JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 1997, p. 596–599 Vol. 35, No. 3
0095-1137/97/$04.00+0

Large-Restriction-Fragment Analysis of *Mycobacterium kansasii* Genomic DNA and Its Application in Molecular Typing

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Received 30 July 1996/Returned for modification 12 September 1996/Accepted 16 December 1996

Large-restriction-fragment (LRF) polymorphisms in *Mycobacterium kansasii* isolates from 84 patients with bronchopulmonary infections in Japan between the 1960s and 1995 were studied by pulsed-field gel electrophoresis (PFGE). Chromosomal fragments digested with *Vsp*I were most suitable for PFGE separation of 16 to 21 fragments of between 40 and 550 kbp. All 84 isolates and the type strain *M. kansasii* ATCC 12478 were successfully typed by LRF analysis with *Vsp*I digestion. Twenty-one distinctive LRF types were identified, and the LRF patterns tested over time were reproducible and stable. A computer-assisted dendrogram of the percent similarity demonstrated that isolates of 18 LRF types had relatively close genetic relatedness, while isolates of the remaining 3 types showed divergence. Sequence analysis of the 16S rRNA gene in the isolates showing divergent genetic relatedness revealed a sequence identical to that of a previously reported subspecies of *M. kansasii*. In the Chugoku district of Japan, 11 cases of *M. kansasii* infection which occurred in workers in a coastal industrial zone between 1982 and 1993 were caused by one particular strain tentatively named LRF type M. When both detailed demographic data for the patients and ecologic data for the *M. kansasii* isolates are obtained, LRF typing may be of potential use for investigating the source and transmission of *M. kansasii* infection.

Mycobacterium kansasii is an opportunistic pathogen which causes pulmonary infections (28). Disseminated disease rarely occurs, but it is frequently associated with AIDS (4, 13). An epidemiologic investigation in Japan showed an increasing annual incidence of *M. kansasii* pulmonary infections, starting as low as 0.11 or fewer cases per 10^5 people until 1977; it then increased to 0.45 cases per 10^5 people in 1984 (25). Infections due to *M. kansasii* in Japan are found mainly in urban areas, especially along the southeastern industrial coastal districts. In the United States this infection is found predominantly in the southeastern and southern coastal states and in central industrial states (27). The prevalence of this infection has been suggested to be related to air pollution, dust exposure, and contaminated water systems (7, 11, 20, 25, 28). However, the infection route of this organism is poorly understood because of the lack of an effective typing methodology for *M. kansasii*. Bacteriophage typing of *M. kansasii* is technically complicated and has a limited ability to type *M. kansasii* isolates and limited reproducibility (2, 5, 6). Molecular typing methods are used in many epidemiologic investigations of infectious diseases, including mycobacterial infections, with excellent type discrimination and reproducibility (23). Chromosomal DNA large-restriction-fragment (LRF) patterns produced by pulsed-field gel electrophoresis (PFGE) have been applied to mycobacterial typing (1, 3, 14, 15, 21, 30) and have been proposed as the standard method for typing *M. avium* complex isolates. Southern blot hybridization with DNA probes has also been used to type *M. kansasii* strains (8, 9, 17, 18, 29); one report found a variant sequence of 16S rRNA, indicating a subspecies of *M. kansasii* (17). However, little information is available concerning the ability of the LRF method to type *M. kansasii* strains.

The aims of this study were to examine the typeability, reproducibility, and discriminatory power of the LRF patterns for *M. kansasii* strains and to evaluate the potential usefulness of this method for typing *M. kansasii* strains.

MATERIALS AND METHODS

Organisms. The type strain *M. kansasii* ATCC 12478 was used as a standard. Eighty-four clinical isolates of *M. kansasii* were obtained mainly from the members of Mycobacteriosis Research Group of the Japanese National Chest Hospitals between the 1960s and 1995 in five districts (25). All of the clinical isolates were recovered from 84 patients with bronchopulmonary infections. The 84 isolates consisted of 14 from the Kanto district, 12 from the Chubu district, 20 from the Kinki district, 25 from the Chugoku district, and 13 from the Kyushu district. Among the 25 isolates from the Chugoku district, 16 were from patients with pulmonary infections in the Mizushima coastal industrial zone (Fig. 1). Each isolate was characterized and identified by biologic and biochemical tests (24) and/or the DNA-DNA hybridization technique by using DDH Mycobacteria (Kyokuto Pharmaceutical Co., Tokyo, Japan) (12). These isolates and the type strain were stored at −80°C in 50% glycerol until they were tested.

DNA preparation. Genomic DNA was prepared by a modification of a previously reported method (21). Isolates were grown in 20 ml of 7H9 broth supplemented with 0.5 M sucrose, 0.05% (wt/vol) Tween 80, and M supplement (Becton Dickinson, Cockeysville, Md.) at 37°C for 7 to 14 days to an optical density of 0.20 at 600 nm. Cycloserine (1 mg/ml) was added, and the cultures were incubated for an additional 24 h prior to harvesting. After centrifugation at 1,000 × *g* for 20 min at 4°C, cells were resuspended in 400 μl of TS buffer (50 mM Tris, 0.5 M sucrose [pH 7.6]). The suspension was frozen in dry ice-ethanol and was then thawed. Subsequently, 50 μl of the suspension was mixed with an equal volume of 1.2% low-melting-point agarose, and the mixture was then poured into a plug mold. The plug was incubated in a lysis solution (1 M NaCl, 0.1 M EDTA [pH 8.0], 10 mM Tris-HCl [pH 8.0], 0.5% [wt/vol] Brij, 0.2% [wt/vol] deoxycholate, 0.5% [wt/vol] lauroylsarcosine) containing lysozyme (2 mg/ml) at 37°C for 48 h and thereafter was treated with proteinase K (0.5 mg/ml).

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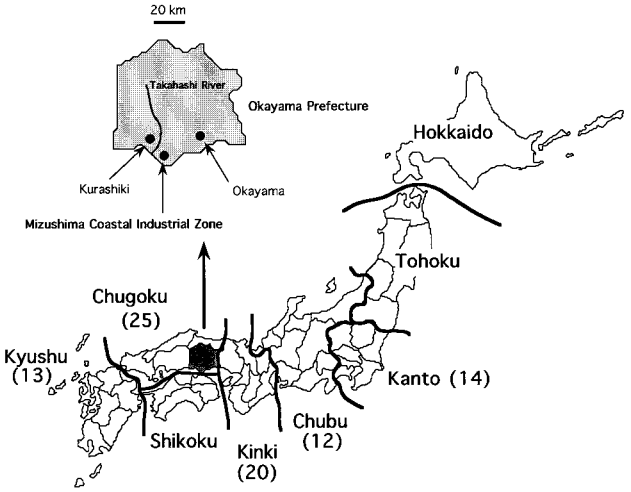

FIG. 1. Map of Japan and numbers of *M. kansasii* strains (in parentheses) collected in each district.

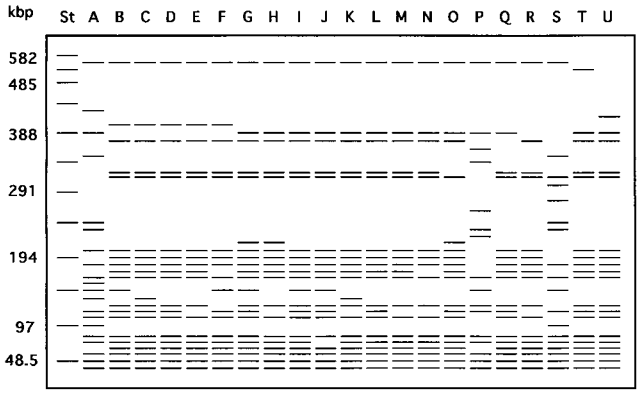

FIG. 2. Schematic representation of the 21 LRF patterns (LRF types A through U) obtained after cleavage of the genomic DNAs with *Vsp*I. St, bacteriophage λ DNA PFGE molecular markers. The positions of λ DNA size markers are indicated on the left.

Restriction endonuclease digestion and PFGE. Restriction endonucleases (*Dra*I, *Xba*I, *Spe*I, *Ssp*I, and *Vsp*I) with rare recognition sites were screened to determine if they provided suitable LRF patterns by PFGE. Each thinly sliced agarose plug was digested with 10 U of each enzyme for 20 h and was electrophoresed through a 1% agarose gel in TBE buffer (0.1 M Tris, 0.1 M boric acid, 2 mM EDTA [pH 8.0]) at 10°C by using a contour-clamped homogeneous electric field system (Pulsaphor Plus; Pharmacia LKB Biotechnology, Uppsala, Sweden). Electrophoresis was performed at 200 V for 20 h with suitable pulse times for each enzyme. The DNA banding pattern was photographed under UV light after ethidium bromide staining. When strains analyzed on different gels appeared to have similar LRF patterns, they were run side by side on a single gel to clarify the band patterns.

Computer-assisted analysis of fingerprints. Dendron software (Solltech, Inc., Oakdale, Iowa) was used to compare *M. kansasii* fingerprints (26). LRF patterns were scanned into the computer. A similarity coefficient between each pair of fingerprints was calculated on the basis of band positions alone, according to the manufacturer's instruction.

Sequence analysis of the 16S rRNA gene. The primers for amplification of bases 29 to 305 of the 16S rRNA gene were 5′-AGA GTT TGA TCC TGG CTC AG-3′ and 5′-AGT CCC AGT GTG GCC GG-3′. DNA (100 ng) was amplified in 50 μl of a buffer containing each primer (1 μM), *Taq* polymerase (1 U; Boehringer Mannheim, Mannheim, Germany), and deoxynucleoside triphosphates (200 mM each). The reaction mixture was heat denatured at 94°C for 2 min, and amplification was performed for 35 cycles at 94°C for 30 s, 57°C for 30 s, and 72°C for 1 min with the GeneAmp PCR System 9600R thermal cycler (Perkin-Elmer, Norwalk, Conn.). PCR products were purified and then cloned by using the pT7Blue T-Vector kit (Novagen, Madison, Wis.). The cloned products were analyzed with the *Taq* Dye Deoxy Terminator Cycle Sequencing kit (Perkin-Elmer/Applied Biosystems Division, Foster, Calif.) and the model 377 Fluorescent Sequencer (Perkin-Elmer/Applied Biosystems Division). At least two independent PCR clones from each strain were sequenced for each genetic analysis.

RESULTS

LRF patterns of the *M. kansasii* strains. All 84 clinical isolates and the ATCC 12478 type strain were successfully typed by PFGE. Among the five restriction enzymes evaluated, *Vsp*I generated LRF patterns with 16 to 21 fragments of between 40 and 550 kbp, most suitable for analysis. Twenty-one LRF patterns (tentatively termed LRF types A through U) were identified among the 85 strains tested (Fig. 2). Although *Spe*I generated LRF patterns with 24 to 28 bands of 30 to 350 kbp, only 16 patterns (tentatively termed LRF types a through p) could be identified. Table 1 presents the LRF types obtained by both *Vsp*I and *Spe*I digestion in relation to the districts where the organisms were isolated. The LRF types obtained with *Vsp*I were more polymorphic than those obtained with *Spe*I, demonstrating that *Vsp*I digestion could subtype identical *Spe*I types d and g into three and five *Vsp*I types, respectively. By contrast, *Spe*I digestion subtyped only *Vsp*I type R into two *Spe*I types. The other enzymes generated less discernible patterns. Type strain ATCC 12478 was typed as type M by *Vsp*I digestion, and type M was the most frequently observed type among the clinical isolates. Since LRF analysis with *Vsp*I was more discriminating, this method was used in the following analyses. Additional strains isolated at different times from each of two patients were examined to test the stability of the LRF typing method. The LRF patterns of each of the two strains from these two patients remained unchanged. The LRF patterns were stable for two clinical isolates and strain ATCC 12478 after more than five successive cultures. The reproducibility of the LRF patterns was also confirmed by repeat testing.

The 21 LRF types obtained by *Vsp*I digestion were subjected to genetic similarity analysis with the Dendron software pro-

TABLE 1. LRF typing results for the 84 *M. kansasii* strains according to the district from which they were isolated

LRF type		No. of isolates from the indicated district					
*Vsp*I	*Spe*I	Kanto	Chubu	Kinki	Chugoku	Kyushu	Total
A	a		1		1		2
B	b		1	6			7
C	c	6	1	1			8
D	d		3		1	3	7
E	d			1			1
F	e			1			1
G	f			1			1
H	g			1			1
I	h	2	2		3		7
J	i			1			1
K	j			2			2
L	g	1	1	2		3	7
M	g		2	4	15	1	22
N	g		1		4		5
O	k					1	1
P	l				1		1
Q	g					2	2
R	d	1					1
R	m	1				1	2
S	n					1	1
T	o					1	1
U	p	3					3

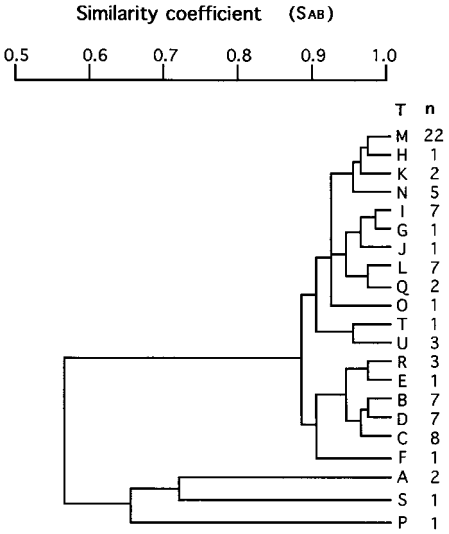

FIG. 3. Dendrogram based on computer-assisted comparison of the 21 LRF types (LRF types A through U) obtained after cleavage of the genomic DNAs with *Vsp*I. The genotype (T) and the number of strains (n) belonging to each LRF type are indicated.

gram. Figure 3 shows the dendrogram of the LRF types. Eighteen of the 21 LRF types formed a cluster showing genetic relatedness with a similarity coefficient of 0.87 or higher. The remaining three LRF types (LRF types A, S, and P) were genetically divergent from the cluster and from one another. These observations were also supported by the LRF patterns obtained by *Spe*I digestion.

Table 1 demonstrates the LRF types of the *M. kansasii* isolates according to the districts where they were isolated. In the Chugoku district, LRF type M ($n = 15$ strains) was most frequently observed, followed by types N ($n = 4$), I ($n = 3$), A ($n = 1$), D ($n = 1$), and P ($n = 1$). Among the 15 LRF type M strains, 11 were recovered from workers in the Mizushima coastal heavy industrial zone (Fig. 1) between 1982 and 1993.

16S rRNA gene sequence analysis. Analysis of the 16S rRNA gene sequences of six strains was performed; two were of LRF type A and one each was of type S, P, B, and M. Strains of LRF types A, S, and P had sequences identical to that of an *M. kansasii* subspecies reported by Ross et al. (17). LRF types B and M had sequences identical to the previously determined sequence of *M. kansasii* DSM 43224 (16).

DISCUSSION

Molecular typing methods should meet several essential requirements including an ability to type strains, good reproducibility and stability of patterns, and good discriminatory power. One epidemiologic study by bacteriophage typing analyzed the relationship between clinical and environmental isolates in an area where *M. kansasii* is endemic, but it had limited discriminatory power (6). Another study of the geographic distribution of the restriction fragment length polymorphism types of *M. kansasii* isolates with a species-specific probe showed marked variability among the isolates from different countries (17). However, in that study, the discriminatory power of the probe method was questionable, demonstrating only 11 patterns among 105 *M. kansasii* strains. Our study is the first to evaluate the potential use of the LRF patterns obtained by PFGE for typing *M. kansasii* strains.

When DNAs from 84 clinical isolates and type strain ATCC 12478 were digested with *Vsp*I, all of the strains were successfully typed and 21 restriction patterns were produced. The reproducibility and stability of this method were also verified by repeat testing of the strains. Analysis of the 16S rRNA gene sequences of strains of LRF types A, S, and P revealed that these strains had sequences identical to that of a subspecies of *M. kansasii* which had been isolated principally in Europe (17). PFGE revealed greater genetic diversity in this subspecies. By contrast, two strains (LRF types B and M) which fell into the cluster had 16S rRNA genes typical of that of *M. kansasii*. The low level of genetic diversity of *M. kansasii* strains except for those of the subspecies demonstrated in this study is consistent with previously reported evidence of homologous Southern blot hybridization patterns (17, 29) and the antigenic structure of *M. kansasii* (19).

Several investigators have provided possible infection sources and transmission routes of *M. kansasii* (7, 28). Contamination of the environment, especially the water supply, by *M. kansasii* may serve as a source for infections (5, 10, 22). Similarly, iron manufacturing, which requires large quantities of water, may facilitate mycobacterial colonization (11, 20). On the other hand, host factors such as preexisting pulmonary diseases including pneumoconiosis, chronic obstructive pulmonary disease, impaired ventilatory function, and dust inhalation can put an individual at risk of *M. kansasii* infection (7). The aggregation of LRF type M strains among workers in the Mizushima coastal heavy industrial zone from 1982 may be compatible with a common source of infection in the environment, since all factories in this industrial zone use water supplied from the Takahashi River (Fig. 1). However, the source or infection route of the *M. kansasii* isolates was not determined in this study because of the lack of detailed information on the case histories of the patients and the ecology of the *M. kansasii* isolates.

In summary, the LRF patterns obtained by PFGE of *M. kansasii* strains are typeable, reproducible, and discriminative. Therefore, when detailed demographic information about the patients and ecologic data for the infecting *M. kansasii* strains, are obtained, this typing method may be used to investigate the source and route of transmission of *M. kansasii* infections. Further epidemiologic investigations are necessary to identify how *M. kansasii* infects humans, especially because this organism may cause disseminated disease in association with AIDS (4, 13).

ACKNOWLEDGMENTS

We are grateful to Hidehiko Saito, First Department of Internal Medicine, Nagoya University School of Medicine, for encouragement. We thank M. Sakatani (head of the Mycobacteriosis Research Group of the Japanese National Chest Hospitals), Kinki-Chuo National Hospital, Osaka, Japan; Y. Sasaki, Chiba-Higashi National Hospital, Chiba, Japan; and S. Harada, Omuta National Hospital, Fukuoka, Japan, for providing the clinical *M. kansasii* isolates.

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