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Genomic Heterogeneity in Clinical Strains of *Mycobacterium tuberculosis*, *M. terrae* Complex, *M. gordonae*, *M. avium-intracellulae* Complex and *M. fortuitum* by Pulsed-Field Gel Electrophoresis

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Abstract: Clinical strains of *Mycobacterium tuberculosis, M. terrae* complex, *M. gordonae, M. avium-intracellulae* complex, and *M. fortuitum* from Korean patients were isolated and analyzed by comparing large restriction fragment (LRF) patterns produced by digestion of genomic DNA with infrequent-cutting endonucleases like *Asn*I and *Xba*I, and pulsed-field gel electrophoresis (PFGE). Three *M. tuberculosis*, two *M. terrae* complex, two *M. gordonae*, two *M. avium-intracellulae* complex, and two *M. fortuitum* strains were compared by using *Asn*I and *Xba*I, and this allowed easy visual separation of all epidemiologically unrelated strains. PFGE exhibits different DNA restriction patterns which are easy to compare. Genome size of the strains roughly ranged from 3020 to 3335 kb. The LRF patterns are useful for epidemiologic studies of tuberculosis with regard to drug resistance.

Key words: genome, mycobacteria, pulsed-fielded gel electrophoresis.

Pulsed-field gel electrophoresis (PFGE) allows the separation of large DNA fragments. The technique, originally developed for the separation of yeast chromosomes (Schwartz *et al.*, 1983), has been applied to the analysis of bacterial genomes. After digestion by low-frequency cleavage restriction endonucleases, bacterial chromosomes yield DNA patterns composed of a few, usually well-separated fragments. The RAPD (randomly amplified polymorphic DNA) technique was also used for a faster and easier approach to explore genetic polymorphism (Cho *et al.*, 1994a, 1994b).

Epidemiologic studies of tuberculosis can be greatly facilitated by the application of strain-specific markers. Due to the resurgence of tuberculosis, the molecular fingerprinting of mycobacterial isolates by restriction fragment length polymorphism (RFLP) analysis (Varnerot *et al.*, 1992; Zhang *et al.*, 1992) is gaining importance in mycobacterial research and epidemiology. Burns *et al.* (1991) 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. 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* (Hector *et al.*, 1992), *Enterococcus* species (Murray *et al.*, 1992), *Escherichia coli* (Arbeit *et al.*, 1990), *Pseudomonas aeruginosa* (Grothues *et al.*, 1988; Allardet-Servent *et al.*, 1989), *Campylobacter jejuni* and *Campylobacter coli* (Yan *et al.*, 1991). However, no other studies on the genetic organization of mycobacterial strains have been done to date with respect to drug susceptibility. Therefore, there appears to be a need for correlation of the phenotypic and genomic differences existing between strains susceptible or resistant to drugs.

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Previously, we reported a rapid and gentle method to extract a sufficient quantity of unsheared genomic DNA from mycobacterial cells. Treating mycobacterial cells with a mixture of lysozyme and N-acetylglucosaminidase remarkably facilitated the successive digestion of these cells as well as further extraction. This method was devised from agarose-embeded bacteria in order to prevent mechanical, non-specific chromosomal fragmentation, and a gentle lysis procedure has been developed which ensures suitable yields of entire DNA from immobilized, non-dividing cells (Choi *et al.*, 1996). The method demonstrated that the 10 *M. tuberculosis* subspecies presented a homogeneous genomic organization producing characteristic profiles, whereas other mycobacterial strains displayed diverse restriction patterns. We compared the LRF patterns of *T. tuberculosis* H37 Rv and 10 clinical *T. tuberculosis* isolates, which show different drug resistance or sensitivity.

In the present study we applied the PFGE technique to some representative tubercle bacilli in order to differentiate them at the genome level. Three *M. tuberculosis* showing different drug resistance, two *M. terrae* complex, two *M. gordonae*, two *M. avium*-intracellulae complex, and two *M. fortuitum* strains were compared by using *Asn*I and *Xba*I, the purpose of this work being the characterization of mycobacterial strains recovered from Korean clinical sources by PFGE.

Materials and Methods

Organisms and culture

Three *M. tuberculosis* isolates, two *M. gordonae*, two *M. avium-intracellulae* complex, and two *M. fortuitum* strains 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, were identified to species by utilizing standard biochemical methods. The isolates were passaged no more than twice before being frozen at −70℃ in tryptic soy broth with 15% glycerol until needed for further study. One culture of H37Rv (ATCC 27394) was studied. The strain was obtained in 1991 from the American Type Culture Collection (ATCC) in Rockville, Md., USA. This was passaged only once in our laboratory prior to −70℃ storage.

Liberation and preparation of intact mycobacterial DNA in agarose plugs

Agarose plugs were made with heat-treated cells by methods described previously (Park *et al.*, 1994; Choi *et al.*, 1996). Briefly, mycobacterial 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 (0.01 M Tris/HCl, pH 8.0, 0.1 M Na-EDTA, 0.02 M NaCl), the suspension was warmed in an incubator at 30 to 40℃, then diluted with an equal volume of 1% low melting temperature agarose (FMC Bio-Products, Rockland, Maine, USA) made up in sterile water at 42℃. The resulting solution was then poured into a mould chamber (Bio-Rad, Richmond, USA). Solidified blocks were incubated at 37℃ for 1 h in lysozyme (1 mg/ml)-*N*-acetylglucosaminidase (1 mg/ml) (Boehringer Mannheim, Mannheim Germany)-RNase A (50 μg/ml) (Sigma, St. Louis, USA) solution (in TC lysis buffer), and it was kept at 60℃ for 30 min in a slow speed shaker water bath to remove the bound polysaccharides. It was then treated overnight at 50℃ 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℃.

Restriction endonuclease digestion and PFGE

For digestion of DNA in agarose plug, 10 U restriction endonucleases (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℃ in 0.5×TBE buffer (10 mM Tris-borate, 1 mM EDTA). The gel was electrophoresed at 14℃ in a CHEF DR II apparatus (Bio-Rad). The gel was run for 24 h at 200 V with a ramped pulse time from 5 to 25 s. *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

Restriction endonuclease analysis of mycobacterial chromosomal DNAs

In order to compare the restriction patterns of mycobacterial genomes, the restriction endonucleases were assayed: *Not*I, *Sfi*I, *Sma*I, *Dra*I, *Spe*I, *Swa*I, *Pac*I, *Xba*I and *Asn*I. Among all of them we decided to utilize the restriction endonucleases *Asn*I and *Xba*I because they gave the best resolution pattern in PFGE. Each mycobacterial isolate gave a readily discernible LRF pattern when its genomic DNA was digested with AsnI and subjected to PFGE. Fig. 1 shows the *Asn*I restriction patterns of the different mycobacterial strains of *M. tuberculosis*, *M. terrae* complex, *M. gordonae*, *M. avium-intracellulae* complex, and *M. fortuitum* from Korean patients, listed in Table 1, compared to those of yeast chromosomal markers. When *M. fortuitum* and *M. tuberculosis* DNAs were cleaved with restriction enzyme *Asn*I and then subjected to PFGE at different pulse times (from 5~25 s), 10 (lane 1 in Fig. 1) and 19 (lane 6 in Fig, 2A) bands were identified, respectively. In the cases of *M. terrae* complex 545 and 479, the restriction endonuclease *Asn*I generated 9 (lane 3 in Fig. 1) and 13 (lane 3 in Fig. 2A) bands, respectively.

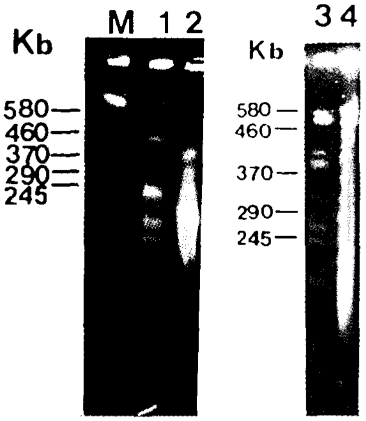
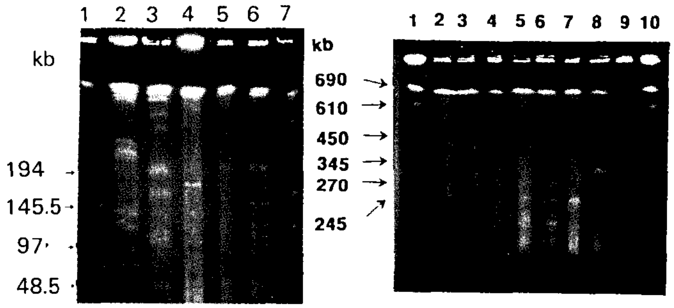


Fig. 1. Pulsed-field gel electrophoresis of mycobacterial DNA digested by *Asn*I (run on 1.0% SeaPlaque agarose gel in PFGE). The ramped pulse times were 25~50 s for 24 h at 14℃ and 200 V. Lanes: M, marker; 1, *M. fortuitum* 547; 2, *M. avium-intracellulae*; 3, *M. terrae* complex 545; 4, *M. gordonae* 560. The numbers on the left and center show the positions for the DNA size standard markers of the sizes indicated.

Epidemiology of clinical isolates of *M. tuberculosis*, *M. terrae* complex, *M. gordonae*, *M. avium-intracellulae* complex and *M. fortuitum*



(A) (B)

Fig. 2. Pulsed-field gel electrophoresis of mycobacterial DNA digested by *Asn*I. (A) The ramped pulse times were 5~25 s for 24 h at 14℃ and 200 V. Lanes: 1, marker; 2, *M. terrae* complex 545; 3, *M. terrae* complex 479; 4, *M. gordonae* 571; 5, *M. tuberculosis* R68; 6, *M. tuberculosis* KIT10110; lane 7, *M. avium-intracellulare* complex 573. (B) The ramped pulse times were 30~40 s for 24 h at 14℃ and 200 V. Lanes 1, marker; 2, *M. terrae* complex 545; 3, *M. terrae* complex 479; 4, *M. gordonae* 571; 5, *M. tuberculosis* R68; 6, *M. tuberculosis* KIT10110; 7, *M. avium-intracellulare* complex 573; 8, *M. avium-intracellulare* complex 569; 9, *M. gordonae* 560; 10, marker. The numbers on the left show the positions for the DNA size standard markers of the sizes indicated.

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

Strians	Drug resistance	Sources
M. tuberculosis		
R68	H,R,S,E,K,TH	clinical isolate
H23	H,R,E	clinical isolate
KIT10110	sensitive	clinical isolate
M. avium-intracellulae complex		
573	H,R,S,E,K,EVM,PTH,CS,PAS,OFX,PZA	clinical isolate
569	NT[a]	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[a]	clinical isolate
571	NT[a]	clinical isolate
M. terrae complex		
545	H,R,S,E,TH,CS,PAS,PZA	clinical isolate
479	H,K,CS,PAS,OFX,PZA	clinical isolate

H: isoniazid resitance; R: rifampicin resistance; PZA: pyrazinamide resistance; S: streptomycin resistance; E: ethambutol resistance; PAS: para-aminosalicylic acid resistance; K: kanamycin resistance; TH: ethionamide resistance; CS: cycloserine resisrance; EVM: enviomycin resistance; PTH: prothionamide resistance; OFX: ofloxacin resistance.

[a] NT: not tested.

Table 2. Number and sizes of *AsnI* restriction fragments of mycobacterial genomic DNAs

	M. tuberculosis KIT10110	*M. terrae* complex		*M. fortuitum* 547
	M. tuberculosis R68	545	479	*M. fortuitum* 551
	M. tuberculosis H23			
	600	600	600	660
	580	580	580	540
	300	400	400	440
	225	350	330	435
	220	280	220	390
	170	240	200	240
	150	230	170	235
	140	140	165	45
	135	70	130	40
	132		125	30
	130		110	
	110		90	
	95		50	
	93			
	55			
	50			
	49			
	48			
	47			
Total (kb)	3,335	3,020	3,470	3,275

The sizes of the chromosomes were estimated by summing the individual fragment lengths in each restriction endonuclease digest. For each restriction analysis of a strain, the whole range of fragment size was subdivided into several groups, and band positions were determined from the gel with optimum resolution in the respective molecular mass range. The size of each DNA fragment was determined by calibration using linear DNA molecular markers as a reference. Chromosomal DNAs of *M. tuberculosis* and *M. fortuitum* digested with *AsnI* generated fragments ranging from 45 to 600 kb or 30 to 660 kb, respectively (Table 2). In the case of *M. terrae* complex 545 and 479, *AsnI* generated fragments ranging from 70 to 600 and 50 to 600 kb, respectively (Table 2). The total chromosome size of these strains were calculated from the sum of the fragment sizes generated by the digested chromosomal DNA. The sizes of the genomes of *M. tuberculosis*, *M. terrae* complex 545, *M. terrae* complex 479, and *M. fortuitum* were 3,335, 3020, 3470, and 3275 kb.

On the other hand, restriction digestion patterns of drug resistant strains such as *M. tuberculosis* R68 (Fig. 2A, lane 5 and Fig. 2B, lane 5), which is resistant to isoniazid (H), refampicin (R), streptomycin (S), ethambutol (E), kanamycin (K) and ethionamide (TH) and *M. tuberculosis* H23 (Fig. 3, lane 4) showing resistances to H, R, and E could not be differentiated from the *M. tuberculosis* KIT10110 (Fig. 2A, lane 6, Fig. 2B, lane 6 and Fig. 3, lane 5) strain showing drug sensitivities when DNAs were treated with *AsnI*. The same results were also obtained when the genomic DNAs were treated with *XbaI* (Fig. 3, lanes 8 and 9) or *AsnI-XbaI* (Fig. 3, lanes 12 and 13).

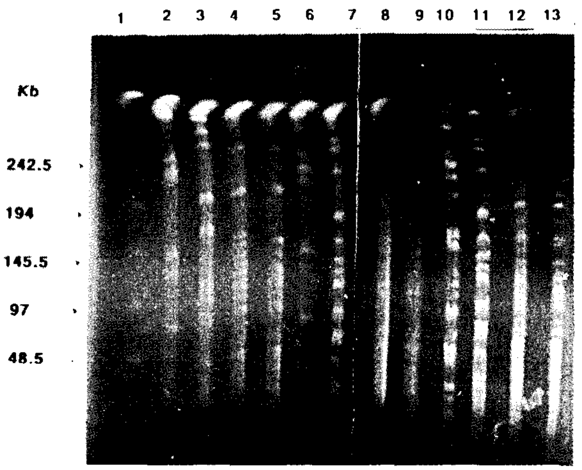


Fig. 3. Pulsed-field gel electrophoresis of mycobacterial DNA digested by *AsnI*, *XbaI* and *AsnI-XbaI*. The ramped pulse times were 5~20 s for 24 h at 14℃ and 200 V. Lane 1, marker. Lanes 2~5 for *AsnI* digestion. Lane 2, *M. terrae* complex 545; 3, *M. terrae* complex 479; 4, *M. tuberculosis* H23; 5, *M. tuberculosis* KIT10110. Lanes 6-9 for *XbaI* digestion. Lanes 6, *M. terrae* complex 545; 7, *M. terrae* complex 479; 8, *M. tuberculosis* H23; 9, *M. tuberculosis* KIT10110. Lanes 10-13 for *AsnI-XbaI* double digestion. The numbers on the left show the positions for the DNA size standard markers of the sizes indicated.

However, when DNAs from *M. terrae* complex 545 and 479 strains were treated with the same restriction enzyme *AsnI*, different patterns were observed (Fig. 2A, lanes 2, 3 and Fig. 2B, lanes 2 and 3). Seemingly, *XbaI* (Fig. 3, lanes 6 and 7) or *AsnI-XbaI* (Fig. 3, lanes 10 and 11) digestion showed different restriction patterns in each strain. The only difference between the two strains is drug resistance: *M. terrae complex* 545 shows resistance to drugs of H, R, S, E, TH, cycloserine (CS), para-aminosalicylic acid (PAS), and pyrazinamide (PZA), and *M. terrae* complex 479 shows resistance to drugs of H, K, CS, PAS, ofloxacin (OFX) and PZA.

In the case of *M. gordonae* 571 and 560, a different restriction pattern was also shown, even though the drug resistance of the two strains was not tested (Fig. 2A, lane 4 and Fig. 2B lanes 4,9). *M. avium-intracellulae* complex strains 573 and 569 also showed different patterns of restriction digestion (Fig. 2B, lanes 7,8). Strains isolated from patients suffering from disease were found to be distinguishable. The heterogeneity of Korean-type mycobacteria was obvious, as all strains gave a different pattern with different drug resistance.

The pulsed-field electrophoretic restriction patterns shown in the present study confirm analyses of restriction fragment length polymorphism (Burns *et al.*, 1991; Kochi *et al.*, 1991; Hector *et al.*, 1992; Zhang *et al.*, 1992), as different profiles were found for Korean epidemiological mycobacterial strains with different characteristics to drugs. Thus, this technique demonstrated the heterogeneity of Korean-type mycobacterial strains. Genomic typing methods investigated were also capable of differentiating mycobacterial strains from Korean clinical isolates.

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