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IS*6110*-Restriction Fragment Length Polymorphism and Spoligotyping Analysis of *Mycobacterium tuberculosis* Clinical Isolates for Investigating Epidemiologic Distribution in Korea

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The Beijing family of *Mycobacterium tuberculosis* has been emerging in the world. However, there are few nationwide data of genotypic distribution in Korea. This study aimed to identify the genotypic diversity of clinical isolates of *M. tuberculosis* and to demonstrate the population of Beijing family in Korea. We collected 96 clinical *M. tuberculosis* isolates from 11 university hospitals nationwide in Korea from 2008 to 2009. We observed 24 clusters in IS*6110*-RFLP analysis and 19 patterns in spoligotyping. Seventy-five isolates were confirmed to be Beijing family. Two isolates of the K strain and 12 isolates of the K family strain were also found. We found that drug resistance phenotypes were more strongly associated with Beijing family than non-Beijing family (P=0.003). This study gives an overview of the distribution of genotypes of *M. tuberculosis* in Korea. These findings indicate that we have to pay more attention to control of *M. tuberculosis* strains associated with the Beijing family.

Key Words: *Mycobacterium tuberculosis*; IS*6110*-RFLP; Spoligotyping; Beijing Family; K Strain

INTRODUCTION

Tuberculosis (TB) remains a worldwide healthcare concern, being characterized as an epidemic by the World Health Organization (WHO). In 2008, there were an estimated 8.9-9.9 million incident cases of TB, 9.6-13.3 million prevalent cases, 1.1-1.7 million deaths among HIV-negative people and an additional 0.45-0.62 million deaths among HIV-positive people (1). In Korea, the prevalence of TB was estimated to be 123 per 100,000 in 2006 (2). The increasing number of drug-resistant *Mycobac-*

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terium tuberculosis isolates, including multidrug-resistant *M. tuberculosis* (MDR-TB) creates serious problems in clinical series and whole-country cohorts (3).

Genotyping has allowed the association of particular strain families of *M. tuberculosis* with demographic and transmission characteristics (4). The IS*6110*-based restriction fragment length polymorphism (RFLP) is the most widely applied test and currently the standard method for comparing the genetic relatedness of *M. tuberculosis* strains. Spoligotyping using the direct repeat locus (DR) is useful for both clinical management and molecular epidemiologic studies of *M. tuberculosis* (5).

For a long time, the Beijing family which may have been endemic in China, has been emerging in other parts of the world (6). The Beijing *M. tuberculosis* strains have highly similar multibanded IS*6110*-RFLP patterns and identical spoligotyping patterns. The family is highly prevalent in East Asia (7, 8). However, there are few data on the nationwide genotypic distribution in Korea (8, 9).

This study aimed to identify the genotypic diversity of clinical *M. tuberculosis* isolates and to demonstrate the prevalence of Beijing family strains in Korea using IS*6110*-RFLP and spoligotyping methods.

MATERIALS AND METHODS

Sample collection and bacterial strains

From 2008 to 2009, we collected 96 clinical *M. tuberculosis* isolates from 11 university hospitals nationwide in Korea (Ajou University Hospital, Cheju National University Hospital, Chonbuk National University Hospital, Chonnam National University Hospital, Hallym University Hospital, Keimyung University Hospital, Konyang University Hospital, Pusan National University Hospital, Ulsan University Hospital, Yonsei University Severance Hospital, and Wonju Christian Hospital). The number of isolates from a given hospital was roughly proportional to the prevalence of TB in that area, as judged by the annual report on TB patient notification in Korea 2007 (10). The source patients were 58 men and 38 women TB patients with a mean age of 45 yr (range 21-78 yr). There were 82 primary TB infections, and 12 had a history of treatment. In two cases, the status was unknown because of follow-up loss. The sites of infection were not specified. Duplicated isolates from the same patient were excluded.

DNA extraction

Chromosomal DNA was purified by the standardized method from Löwenstein-Jensen (L-J) slant-grown colonies (11). Briefly, lysozyme (1 mg/mL) was added colony-containing tube and the mixture was incubated for 1 hr at 37°C. Next, proteinase K (10 mg/mL) and 70 μL of 10% sodium dodecyl sulfate were added, and incubation was continued for 10 min at 65°C. Then N-acetyl-N,N,N-trimethyl ammonium bromide was added, and the mixture was incubated for 10 min at 65°C. An equal volume of chloroform-isoamyl alcohol (24:1 vol/vol) was added. After centrifugation for 5 min, 0.6 volume of isopropanol was added to the supernatant liquid to precipitate the DNA. After 20 min at -20°C and centrifugation for 15 min, the pellet was washed once with 70% ethanol, and the air-dried pellet was dissolved in 20 μL of 0.1×Tris-EDTA (TE) buffer.

IS*6110*-RFLP

All isolates were subjected to IS*6110*-RFLP analysis by an internationally standardized method (12). Briefly, the chromosomal DNA was restricted with *Pvu*II for RFLP analysis. The digested DNA was separated overnight by gel electrophoresis and transferred from the gel to a positively charged nylon membrane (Hybond N1) (Amersham, Buckinghamshire, UK). After hybridization for repetitive elements with labeled DNA probes, the bound probes were detected with an enhanced chemiluminescence direct nucleic acid system (Amersham) according to the manufacturer's recommendations. The RFLP patterns were analyzed by GelComparII software (Applied Maths, Korthrijk, Belgium) (9).

Spoligotyping

Spoligotyping was carried out using a Combi™ chip spoligotyping kit (GeneIn, Inc., Busan, Korea) according to the manufacturer's instructions. Briefly, the DNAs of the whole DR region were amplified, and the amplified DNAs were hybridized with 43 spoligotyping probes as described (13). After the results had been converted with binary and octal code, the spoligotypes were designated as a cluster and assigned a shared international type number (SIT) according to the international database, SpolDB4 (14).

Determination and analysis of Beijing family and K strains

We determined Beijing family strains on the basis of several genetic characteristics. By definition, they have an IS*6110* insertion A1 band in the origin of replication (corresponding to a 3.36-kb band) and show an IS*6110* banding pattern similar to that of strain Beijing (copy number range 15-26) (15). They contain nine spacers from 35 to 43 in the spoligotype pattern (16). The Beijing-like family was identified by SpolDB4. K strain (Korean *M. tuberculosis* strain) was also identified, which was previously defined as a sublineage of the Beijing strain and has a unique ten IS*6110* RFLP band pattern (17). The K family was identified by the characteristics that had eight to twelve IS*6110* bands, and more than five bands were as same those of as K strains (17).

Drug susceptibility testing

Antibiotic susceptibility testing was performed at the Korean Institute of Tuberculosis by the proportion method using L-J medium against 11 antibiotics: isoniazid (INH), rifampin (RIF),

ethambutol, streptomycin, capreomycin, kanamycin, ofloxacin, prothionamide, cycloserine, *p*-aminosalicylic acid, and rifabutin (18). The drug susceptibility test for pyrazinamide was determined using the pyrazinamidase assay (19).

Statistical analysis

Data were analyzed with SPSS 12.0 (SPSS Inc., Chicago, IL, USA). Categorical variables were compared by Fisher's exact test. Significance was defined as a *P* value of <0.05.

Ethics statement

This research was exempted from full committee review of Institutional Review Board at Pusan National University Hospital because cultured bacterial isolates were used without any identifiers linked to the subjects.

RESULTS

IS*6110*-RFLP patterns of 96 strains

The IS*6110* copy numbers ranged from 5 to 21 (mode=10) (Fig. 1).

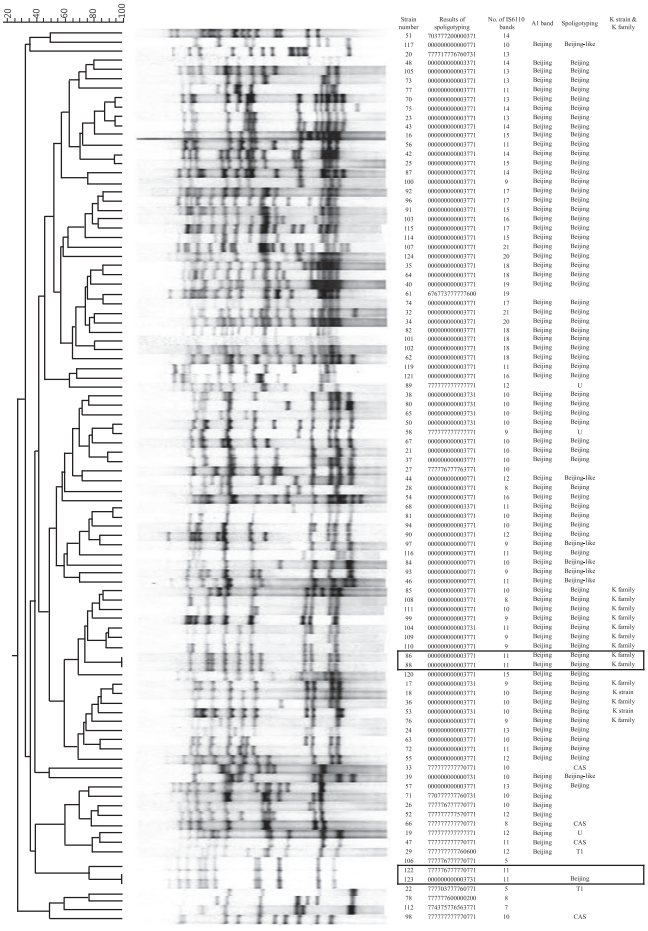

Fig. 1. Results of RFLP and spoligotyping of 96 *M. tuberculosis* isolates. Two solid boxes indicate that the two pairs of four isolates showed identical IS*6110*-RFLP patterns in each pair.

The majority of the strains (45/96, 46.9%) contained 9-11 copies. With a 60% similarity threshold, 24 clusters were observed in the dendrogram based IS*6110*-RFLP. A total of 86 isolates (89.6%) displayed one of 14 clusters and 10 isolates (10.4%) displayed unique patterns. Two pairs of four isolates showed identical IS*6110*-RFLP patterns in each pair (solid boxes on right in Fig. 1); the source patients for each pair had no epidemiologic linkage.

Spoligotyping patterns of 96 strains

Eighteen spoligotyping patterns were observed. Among the isolates, 85 (88.5%) displayed one of 7 spoligotypes and 11 (11.4%) displayed unique spoligotypes. According to the SpolDB4, 85 isolates (88.5%) were classified into nine shared international types (SITs), whereas 11 isolates were not classified. Among the nine clusters identified by SpolDB4 analysis, the SIT1 cluster contained a majority of the isolates (62/96, 64.6%). When considering all spoligotypes, the Beijing, including the SIT1 cluster, was the most prevalent (69/96, 71.9%), followed by seven (7.3%) Beijing-like spoligotypes, four (4.2%) CAS family, three (3.1%) U family, and two (2.1%) T1 family (Table 1).

Distribution of Beijing and K strains

When analyzing IS*6110*-RFLP patterns, 83 of 96 isolates (86.5%)

Table 1. Results of spoligotyping

Spoligotyping octal codes	SpolDB4 analysis		No. of strains		
	SIT	Label	Total	A1 band	K strain/ K family
000000000003771	1	Beijing	61	61	1/10
000000000003731	190	Beijing	6	5	1/2
000000000003371	265	Beijing	2	2	
000000000000771	269	Beijing like	6	6	
000000000000731	406	Beijing like	1	1	
777777777770771	1,378	CAS	4	2	
777777777777771	523	U	3	2	
777777777760600	243	T1	1	1	
777703777760771	1,223	T1	1		
777777777570771	Unclassified		1	1	
777777600000200	Unclassified		1		
777776777770771	Unclassified		3	1	
777776777763771	Unclassified		1		
777717776760731	Unclassified		1		
774375776563771	Unclassified		1		
770777777760731	Unclassified		1	1	
703777200000371	Unclassified		1		
676773777777600	Unclassified		1		

SIT, shared international type from international spoligotype database SpolDB4 (http://www.pasteur-eloupe.fr:8081/SITVITDemo/); Label, spoligotype families as assigned in SpolDB4; A1 band, the number of isolates including IS*6110* insertion A1 band in the origin of replication (corresponding to a 3.36-kb band); K strain, the number of isolates indicating a unique 10 IS*6110* RFLP band pattern; K family, the number of isolates having 8 to 12 IS*6110* bands and more than five bands in the K strains.

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Table 2. Spoligotype and drug resistance in 96 *M. tuberculosis* clinical isolates

Spoligotype	No. of isolates (%)	MDR (%)	Any drug (%)
Beijing family	76 (79.1)	9 (11.8)*	20 (26.3)*
Non-Beijing family	20 (20.9)		1 (5.0)
Total	96 (100)	9 (9.4)	21 (21.9)

*MDR and any drug resistance rates are significantly higher in Beijing family than in non-Beijing family.
MDR, multi-drug resistant; Any drug, resistance to at least one drug, including INH and RIF.

that contained the Beijing-specific A1 insertion band were determined to be Beijing family. However, 75 of these 86 isolates, and another isolate with no A1 band were confirmed to be Beijing family by spoligotyping (Fig. 1). These isolates each had 8 to 21 bands (mode 10). The unique IS*6110*-RFLP pattern of K strain was found in 2 isolates (2.1%), and 12 isolates (12.5%) closely resembled the K family strain. Of the 76 Beijing family strains, K family strains accounted for 18.4%.

Relation between genotypes and drug resistances

In this study, 21 isolates were resistant to at least one anti-tuberculosis drug. Nine of these were MDR-TB. Nineteen of 21 isolates resistant to at least one drug and all nine MDR-TB isolates belonged to the Beijing family, meaning there is a significantly higher rate of MDR or any drug resistance in the Beijing family (P=0.003) (Table 2). The differences in the resistance rates between K family and non-K Beijing family were not significant. Drug resistance rates in the study population were 9.4% (9/96) for MDR-TB and 21.9% (21/96) for at least one drug, including INH and RIF. For primary TB cases, the drug resistance rates were 2.4% (2/82) for MDR-TB and 15.9% (13/82) for at least one drug, including INH and RIF.

DISCUSSION

The Beijing family of *M. tuberculosis* strains may have been endemic in China for a long time (6) and is now emerging in other parts of the world. This family is dominant in Asian countries such as China (86%), Mongolia (50%), Japan (73%), Indonesia (34%), Thailand (44%), and Vietnam (54%) (7, 8, 20-22). It also is present in Korea. In a 1995 report, 43% of tested strains (6/14) were the Beijing family (8). In another report, 72% (99/138) were the Beijing family, which was defined by IS*6110*-RFLP patterns (9). The current study shows a higher rate of the Beijing family (79%), nearly same as the rates in China and Japan. In the first report from Korea (8), the selection criteria for the tested strains were not specified, and the test volume was small. In contrast, the two other studies including the current study, examined strains from several areas in Korea, and the number of tested strains was roughly proportional to the number of TB patients reported from each area. Very recently, one report claimed that nearly all the isolates in one Korean tertiary TB hospital were Beijing family (23). However, this hospital normally recruits drug-resistant or treatment failure cases from the entire country, so the strain population could be distorted. Therefore, our data appear to represent the genuine distribution of the Beijing family in Korea. Although isolates were collected from 11 university hospitals throughout the country, we did not find any association between the Beijing family and region. Also, no correlation between Beijing family and the sex or age of the patients was found. Two pairs of isolates from each of two university hospitals showed identical IS*6110*-RFLP patterns, but we did not find an epidemiological relation between the genotypes of the strains. That means that identical IS*6110*-RFLP patterns do not always indicate strains of the same origin.

The Beijing family organisms have multiple copies of IS*6110* bands (15 to 26), and therefore, differentiation between strains is not easy (24, 25). Many other characteristics have been demonstrated to identify the Beijing family by IS*6110*-RFLP analysis. In the current study, the A1 band in the IS*6110* RFLP pattern was used to identify 83 strains presumablely as Beijing family, of which 76 were confirmed as such by spoligotyping. However, 19 Beijing reference strains suggested by Kremer et al. and at least 450 IS*6110* profiles reported by the Public Health Research Institute (PHRI) TB Center database were not useful in detecting Beijing strains in this study (data not shown) (24, 26). A dendrogram of the IS*6110*-RFLP band pattern was diverse even within the Beijing family, showing that the 76 strains belonged to 18 clusters. Interestingly, the Beijing family strains tested in this study harbored relatively small numbers of IS*6110* bands (8 to 21), indicating that the characteristics of the Beijing family isolated in Korea are different from those isolated in other countries. Mycobacterial interspersed repetitive units (MIRU)-variable number of tandem repeats (VNTR), another molecular epidemiologic tool for *M. tuberculosis*, was introduced to genotype *M. tuberculosis* isolates from Korea (4, 27). The MIRU-VNTR is highly discriminatory and easy to use, and can differentiate strains belonged to the Beijing family (4). However, it is not suitable for demonstrating the population of Beijing strains because it cannot provide any information distinguishing Beijing from non-Beijing family strains. In previous studies, the dominant *M. tuberculosis* strains in Korea were defined as the K strain, a sublineage of the Beijing strain (17). According to the definition, two isolates (2.1%) were identified with K strains and 12 isolates (12.5%) with the K family in the current study. These figures are slightly lower than those in the report of Park et al. (18.8%) from Korea (28). However, these data were developed with strains from one area, Gyeonggi Province; to estimate the population of the K family throughout Korea, a large-scale study using nationwide collection would be necessary.

The effect of Beijing family strains on drug resistance is not clear (29), but the Beijing family is reported to have a higher rate of resistance (25). We also demonstrated that drug resistance

was strongly associated with Beijing family strains in Korea. All MDR-TB isolates and the majority (19/21) of the isolates with at least one drug resistance belonged to the Beijing family. One recent study reported interesting data that in one Korean TB hospital, which specializes in caring for patients with drug-resistant TB, 97% of the isolates belonged to the Beijing family (23). This suggests a close connection between drug resistance and Beijing family membership. Therefore, patients found to be infected with *M. tuberculosis* of the Beijing family should be monitored closely for drug-taking compliance or response to therapy. Spoligotyping analysis could easily classify and be useful to detect Beijing strains. The 76 isolates of the Beijing family belonged to only five clusters according to the international spoligotype database, SpolDB4. Therefore, when considering that it is important to identify Beijing family members, spoligotyping can be performed more easily and rapidly than IS*6110*-RFLP analysis. Another issue regarding drug resistance is the primary or acquired resistance rate of *M. tuberculosis*. Bai et al. (30) reported that in 2004, the resistance rates were 2.7% for MDR and 12.8% for any one drug resistance in new TB cases and 14% for MDR and 27.7% for any one drug resistance among patients with a history of treatment in Korea. In the current study, the drug resistance rates in primary TB cases appeared 2.4% for MDR-TB and 15.9% for any one drug resistance, representing a similar result with the previous report.

In summary, this study gives an overview of the distribution of genotypes of clinical *M. tuberculosis* isolates in Korea. Our data showed that the Beijing strain currently is the most prevalent. Especially, we found an association between Beijing family strains and drug resistance phenotypes. These findings indicate that we have to pay more attention to control of *M. tuberculosis* strains associated with the Beijing family. Our data also indicate that a poor drug treatment outcome may occur more commonly in Beijing family strains.

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