiology. J. Infect. Dis. **171:**1504–1513.

12. **Ho, T. B., B. D. Robertson, G. M. Taylor, R. J. Shaw, and D. B. Young.** 2000. Comparison of *Mycobacterium tuberculosis* genomes reveals frequent deletions in a 20 kb variable region in clinical isolates. Yeast **17:**272–282.

13. **Kamerbeek, J., L. Schouls, A. Kolk, M. van Agterveld, D. van Soolingen, S. Kuijper, A. Bunschoten, H. Molhuizen, R. Shaw, M. Goyal, and J. van Embden.** 1997. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. J. Clin. Microbiol. **35:**907–914.

14. **Kolk, A. H., A. R. Schuitema, S. Kuijper, J. van Leeuwen, P. W. Hermans, J. D. van Embden, and R. A. Hartskeerl.** 1992. Detection of *Mycobacterium tuberculosis* in clinical samples by using polymerase chain reaction and a nonradioactive detection system. J. Clin. Microbiol. **30:**2567–2575.

15. **Kox, L. F., D. Rhienthong, A. M. Miranda, N. Udomsantisuk, K. Ellis, J. van Leeuwen, S. van Heusden, S. Kuijper, and A. H. Kolk.** 1994. A more reliable PCR for detection of Mycobacterium tuberculosis in clinical samples. J. Clin. Microbiol. **32:**672–678.

16. **Kremer, K., D. van Soolingen, R. Frothingham, W. H. Haas, P. W. Hermans, C. Martin, P. Palittapongarnpim, B. B. Plikaytis, L. W. Riley, M. A. Yakrus, J. M. Musser, and J. D. van Embden.** 1999. Comparison of methods based on different molecular epidemiological markers for typing of *Mycobacterium tuberculosis* complex strains: interlaboratory study of discriminatory power and reproducibility. J. Clin. Microbiol. **37:**2607–2618.

17. **Kurepina, N. E., S. Sreevatsan, B. B. Plikaytis, P. J. Bifani, N. D. Connell, R. J. Donnelly, D. van Soolingen, J. M. Musser, and B. N. Kreiswirth.** 1998. Characterization of the phylogenetic distribution and chromosomal insertion sites of five IS*6110* elements in *Mycobacterium tuberculosis:* non-random integration in the *dnaA-dnaN* region. Tubercle Lung Dis **79:**31–42.

18. **Magdalena, J., A. Vachee, P. Supply, and C. Locht.** 1998. Identification of a new DNA region specific for members of *Mycobacterium tuberculosis* complex. J. Clin. Microbiol. **36:**937–943.

19. **Manca, C., L. Tsenova, A. Bergtold, S. Freeman, M. Tovey, J. M. Musser, C. E. Barry III, V. H. Freedman, and G. Kaplan.** 2001. Virulence of a *Mycobacterium tuberculosis* clinical isolate in mice is determined by failure to induce Th1 type immunity and is associated with induction of IFN-alpha/beta. Proc. Natl. Acad. Sci. USA **98:**5752–5757.

20. **Mazars, E., S. Lesjean, A. L. Banuls, M. Gilbert, V. Vincent, B. Gicquel, M. Tibayrenc, C. Locht, and P. Supply.** 2001. High-resolution minisatellite-based typing as a portable approach to global analysis of *Mycobacterium tuberculosis* molecular epidemiology. Proc. Natl. Acad. Sci. USA **98:**1901–1906.

21. **Mistry, N. F., A. M. Iyer, T. D'Souza D, G. M. Taylor, D. B. Young, and N. H. Antia.** 2002. Spoligotyping of *Mycobacterium tuberculosis* isolates from multiple-drug-resistant tuberculosis patients from Bombay, India. J. Clin. Microbiol. **40:**2677–2680.

22. **Olive, D. M., and P. Bean.** 1999. Principles and applications of methods for DNA-based typing of microbial organisms. J. Clin. Microbiol. **37:**1661–1669.

23. **Parsons, L. M., R. Brosch, S. T. Cole, A. Somoskovi, A. Loder, G. Bretzel, D. Van Soolingen, Y. M. Hale, and M. Salfinger.** 2002. Rapid and simple approach for identification of *Mycobacterium tuberculosis* complex isolates by PCR-based genomic deletion analysis. J. Clin. Microbiol. **40:**2339–2345.

24. **Radhakrishnan, I., M. Y. K, R. A. Kumar, and S. Mundayoor.** 2001. Implications of low frequency of IS*6110* in fingerprinting field isolates of *Mycobacterium tuberculosis* from Kerala, India. J. Clin. Microbiol. **39:**1683.

25. **Soini, H., X. Pan, A. Amin, E. A. Graviss, A. Siddiqui, and J. M. Musser.** 2000. Characterization of *Mycobacterium tuberculosis* isolates from patients in Houston, Texas, by spoligotyping. J. Clin. Microbiol. **38:**669–676.

26. **Sola, C., I. Filliol, M. C. Gutierrez, I. Mokrousov, V. Vincent, and N. Rastogi.** 2001. Spoligotype database of *Mycobacterium tuberculosis:* biogeographic distribution of shared types and epidemiologic and phylogenetic perspectives. Emerg. Infect. Dis. **7:**390–396.

27. **Sreevatsan, S., X. Pan, K. E. Stockbauer, N. D. Connell, B. N. Kreiswirth, T. S. Whittam, and J. M. Musser.** 1997. Restricted structural gene polymorphism in the *Mycobacterium tuberculosis* complex indicates evolutionarily recent global dissemination. Proc. Natl. Acad. Sci. USA **94:**9869–9874.

28. **Supply, P., S. Lesjean, E. Savine, K. Kremer, D. van Soolingen, and C. Locht.** 2001. Automated high-throughput genotyping for study of global epidemiology of *Mycobacterium tuberculosis* based on mycobacterial interspersed repetitive units. J. Clin. Microbiol. **39:**3563–3571.

29. **Supply, P., J. Magdalena, S. Himpens, and C. Locht.** 1997. Identification of novel intergenic repetitive units in a mycobacterial two-component system operon. Mol. Microbiol. **26:**991–1003.

30. **Supply, P., E. Mazars, S. Lesjean, V. Vincent, B. Gicquel, and C. Locht.** 2000. Variable human minisatellite-like regions in the *Mycobacterium tuberculosis* genome. Mol. Microbiol. **36:**762–771.

31. **Supply, P., R. M. Warren, A. L. Banuls, S. Lesjean, G. D. Van Der Spuy, L. A. Lewis, M. Tibayrenc, P. D. Van Helden, and C. Locht.** 2003. Linkage disequilibrium between minisatellite loci supports clonal evolution of *Mycobacterium tuberculosis* in a high tuberculosis incidence area. Mol. Microbiol. **47:**529–538.

32. **van Soolingen, D., P. E. de Haas, P. W. Hermans, P. M. Groenen, and J. D. van Embden.** 1993. Comparison of various repetitive DNA elements as genetic markers for strain differentiation and epidemiology of *Mycobacterium tuberculosis*. J. Clin. Microbiol. **31:**1987–1995.

33. **van Soolingen, D., P. W. Hermans, P. E. de Haas, D. R. Soll, and J. D. van Embden.** 1991. Occurrence and stability of insertion sequences in *Mycobacterium tuberculosis* complex strains: evaluation of an insertion sequence-dependent DNA polymorphism as a tool in the epidemiology of tuberculosis. J. Clin. Microbiol. **29:**2578–2586.

34. **van Soolingen, D., L. Qian, P. E. de Haas, J. T. Douglas, H. Traore, F. Portaels, H. Z. Qing, D. Enkhsaikan, P. Nymadawa, and J. D. van Embden.** 1995. Predominance of a single genotype of *Mycobacterium tuberculosis* in countries of east Asia. J. Clin. Microbiol. **33:**3234–3238.

35. **Vera-Cabrera, L., M. A. Hernandez-Vera, O, Welsh, W. M. Johnson, and J. Castro-Garza.** 2001. Phospholipase region of *Mycobacterium tuberculosis* is a preferential locus for IS6110 transposition. J. Clin. Microbiol. **39:**3499–3504.

36. **Zink, A. R., C. Sola, U. Reischl, W. Grabner, N. Rastogi, H. Wolf, and A. G. Nerlich.** 2003. Characterization of *Mycobacterium tuberculosis* complex DNAs from Egyptian mummies by spoligotyping. J. Clin. Microbiol. **41:**359–367.