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Genomic Polymorphism in Clinical Mycobacterial Strains Analyzed by Pulsed-Field Gel Electrophoresis

Jeong-Ran Kim and Cheorl-Ho Kim*

Department of Biochemistry and Molecular Biology, College of Oriental Medicine, DongGuk University, Kyungpook 780-714, Korea

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Ten *Mycobacterium tuberculosis* clinical isolates in Korea, showing different drug resistances, were analyzed by comparing large restriction fragment (LRF) patterns produced by digestion of genomic DNA with infrequent-cutting endonucleases of *Spe*I, *Asn*I and *Xba*I, and pulsed-field gel electrophoresis (PFGE). *Spe*I and *Asn*I allowed easy visual separation of all epidemiologically unrelated strains. When DNAs were digested with *Asn*I and *Spe*I, strains yielded an absolutely identical pattern for Korean type's mycobacteria even though they showed different drug resistance. However, when three *M. tuberculosis* strains, showing drug resistance, were digested with *Xba*I, patterns were different from those of the other *M. tuberculosis* strains which are susceptible to drugs. This study reveals that the comparison of chromosomal restriction patterns is very useful as an additional aid for the differentiation and identification of *M. tuberculosis* strains showing drug resistances.

Key words: Mycobacteria, genome, pulsed-field gel electrophoresis

The pulsed-field gel electrophoresis (PFGE) technique, which allows the separation of large DNA fragments, was originally developed for the separation of yeast chromosomes and has been applied to the analysis of bacterial genomes (19).

Due to the resurgence of tuberculosis, the molecular fingerprinting of mycobacterial isolates by restriction fragment length polymorphism (RFLP) analysis (21, 24) is gaining importance in mycobacterial research and epidemiology. Levy-Frebault *et al.* (15) used infrequent-cutting endonucleases and field inversion gel electrophoresis (a prototype of PFGE) to demonstrate DNA polymorphisms in several mycobacterial species. The restriction fragment patterns were easily compared where LRF patterns created by using infrequent-cutting restriction endonucleases and PFGE have been used to study the epidemiology of several bacterial species, including *Mycobacterium fortuitum* (8), *Enterococcus* species (16), *Escherichia coli* (2), *Pseudomonas aeruginosa* (1, 7), *Campylobacter jejuni* and *Campylobacter coli* (23).

We reported a rapid and gentle method to extract genomic DNA from mycobacterial cells, treating mycobacterial cells with a mixture of lysozyme and *N*-acetylglucosaminidase in a previous paper (5). We also applied the method and PFGE technique to some representatives of Korean type's tubercle bacilli in order to characterize at the genome level (12).

In this report, we compared the LRF patterns of H37Rv and 10 clinical *Mycobacterium tuberculosis* isolates, which showed either a different drug resistance or a different sensitivity. The purpose of this work was to characterize *M. tuberculosis* strains recovered from Korean clinical sources by PFGE. Studying the characteristics of resistant and sensitive strain, to drug may be useful for a better insight into what still is poorly understood resistance of *M. tuberculosis*.

Materials and Methods

Strains and growth conditions

Ten *M. tuberculosis* isolates were obtained from the clinical laboratory of the Korean Institute of Tuberculosis. These isolates, which were originally recovered from patient specimens on Middlebrook 7H10 and/or Lowenstein-Jensen agar (12), were identified to species by utilizing standard biochemical methods. The isolates were passaged no more than twice before being frozen at -70°C in tryptic soy broth with 15% glycerol until needed for study.

One culture of H37Rv (ATCC 27394) was used. The strain was obtained in 1991 from the American Type Culture Collection (ATCC) in Rockville, Md. This strain was passaged only once in our la-

* To whom correspondence should be addressed

boratory prior to freezer stoage.

Preparation of intact mycobacterial DNA in agarose plugs

Agarose plugs were made with heat-treated cells prepared as described (5, 12, 17). Briefly, mycobacterial cells cultured in Middlebrook 7H9 broth were harvested at the early exponential phase. Cells (100 mg wet weight) were washed twice with 10 ml of TC lysis buffer (10 mM Tris-HCl, 1 M NaCl, pH 7.6) containing 1% sodium lauryl sarcosine, followed by centrifugation. After resuspension of the cells in 2 ml of suspension buffer (10 mM Tris-HCl, pH 8.0, 0.1 M Na-EDTA, 0.02 M NaCl), the suspension was incubated in an incubator at 30 to 40°C, then diluted with an equal volume of 1% low melting temperature agarose (FMC Bio-Products, Rockland, Maine) made up in sterile water at 42°C. The resulting solution was then poured into a mould chamber (Bio-Rad). Solidified blocks were incubated at 37°C for 1 h in lysozyme (1 mg/ml)-*N*-acetyl-β-D-glucosaminidase (1 mg/ml)(Boehringer Mannheim Korea)-RNase A (50 μg/ml) (Sigma) solution (in TC lysis buffer), and they were agitated at 60°C for 30 min in a slow speed shaker water bath to remove the bound polysaccharides. It was then treated overnight at 50°C with an equal volume of buffer containing Proteinase K (1 mg/ml, Boehringer Mannheim, Germany), 0.5% *N*-laurylsarcosine (Sigma), and 1 mM EDTA, pH 8.0. Proteinase activity was inhibited by washing the blocks twice for 1 h at room temperature in phenylmethylsulphonyl fluoride (40 μg/ml, PMSF). The blocks were then stored in 0.05 M Na-EDTA (pH 8.0) at 4°C.

Restriction enzyme digestion and PFGE

For digestion of DNA in agarose plug, 10 U restriction endonucleases of *Spe*I, *Xba*I or *Asn*I (Boehringer Mannheim) in buffer as recommended by the manufacturer (total volume of 50 μl) were used. After digestion, blocks were mounted on the teeth of an electrophoresis comb. The gel was cast with 1.0% (w/v) SeaPlaque agarose (FMC) at 55°C in 0.5×TBE buffer (10 mM Tris-borate, 1 mM EDTA). The gel was electrophoresed at 14°C in a CHEF DR II apparatus (Bio-Rad). The gel was run for 24 h at 200 V with the given ramped pulse times. *Saccharomyces cerevisiae* chromosomes (Bio-Rad) and λ-DNA concatamer (Bio-Rad) were used as size markers for high-molecular mass DNA fragments.

Results and Discussion

Each *M. tuberculosis* isolate gave a readily discernible LRF pattern when its genomic DNA was digested with *Spe*I, *Asn*I or *Xba*I and subjected to PFGE. Restriction patterns of the different clinical strains of *M. tuberculosis* from Korean patients including the type strain ATCC 27294, listed in Table 1, were compared to those of lambda concatamers. As the molecular weight of a single phage lambda is about 50 kb, the upper limit of resolution equivalent to 14 concatamerized lambda phages corresponds roughly 600 Kb.

*Asn*I digestion produced 15 to 18 bands between 30 and 600 kb (Fig. 1). *Spe*I generated 16 to 18 bands between 40 and 290 kb (Fig. 2). When DNA from 10 random isolates and H37Rv (ATCC 27294) were digested with *Spe*I, exactly the same LRF patterns were produced (Fig. 2, lanes 1 through 10). *Spe*I restriction digestion patterns of drug resistant

Table 1. Mycobacterial strains used in this study and drug resistances

Strains	Drug resistance	Sources
M. tuberculosis	sensitive	ATCC 27294
H37Rv	H[a], R[b], S[c], E[d], K[e], TH[f]	clinical isolate
R68	H, R, S, E, K, PZA[g], PAS[h]	clinical isolate
K30	H,R,E,	clinical isolate
H23	sensitive	clinical isolate
KIT 10181	sensitive	clinical isolate
KIT 10202	sensitive	clinical isolate
KIT 10211	sensitive	clinical isolate
KIT 10215	sensitive	clinical isolate
KIT 10468	sensitive	clinical isolate
KIT 10110	sensitive	clinical isolate
M. avium-intracellurae complex		
573	H, R, S, E, K, EVM[i], PTH[j], CS[k], PAS, OFX[l], PZA	clinical isolate
569	NT[m]	clinical isolate
M. fortuitum		
551	H, S, E, EVM, PTH, CS, PAS, PZA	clinical isolate
547	H, R, S, E, K, TH, CS, PAS, PZA	clinical isolate
M. gordonae		
560	NT[m]	clinical isolate
571	NT[m]	clinical isolate
M. terrae complex		
545	H, R, S, E, TH, TUM, CS, PAS, PZA	clinical isolate
479	H, K, CS, PAS, OFX, PZA	clinical isolate

[a]H, isoniazid resistance; [b]R, rifampicin resistance; [c]S, streptomycin resistance; [d]E, ethambutol resistance; [e]K, kanamycin resistance; [f]TH, ethionamide resistance; [g]PZA, pyrazinamide resistance; [h]PAS, para-aminosalicylic acid resistance; [i]EVM, enviomycin resistance; [j]PTH, prothionamide resistance; [k]CS, cycloserine resistance; [l]OFX, ofloxacin resistance; [m]NT: not tested.

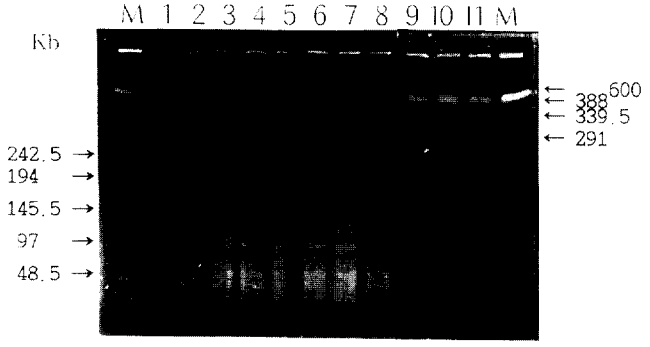

Fig. 1. Pulsed-field gel electrophoresis of mycobacterial DNA digested by *Asn*I (run on 1.0% agarose gel in PFGE). Lanes: 1 and 2, *M. tuberculosis* H37Rv (ATCC27294); 3, M. tuberculosis R68; 4, *M. tuberculosis* K30; 5, *M. tuberculosis* H23; 6, *M. tuberculosis* KIT10181; 7, *M. tuberculosis* KIT 10202; 8, *M. tuberculosis* KIT10211; 9, *M. tuberculosis* KIT 10215; 10, *M. tuberculosis* KIT10468; 11, *M. tuberculosis* KIT10110. The numbers in the middle show the positions for the DNA size standard markers. The ramped pulse times were 5-25 s for 24 h at 14°C and 200 V. The gel was 1. 0% SeaPlaque agarose in 0.5×TBE.

strains such as *M. tuberculosis* R68, which is resistant to isoniazid (H), rifampicin (R), streptomycin (S), ethambutol (E), kanamycin (K) and ethionamide (TH), *M. tuberculosis* K30 showing resistance to H, R, S, E, K, TH, and *M. tuberculosis* H23 showing H, R, E resistance, could not be differentiated from the *M. tuberculosis* strains showing drug sensitivities. In contrast, when DNAs from *M. gordonae* (lane 11), *M. fortuitum* (lane 12), *M. avium* intracellulae (lane 13) and *M. terrae* complex (lane 14), listed in Table 1, were digested with *Spe*I, the pattern of these strains differed from each other.

On the other hand, when DNAs from eight clinical isolates and H37Rv (ATCC 27294) were digested with XbaI, three different LRF patterns were produced (Fig. 3). Two of the clinical isolates (KIT 10181 and 10202), which were sensitive to all of the drugs, gave distinct LRF patterns (Fig. 3, lane 3 and 4), while three of the isolates (lane 6), which showed drug resistances, gave identical *Xba*I LRF patterns (Table 2).

Pulsed-field electrophoretic restriction patterns confirm the analyses of restriction fragment length polymorphism (4, 8, 13, 24), as identical profiles were found for Korean epidemiological *M. tuberculosis* strains even though they showed different characteristics to drugs. Thus, this technique demonstrated the homogeneity of Korean types of the mycobacterial strains. Phenotypic methods investigated were capable of differentiating the Korean clinical isolates.

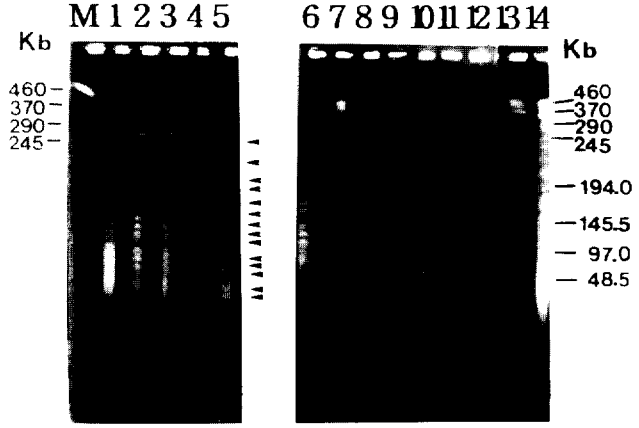

Fig. 2. Pulsed-field gel electrophoresis of mycobacterial DNA digested by *Spe*I (run on 1.0% agarose gel in PFGE). Lanes: 1, *M. tuberculosis* H37Rv(ATCC27294); 2, *M. tuberculosis*; 3, *M. tuberculosis* K30; 4, *M. tuberculosis* H23; 5, *M. tuberculosis* KIT10181; 6, *M. tuberculosis* KIT10202; 7, *M. tuberculosis* KIT10211; 8, *M. tuberculosis* KIT10215; 9, *M. tuberculosis* KIT10468; 10, *M. tuberculosis* KIT10110; 11, *M. gordonae*; 12, *M. fortuitum* 547; 13, *M. avium-intracellulae* complex; 14, *M. terrae* complex. The numbers in the middle show the positions for the DNA size standard markers. The ramped pulse times were 5-25 s for 24 h at 14°C and 200 V. The gel was 1.0% SeaPlaque agarose in 0.5×TBE. Arrow heads indicate migrated positions of the restriction fragments.

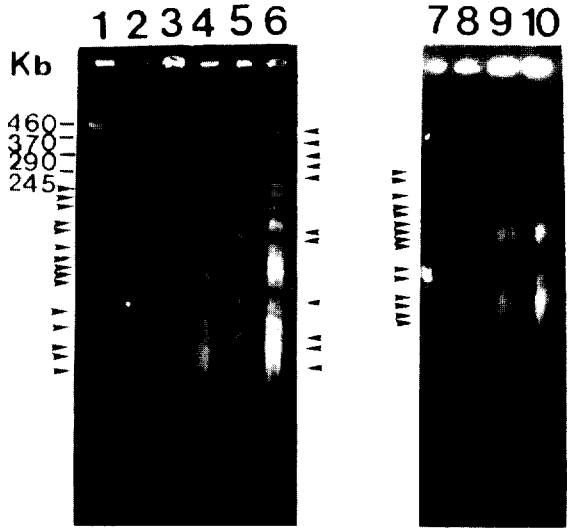

Fig. 3. Pulsed-field gel electrophoresis of mycobacterial DNA digested by *Xba*I (run on 1.0% agarose gel in PFGE). Lanes: 1, maker; 2, *M. tuberculosis* H37Rv; 3, *M. tuberculosis* KIT10181; 4, *M. tuberculosis* 10202; 5, *M. terrae complex* 545; 6, *M. tuberculosis* R68; 7, *M. terrae complex* 479; 8, *M. terrae complex* 479; 9, *M. tuberculosis* H23; 10, *M. tuberculosis* K30. The numbers on the left and right show the positions for the DNA size standard markers. Other conditions are the same as Fig. 1. Arrow heads indicate migrated positions of the restriction fragments.

Table 2. LRF patterns of clinical isolates of *M. tuberculosis*

Strains	LRF pattern code with:		
	*Asn*I[a]	*Spe*I[a]	XbaI[a]
M. tuberculosis			
H37Rv	A	B	C
R68	A	B	D
K30	A	B	D
H23	A	B	D
KIT10181	A	B	C
KIT10202	A	B	C
KIT10211	A	B	C
KIT10215	A	B	C
KIT10468	A	B	C
KIT10110	A	B	C

[a] Each capital letter represents a different LRF pattern.

Studies of *M. tuberculosis* by a number of techniques, including serotyping (10), biotyping (18), and bacteriophage typing (9, 11, 20), have shown isolates of *M. tuberculosis* to exhibit minimal heterogeneity. With respect to drug resistance of *M. tuberculosis* strains, restriction fragment analysis by PFGE has revealed greater heterogeneity between strains. The LRF patterns produced with *Xba*I allowed easy discrimination of drug resistant strains of *M. tuberculosis*.

In summary, LRF patterns provide a reliable and practical method for comparing particular strains of *M. tuberculosis* without the need for radioactive or specific DNA probes. As such, LRF patterns produced by digesting genomic *M. tuberculosis* DNA with infrequent-cutting endonucleases and separating the LRFs by PFGE will aid in the epidemiologic study of tuberculosis.

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