JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 1997, p. 25–32 Vol. 35, No. 1
0095-1137/97/$04.00+0

Genotypic Characterization of Five Subspecies of *Mycobacterium kansasii*

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Received 18 June 1996/Returned for modification 23 July 1996/Accepted 17 September 1996

Different molecular typing methods including restriction fragment length polymorphism (RFLP) analysis with the major polymorphic tandem repeat (MPTR) probe and the IS*1652* probe, pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP) analysis, and PCR restriction analysis of the *hsp-65* gene (PRA) were applied to clinical and water isolates of *Mycobacterium kansasii*. RFLP with the MPTR probe, PRA, PFGE, and AFLP analysis revealed five homogeneous clusters which appeared to be subspecies. RFLP with the MPTR probe and PRA gave patterns specific for each cluster, whereas PFGE and AFLP analysis gave polymorphic patterns. IS*1652* was present in two of the five clusters and provided polymorphic patterns for one cluster only. The two IS*1652*-positive clusters were Accuprobe negative (Accuprobe test; Gen-Probe Inc.), and only two other clusters were Accuprobe positive. A PCR test based on the detection of a species-specific fragment (M. Yang, B. C. Ross, and B. Dwyer, J. Clin. Microbiol. 31:2769–2772, 1993) was positive for all *M. kansasii* strains. This PCR test is an accurate, rapid, and specific *M. kansasii* identification test. No subspecies was particularly more virulent, because all clusters contained clinical strains, from AIDS patients and non-AIDS patients, and environmental strains.

Mycobacterium kansasii, like *M. avium* and *M. xenopi*, is one of the major pulmonary mycobacterial pathogens. The relative incidence of disease due to these pathogens varies between geographical regions. In the United States, *M. avium* and *M. kansasii* are the most frequently isolated mycobacteria, whereas *M. kansasii* is rare in Australia. However, in some European countries, *M. kansasii* and *M. xenopi* infections were more common than *M. avium* infections before the AIDS epidemic (7). *M. kansasii* infections are often clustered, mainly in urban areas, as reported in studies in Texas, the Czech Republic, and other regions (7, 11). *M. avium* predominates in human immunodeficiency virus (HIV)-infected patients, and *M. avium* infection represents the most frequent disseminated infection diagnosed in these patients. However, HIV-infected patients also suffer pulmonary or disseminated infections due to *M. kansasii*. In some areas, for example, Switzerland, the prevalence of mycobacterial disease due to *M. kansasii* has been unchanged by the AIDS epidemic, which was not associated with a shift to a predominance of *M. avium* (7).

Unlike tubercle bacilli, human-to-human transmission has never been established for infections due to other mycobacteria. These mycobacterial infections are considered to be acquired from the environment. *M. kansasii* has been almost exclusively recovered from tap water; other environmental samples are rarely positive (3, 6, 10, 14, 23, 31). *M. kansasii* was found to be able to survive in tap water for up to 12 months, whereas long-term survival in soil could not be shown (10). Because the *M. kansasii* isolates found in drinking-water distribution systems are not contaminants from another source but are able to grow and persist in this environment, the tap water reservoir is probably the source of human infection. The use of typing methods allowing for the precise characterization of water and human isolates would establish the epidemiological relationships between environmental and clinical isolates. The involvement of tap water in human infections could then be confirmed or disproved.

The first typing method developed for *M. kansasii* was phage typing (5). However, the technique was not widely applied because it is laborious and the technical difficulties result in poor reproducibility and interpretation of the results. Other features, especially catalase activity and colonial variation based on surface glycolipid composition, have been used to type *M. kansasii* isolates. Isolates with high catalase activity were considered more virulent, and the rough colonies were shown to persist longer than smooth variants in experimentally infected mice (2, 30). However the genetic basis for these variations has not been investigated. Analysis of the 16S rRNA sequence (21), the amplification of the 16S-23S rRNA spacer region (1), PCR and restriction analysis of the *hsp-65* gene (PRA) (18, 24), and the detection of an insertion sequence-like element, IS*1652* (32), showed that *M. kansasii* contains a subspecies genetically distinct from the typical *M. kansasii* isolates. Conventional biochemical tests could not distinguish between the two groups. The commercially available identification probe (Accuprobe; Gen-Probe Inc.) fails to hybridize with the subspecies isolates (12, 21, 26, 33), whereas a specific DNA probe isolated in plasmid p6123 successfully hybridized with *M. kansasii* isolates, including the genetic subgroup (33).

Two repeated DNA sequences in addition to IS*1652* have been described in *M. kansasii*. Ross et al. (22) have demonstrated the existence of a repeated DNA sequence, designated the polymorphic GC-rich repetitive sequence, which is also found in *M. tuberculosis*, *M. gastri*, and *M. szulgai*; Hermans et al. (9) have described the major polymorphic tandem repeat (MPTR), consisting of 10-bp repeats that are also present in *M. tuberculosis* and *M. gordonae*.

We evaluated DNA-based techniques for *M. kansasii* identification and strain typing. We thus investigated *M. kansasii* strains, including water and clinical isolates from AIDS and non-AIDS patients, by analysis of restriction fragment length polymorphisms (RFLPs) with the MPTR probe and the IS*1652* probe, pulsed-field gel electrophoresis (PFGE), amplified frag-

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TABLE 1. Clinical isolates used in this study and patterns obtained by RFLP analysis with MPTR probe and PFGE

Clinical strain	Hospital	Set[a]	Source	HIV status	Pattern by RFLP analysis with MPTR probe	Pattern by PFGE
c1	ND[b]		Type strain	ND	I	Ic
c2	A		Gastric aspirate	+	I	Ia
c3	B		Abscess	−	I	Ia
c4	C	1	Sputum	−	I	Ib
c5	C	1	Biopsy specimen	−	I	Ia
c6	C	1[c]	Sputum	+	I	Ia
c7	C	1	Gastric aspirate	−	I	Ia
c8	C	1	Sputum	−	I	Ia
c9	D		Gastric aspirate	−	I	Ia
c10	E		Sputum	+	I	Not typeable
c11	E		Sputum	−	I	Ia
c12	F	2[d]	Gastric aspirate	−	I	Id
c13	F	2[d]	Gastric aspirate	−	I	Id
c14	F	2[d]	Gastric aspirate	−	I	Id
c15	F	2	Blood	+	I	Ia
c16	F	2	Sputum	−	I	Ia
c17	F	2	Gastric aspirate	−	I	Ia
c18	G		Gastric aspirate	+	II	IIa
c19	H		Bone	+	II	IId
c20	I		Gastric aspirate	−	II	IIa
c21	E		Gastric aspirate	+	II	IIa
c22	J		Gastric aspirate	−	II	IIa
c23	C	1[c]	Blood	+	II	IIIb
c24	C	1[c]	Sputum	+	II	IIe
c25	C	1	Bone	−	II	IIb
c26	C	1	Sputum	−	II	IIc
c27	C	1[c]	Sputum	+	II	IIc
c28	C	1	Gastric aspirate	−	II	IIc
c29	C	1[c]	Blood	+	II	IIc
c30	C	1	Bone	+	II	IIc
c31	C	1	Gastric aspirate	−	III	IIIa
c32	G		Urine	ND	III	IIIc
c33	K		Gastric aspirate	+	IV	IV
c34	L		Gastric aspirate	−	IV	Not typeable
c35	G		Sputum	−	IV	IV
c36	M		Sputum	ND	IV	Not typeable
c37	N		Sputum	−	V	V
c38	G		Gastric aspirate	−	V	V
c39	L		Gastric aspirate	−	V	V

[a] Sets are defined in Materials and Methods.
[b] ND, not determined.
[c] From patients hospitalized in the same medical unit.
[d] The patients were from the same family.

ment length polymorphism (AFLP) analysis, and PRA and by use of the p6123 probe (33) and the commercially available identification probe (Accuprobe test; Gen-Probe Inc.).

MATERIALS AND METHODS

Mycobacterial isolates. The type strain and 62 *M. kansasii* isolates including 38 clinical strains and 24 strains from water samples, all isolated in France, were studied (Tables 1 and 2). The strains were identified as *M. kansasii* on the basis of conventional tests.

The clinical strains were from patients attending 14 hospitals (hospitals A to N) located in eight French towns; they included both random isolates and sets of possibly epidemiologically related strains. All clinical strains were single patient isolates, and 14 were from AIDS patients (Table 1).

The water isolates were recovered from tap water sampled in six different hospitals (hospitals F and O to S) in and around Paris, France. Strains were recovered from both hot and cold water sampled in wards, laboratories, patients' rooms, water tanks, and the water mains (Table 2).

Several sets of strains were possibly epidemiologically related.

Set 1 comprised 14 clinical strains isolated from patients attending hospital C (strains c4 to c8 and c23 to c31). In 1994, during a 3-month period, five patients hospitalized in the same medical unit presented with infections due to *M. kansasii* (strains c6, c23, c24, c27, and c29). To determine whether a single strain was involved, we compared the isolates from these five patients and those isolated from nine patients hospitalized in different departments of the hospital from 1991 to 1994.

Set 2 included 13 strains isolated and identified in the same hospital microbiology laboratory. They included three clinical strains (strains c12 to c14), agents of clinically confirmed pulmonary infections in three patients from a single family, a strain (strain w40) recovered from the water of the family's home, and three clinical strains (strains c15 to c17) and six isolates (strains w41 to w46) from water sampled in the hospital.

Sets 3 to 6 corresponded to strains isolated from tap water sampled in a single hospital, i.e., hospitals O, P, Q, and S, respectively, as indicated in Table 2.

The remaining strains (19 clinical isolates and 1 water isolate) were considered epidemiologically unrelated.

PCR assays. We defined primers K1 (5′-GTG CCA CAC CGA CGT TGC-3′) and K2 (5′-GGT AGT GGG CTC GGA TAT GGA-3′) internal to the sequence of the insert from plasmid p6123 isolated by Yang et al. (33). These primers were used for a PCR-based identification test for *M. kansasii*, relying on the amplification of a 268-bp fragment.

To generate an IS*1652* probe for RFLP analysis, we used primers IS2 and IS1, corresponding to positions 690 to 709 and 886 to 905 of IS*1652*, respectively, as described previously by Yang et al. (32).

Amplification reactions were performed in volumes of 50 μl containing 1× *Taq* polymerase buffer (50 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% [wt/vol] gelatin), 5 μl of dimethyl sulfoxide, 200 μM (each) deoxynucleoside triphosphate, 1 μM (each) primer, and 2 U of *Taq* polymerase, covered with mineral oil, for 35 cycles: 1 min at 95°C, 1 min at 60°C, and 1 min at 72°C,

TABLE 2. Environmental isolates used in this study and patterns obtained by RFLP analysis with MPTR probe and PFGE

Water isolate	Hospital or other site	Set[a]	Sampling site	Pattern by RFLP analysis with MPTR probe	Pattern by PFGE
w40	Home	2[b]	Shower	I	Id
w41	F	2	Mycobacteria laboratory	I	Ia
w42	F	2	Mycobacteria laboratory	I	Ia
w43	F	2	Mycobacteria laboratory	I	Ia
w44	F	2	Mycobacteria laboratory	I	Ia
w45	F	2	Endoscopy ward	I	Ia
w46	F	2	Endoscopy ward	I	Ia
w47	O	3	Surgery ward	I	Ia
w48	P	4	Hot water tank	II	IIa
w49	P	4	Hot water tank	II	IIa
w50	P	4	Hot water tank	II	IIa
w51	P	4	Water distribution system	II	IIa
w52	O	3	Surgery ward	II	IIc
w53	Q	5	Internal medicine department	II	IIc
w54	Q	5	Internal medicine department	II	IIc
w55	Q	5	Endoscopy ward	III	IIIa
w56	Q	5	Cardiology ward	III	IIIa
w57	R		Patient room	III	IIIa
w58	Q	5	Internal medicine department	IV	IV
w59	Q	5	Endoscopy ward	IV	IV
w60	S	6	Mycobacteria laboratory	IV	IV
w61	S	6	Mycobacteria laboratory	IV	IV
w62	S	6	Mycobacteria laboratory	IV	IV
w63	P	4	Surgery ward	V	V

[a] Sets are defined in Materials and Methods.
[b] From home of patients infected with strains c12 to c14.

followed by 10 min of extension at 72°C. DNA in crude lysates was used. The lysates were obtained by resuspending a loopful of mycobacteria in 100 μl of TE (10 mM Tris, 1 mM EDTA [pH 8]) containing 1% (vol/vol) Triton X-100 and heating at 100°C for 30 min. Amplification products were analyzed by electrophoresis in 1.5% agarose gels and were detected by ethidium bromide staining.

Southern blot hybridization. Genomic DNA was prepared as described previously (17).

RFLP analysis with the MPTR probe. Two-microgram aliquots of mycobacterial DNA were digested with 10 U of *Bst*EII in a reaction mixture of 30 μl. The DNA fragments were resolved by overnight electrophoresis in a 1% agarose gel at 1.5 V/cm. Then, the DNA was transferred to a nylon membrane (N^+-Hybond; Amersham International, Amersham, United Kingdom) by the method of Southern. Probe TR2, a trimer of the MPTR sequence, was obtained by chemical synthesis (9). The membrane was hybridized overnight at 40°C with the TR2 oligonucleotide probe labeled with horseradish peroxidase by using the ECL kit (Amersham). The membrane was then washed twice for 20 min at 35°C with 2× SSC (1× SSC corresponds to 0.15 M sodium chloride and 0.015 M sodium citrate) containing 0.1% (wt/vol) sodium dodecyl sulfate and was then washed twice for 5 min at room temperature with 2× SSC. Binding of the peroxidase-labeled DNA probe was revealed with the ECL detection system (Amersham).

IS*1652* RFLP analysis. For IS*1652* RFLP analysis, previously prepared membranes carrying *Bst*EII-digested DNA were reused and hybridized overnight at 42°C with the IS*1652* probe prepared by PCR as described above and were labeled by using the ECL kit (Amersham). Then, the membranes were washed twice for 10 min each time at 55°C with 0.5× SSC containing 0.4% (wt/vol) sodium dodecyl sulfate and were finally washed twice for 5 min each time at room temperature with 2× SSC. The peroxidase-labeled probe was revealed with the ECL detection system (Amersham).

p6123 hybridization. To check for the presence of the *M. kansasii* species-specific fragment isolated by Yang et al. (33), 2 μg of mycobacterial DNA was digested with *Eco*RI. Electrophoresis and transfer to a nylon membrane were as described above. The 268-bp probe within the p6123 species-specific fragment was prepared by PCR as described above, purified from the agarose gel by the GeneClean procedure (Bio101, La Jolla, Calif.), and then labeled with [α-^{32}P]dCTP with the Megaprime DNA labeling kit (Amersham). Hybridization and washing steps were performed as previously described (16).

PFGE. The *M. kansasii* isolates were cultivated in 5 ml of Middlebrook 7H9 broth. These cultures were used to inoculate 40 ml of fresh medium at an optical density at 650 nm of 0.08, and the fresh cultures were incubated at 37°C until an optical density of 0.2 was attained. Plugs were prepared as described previously (16) and were digested with 30 U of *Dra*I or *Xba*I. Large restriction fragments were separated by zero-integrated-field electrophoresis with the AutoBase system (Q-Life Systems Inc., Kingston, Ontario, Canada) for 65 h at room temperature with the 8- to 200-kb ROM card and/or the 8- to 500-kb ROM card.

Cluster analysis of PFGE data. After electrophoresis, the gels were stained with ethidium bromide and were photographed on a UV transilluminator. The *Dra*I profiles were scanned and analyzed by using the Taxotron package (Institut Pasteur Taxolab, Paris, France). This package comprises the RestrictoScan, RestrictoTyper, Adanson, and Dendrograph programs. Lanes and bands were detected with the RestrictoScan program. Fragment lengths were interpolated by the Spline algorithm (implemented with the RestrictoTyper software). The similarity index was calculated by the RestrictoTyper program with the fragment length error tolerance set at 5%. The single linkage was computed with the Adanson program, and a dendrogram was drawn by the Dendrograf program.

AFLP analysis. The restriction-ligation reaction and PCR were performed as described previously by Valsangiacomo et al. (27). Briefly, about 500 ng of genomic DNA was digested with *Pst*I, and restriction fragments were ligated to *Pst*I adapter oligonucleotide LG1 (5′-CTC GTA GAC TGC GTA CAT GCA-3′) and LG2 (5′-TGT ACG CAG TCT AC-3′) with T4 DNA ligase (Gibco BRL). The DNA was precipitated by the addition of salt and chilled absolute ethanol. The DNA pellet was dissolved in 50 μl of H_2O. PCR was performed with the primers AMP2 (5′-GAC TGC GTA CAT GCA GGC-3′) and AMP4 (5′-GAC TGC GTA CAT GCA GAT TAG-3′) (the underscoring indicates selective nucleotides). Amplification procedures were performed as described previously (27). Amplified fragments were analyzed by electrophoresis in a 2% agarose gel (MetaPhor agarose; FMC BioProducts, Rockland, Maine) in 1× TAE buffer (40 mM Tris acetate, 1 mM EDTA [pH 8]) and were visualized by ethidium bromide staining.

Accuprobe test. The Accuprobe culture identification test for *M. kansasii* was performed as described by the manufacturer (Gen-Probe Inc.) and was performed with all isolates (Table 3).

PRA. PRA of the *hsp-65* gene was performed as described previously (24). Briefly, a 439-bp fragment was amplified by PCR with primers Tb11 and Tb12. The fragment was then digested with either *Bst*EII or *Hae*III, and the restriction fragments were analyzed by electrophoresis on a 4% agarose gel (MetaPhor agarose; FMC BioProducts).

RESULTS

RFLP analysis with the MPTR probe. The MPTR probe was used for all clinical and water isolates. The RFLP patterns generated five clusters (clusters I to V). Figure 1 shows the RFLP patterns for a representative strain of each cluster. The largest group, cluster I, included 25 strains, including the reference strain, 17 clinical strains, and 8 water isolates. Cluster II consisted of 20 strains, including 13 clinical isolates and 7 water

TABLE 3. Comparison of K1-K2 PCR, Accuprobe test, IS*1652* detection, and PRA results for *M. kansasii* subspecies and *M. gastri*

M. kansasii clusters and *M. gastri*	No. of isolates		K1-K2 PCR result	*M. kansasii* Gen-Probe result	IS*1652* result	Bands by PRA	
	Clinical (n = 39)	Water (n = 24)				*Hae*III	*Bst*EII
I[a]	17[b] (5[c])	8	+	+	−	140, 105, 80	245, 220
II	13 (8)	7	+	−	+	140, 105	245, 145, 85
III	2 (0)	3	+	−	+	140, 100, 70	245, 145, 85
IV	4 (1)	5	+[d]	−	−	140, 115, 70	245, 125, 85
V	3 (0)	1	+	+	−	140, 100, 80	325, 125
M. gastri (type strain)			−	−	−	140, 105, 70	245, 145, 85

[a] The five *M. kansasii* clusters were defined on the basis of RFLP analysis with the MPTR probe and PFGE patterns.
[b] Including the *M. kansasii* type strain.
[c] The numbers of *M. kansasii* strains isolated from AIDS patients are indicated in parentheses.
[d] Strains of this cluster presented bands in addition to the expected 268-bp product.

isolates. The 18 remaining strains were distributed in the three other clusters (Tables 1 and 2).

PFGE analysis. Mycobacteria have a high GC content. We therefore evaluated the potential usefulness of various enzymes recognizing AT-rich restriction sites. All patterns generated by *Xba*I were complex, with more than 30 large restriction fragments (LRFs) (data not shown). *Dra*I produced simple, easily readable patterns, with up to 15 LRFs (Fig. 2), the largest of which was 400 kb. Because of the number and distribution of the LRFs, we found that *Dra*I digestion patterns were more suitable for the study of *M. kansasii*. Nevertheless, three strains, strains c10, c34, and c36, repeatedly yielded incompletely restricted or degraded DNA, despite particular care in DNA purification and prevention of nuclease activity (data not shown). Similar difficulties for PFGE analysis of some mycobacterial strains have previously been reported by others (29).

In our hands, the 50- to 300-kb range presented optimal LRF resolution and included the most polymorphic bands. Nevertheless, for many strains, additional LRFs smaller than 50 kb or/and larger than 300 kb were observed (Fig. 2).

The LRF patterns, limited to DNA fragments of 50 to 300 kb, were scanned and compared by numerical analysis. A minimum of seven bands, except for cluster V, in which only three to four bands were found, was included. Cluster analysis of the PFGE profiles yielded the dendrogram in Fig. 3, which shows the degrees of relatedness between strains with various profiles.

Cluster I comprised 24 strains in four subgroups, subgroups Ia to Id (Fig. 3). A major group, group Ia, consisted of 17

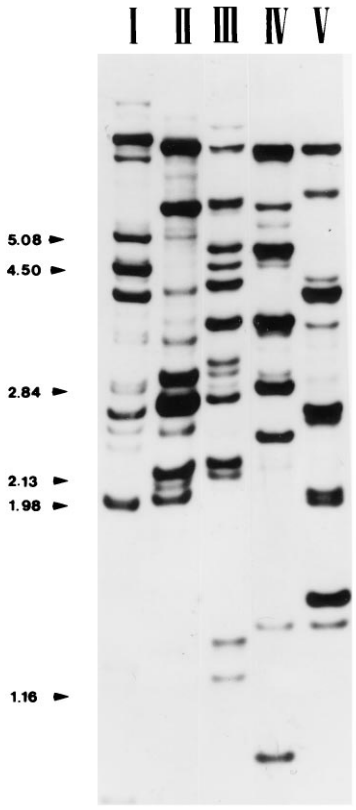

FIG. 1. Southern blot analysis of *Bst*EII-digested DNAs of *M. kansasii* isolates probed with the MPTR element. The molecular size marker (in kilobases) was bacteriophage lambda DNA restricted with *Pst*I.

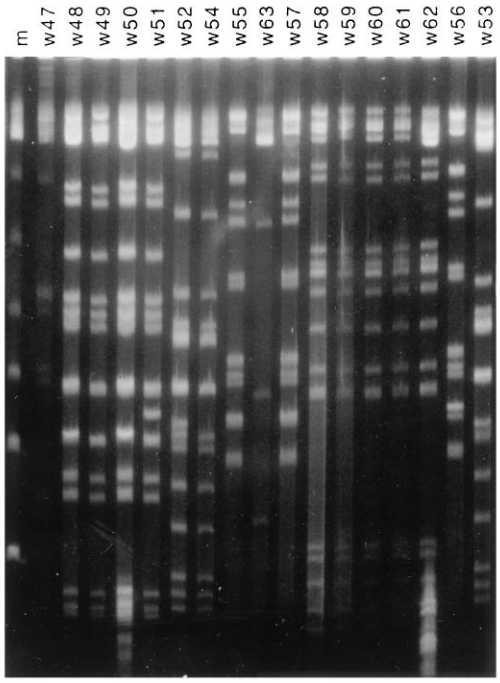

FIG. 2. PFGE separation of *Dra*I-digested genomic DNA from *M. kansasii* water isolates. The molecular size marker (lane m) consisted of bacteriophage lambda DNA concatemers of 50 kb.

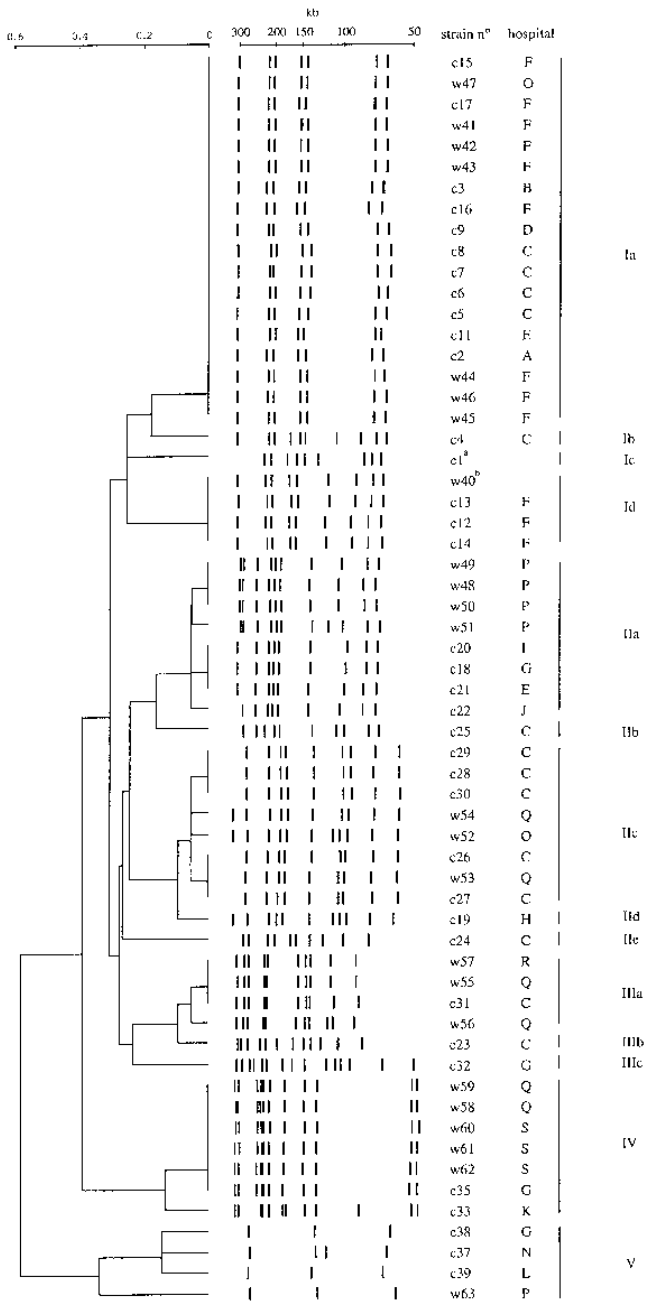

FIG. 3. Dendrogram and schematic representation of pulsed-field electrophoretic types for the *M. kansasii* isolates. Relationships between the *M. kansasii* strains were obtained by numerical analysis of the *Dra*I restriction fragments in the range of 50 to 300 kb (single-linkage clustering). The strain designations are those used in Tables 1 and 2. a, type strain; b, from home of patients from whom strains c12 to c14 were isolated.

strains including 3 clinical strains and 6 water isolates from set 2. The remaining strains consisted of another water isolate, three clinical isolates which were not epidemiologically related, and four strains from set 1 which were isolated at different time periods and from different medical units. Another clinical strain from set 2 constituted subgroup Ib. Subgroup Ic presented four fragment differences with the major subgroup Ia and contained the type strain. The Id subgroup was composed of the clinical strains from set 2 which were isolated from a single family and the water isolate collected at their home.

Cluster II contained 19 strains in five subgroups. Subgroup IIa contained four clinical isolates from patients attending four different hospitals and the set 4 water isolates. Subgroup IIc contained five clinical isolates, all from set 1, and three water isolates, with representatives of sets 3 and 5. Subgroups IIb, IId, and IIe each consisted of single clinical strains; strains from subgroups IIb and IId belonged to set 1.

Cluster III consisted of three clinical isolates and three water isolates and included three subgroups. Two clinical strains from set 1, isolated from patients attending different medical units, were found in subgroups IIIa and IIIb. Subgroup IIIc consisted of a clinical strain unrelated to any set. Water isolates collected in hospitals Q (set 5) and R were in subgroup IIIa. Strain c23, the only member of subgroup IIIb, was in MPTR probe cluster II. This strain was the only strain for which the results of the RFLP analysis with the MPTR probe and PFGE clustering methods disagreed.

Cluster IV was homogeneous and did not display subgroups. It consisted of five water isolates belonging to sets 5 and 6 and two clinical strains which did not belong to any set.

The banding pattern of the cluster V pattern differed from those of the other clusters by a small number of bands (less than or equal to four). Consequently, the internal relatedness within this cluster was lower than that within the other clusters: cluster V covered a relatedness distance of 0.4, whereas the other clusters were at a distance of 0.24. Cluster V consisted of one water isolate from set 4 and of three clinical isolates from three different hospitals.

AFLP analysis. From a set of primers which contained the common sequences of the adapters and a few arbitrary nucleotides on the 3′ extremities (27), we selected primers AMP2 and AMP4. These primers have 2 and 5 bases, respectively, that act as selective oligonucleotides at the 3′ end. They produced discriminant patterns for the 63 strains included in the study and yielded three to eight amplified products of 0.5 to 2.5 kb (Fig. 4).

The reproducibility of the AFLP method was examined by using 20 isolates belonging to epidemiologically related and unrelated strains. Similar patterns were repeatedly obtained for each strain, using independent DNA preparations. However, slight variations in faint bands were observed, probably due to different template DNA concentrations (data not shown).

We ordered the strains according to the clustering yielded by RFLP analysis with the MPTR probe (Fig. 4). Within each cluster except cluster II the fingerprints were very similar or even identical; cluster II presented a greater diversity of patterns. However, seven of the eight strains in PFGE subgroup IIc gave identical AFLP patterns. In each of clusters I, III, and IV, few strains presented patterns different from those shared by most strains. These differences did not in all cases match the PFGE subgroups. For example, strains c4 and c1 (type strain), classified as Ib and Ic by PFGE, fell into a single AFLP subgroup; on the other hand, strain w62, which displayed an AFLP pattern different from those of the other strains of cluster IV, was identical to other cluster IV strains by PFGE. The AFLP patterns within cluster V were very similar. The AFLP patterns of strains untypeable by PFGE were compared with the patterns obtained with the MPTR probe: the AFLP pattern of strain c10 differed from those of strains of cluster I, although strains c34 and c36, belonging to cluster IV, presented AFLP patterns closely related to those of the other members of this cluster. Strain c23, classified in cluster II by RFLP analysis with

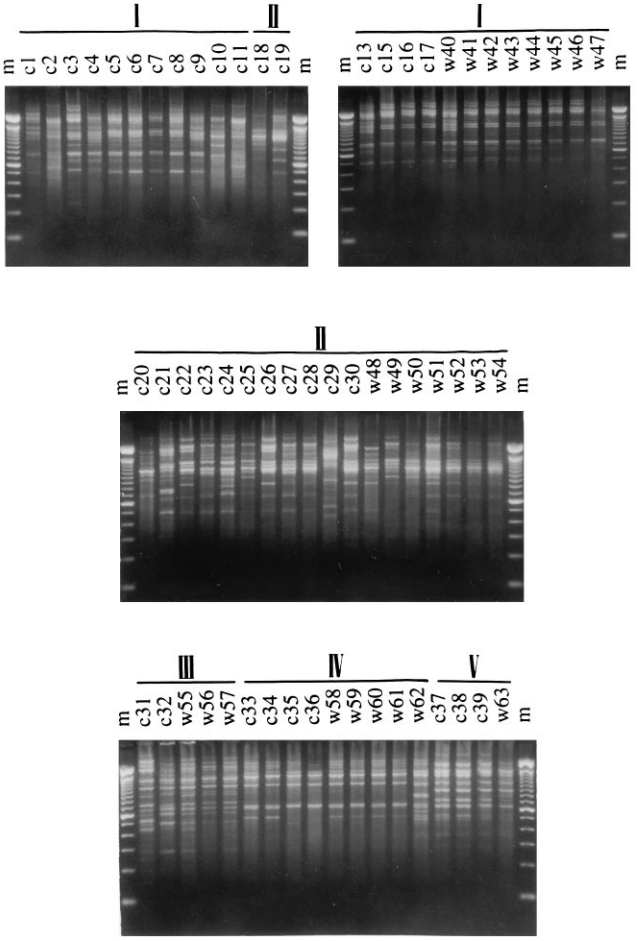

FIG. 4. AFLP patterns of the 63 *M. kansasii* isolates clustered according to RFLP analysis with the MPTR probe. Lane m, DNA molecular size markers consisting of 100-bp ladders.

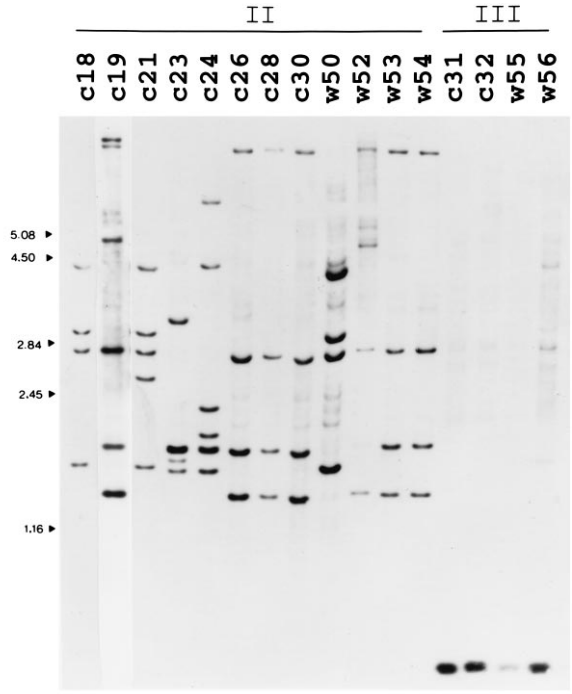

FIG. 5. Southern blot analysis of *Bst*EII-digested DNAs of *M. kansasii* isolates probed with IS*1652*. The molecular size marker (in kilobases) was bacteriophage lambda DNA restricted with *Pst*I.

the MPTR probe and in cluster III by PFGE, presented an AFLP pattern closer to that of members of cluster II.

RFLP analysis with the IS*1652* probe. Southern blot analysis with the IS*1652* probe revealed that this insertion sequence (IS)-like element was present in clusters II and III but could not be detected in clusters I, IV, and V (data not shown). Strains from cluster II carried four to six copies, giving polymorphic RFLP patterns (Fig. 5). Within this cluster, RFLP analysis with the IS*1652* probe showed polymorphism similar to that observed with PFGE, and the results of RFLP analysis with the IS*1652* probe were easier to interpret than those of AFLP analysis. The IS*1652* RFLP patterns correlated with subgroups IIa to IIe obtained by PFGE analysis. Thus, similar IS*1652* patterns, which differed by no more than one band, were displayed by strains from PFGE subgroups IIa (strains c18, c21, and c50) and IIc (strains c26, c28, c30, w52-w54) (Fig. 5). Among strains from patients hospitalized in the same medical unit, only strains c27 and c29 were found to be undistinguishable from each other (data not shown) and also from strains c28 and c30, whereas strains c23 and c24 gave different patterns (Fig. 5).

All strains belonging to cluster III contained a single copy carried by a single, small digestion fragment (Fig. 5). All isolates which contained IS*1652* were Accuprobe negative (Table 3). Moreover, RFLP analysis with the IS*1652* probe confirmed that strain c23 belonged to MPTR cluster II (data not shown).

PRA. PRA was performed with three or four strains from each of the five clusters. Within each cluster, PRA patterns were identical (data not shown). Figure 6 shows the PRA patterns generated by restriction with *Bst*EII and *Hae*III for representative isolates of each cluster and for the *M. gastri* type strain. Use of *Hae*III revealed five distinct patterns for *M. kansasii*, corroborating the clusters obtained by RFLP analysis with the MPTR probe. *Bst*EII was less discriminatory. Again, PRA indicated that strain c23 belonged to cluster II (data not shown).

Identification tests. Positive results by Accuprobe culture identification tests for *M. kansasii* (Accuprobe; Gen-Probe Inc.) were obtained for only 41% of the strains. The sensitivity was similarly low for both clinical and water isolates. No strain from clusters II to IV was positive with the Accuprobe system (Table 3).

To develop a PCR assay for the identification of *M. kansasii*,

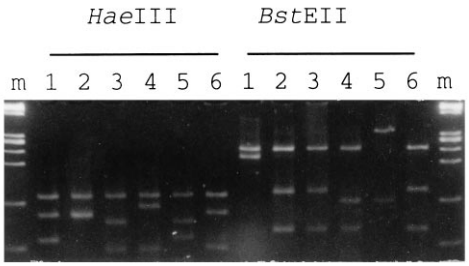

FIG. 6. PRA. Lanes: m, molecular size markers; 1 to 5, representative strains from clusters I to V, respectively (Table 3); 6, *M. gastri* type strain. The sizes of the restriction fragments are indicated in Table 3.

we selected primers K1 and K2, internal to the sequence of recombinant plasmid p6123 described by Yang et al. (33). All isolates were positive, yielding a single amplified product of 268 bp. However, some strains also gave a few faint additional bands (data not shown). These strains belonged to cluster IV, as described below (Table 3). PCR test results were confirmed to be true positive by Southern blot hybridization with the 268-bp amplified product probe on *Eco*RI-digested DNAs (data not shown). Interestingly, the K1-K2 PCR test was negative for the *M. gastri* type strain.

DISCUSSION

Comparison of fingerprinting methods. All the fingerprinting methods tested (RFLP analysis with the MPTR probe, PFGE, AFLP analysis, and PRA) showed excellent typeability and reproducibility.

IS*1652* was found only in clusters II and III (Table 3). For cluster II strains, which harbored several copies of the IS element, RFLP analysis with IS*1652* was more discriminatory than RFLP analysis with the MPTR probe. Although this IS-like element does not contain either a transposase open reading frame or inverted repeats, the polymorphism generated by IS*1652* within cluster II suggests that the element is mobile by transposition or that DNA rearrangement may occur. The relative motility of IS*1652* has already been suggested by comparison with another more stable sequence, polymorphic GC-rich repetitive sequence. Furthermore, the formation of a potential cointegrate has been described previously (32). In contrast, strains from cluster III did not reveal any polymorphism, and each harbored a single IS*1652* copy which may be unable to transpose (Fig. 5).

PFGE and AFLP analysis of *M. kansasii* are particularly useful because they reveal polymorphisms within each cluster. Mycobacterial strain characterization by PFGE has been previously demonstrated to be useful for epidemiological studies to trace sources of contamination and delineate outbreaks (4, 8, 16, 29, 34). Variations in the array of LRFs in both size and number may be due to sequence rearrangements, insertion or deletion of DNA, or base substitution within the restriction sites (25). However, 5% strains were untypeable by PFGE, consistent with PFGE studies applied to other mycobacterial strains (29). Moreover the PFGE protocol is laborious and time-consuming.

The rapidity of AFLP analysis makes it appropriate for preliminary screening, whereas PFGE remains the reference technique for strain characterization. Moreover, PFGE is more suitable for large-scale studies, because relationships between strains may be measured, allowing dendrograms to be drawn.

The five clusters and taxonomic considerations. The five methods used reveal independent polymorphisms (different restriction enzymes were selected for PFGE and AFLP analysis and for the MPTR probe method; the genes for the 65-kDa protein and IS*1652* are unrelated molecular markers). Nevertheless, the clusters indicated by each method were in agreement. This robust cluster delineation strongly suggests the existence of five distinct subspecies within *M. kansasii*. The different molecular markers provided different levels of strain characterization: the MPTR probe method and PRA defined subspecies, whereas AFLP analysis and PFGE, as well as RFLP analysis with the IS*1652* probe, when present, allowed recognition of subgroups within subspecies.

Cluster I corresponds to the typical *M. kansasii* isolates, and cluster II corresponds to the subspecies proposed by Ross et al. (21). Clusters III, IV, and V belong to previously poorly defined groups, especially cluster IV, which corresponds to strains negative by both Accuprobe testing and IS*1652* analysis. Clusters II and III may have a common, recent ancestor. The two clusters appear to be closer to each other than to the remaining clusters because they both contain the IS*1652* element and display the same *Bst*EII PRA pattern. Interestingly, cluster V produced PFGE profiles markedly different from those produced by the other clusters. Strains within this cluster yielded only three to four LRFs after genomic *Dra*I digestion, whereas an average of 7 to 15 LRFs were found for the other clusters. This may reflect large differences in the overall genomic structure. However, the use of *Xba*I led to profiles containing a number of LRFs, similar to those of other clusters (data not shown). Possibly, some *Dra*I sites in strains of cluster V are protected against cleavage, possibly by methylation, as demonstrated for *Pvu*II sites in *M. tuberculosis* (28).

The few fingerprinting patterns generated by RFLP analysis with the MPTR probe, PFGE, and AFLP analysis also suggest that the genotypic divergence between strains within each subspecies is relatively low. Nevertheless, strains isolated from patients from the same hospital or isolates recovered from the water distribution system of the same hospital were frequently in different clusters, indicating that the techniques are discriminatory enough for epidemiological purposes.

The study allowed us to evaluate the sensitivity and the specificity of various probes for *M. kansasii* identification. The Accuprobe test was positive for clusters I and V only, and IS*1652* was detected in clusters II and III only. In contrast, the K1-K2 PCR test detected all 63 strains included in the study, thus giving 100% sensitivity. The K1-K2 PCR test was negative for *M. gastri*, whereas the 16S rRNA sequences of *M. kansasii* and *M. gastri*, which is not clinically significant, are identical (20).

The high rate of isolation of Accuprobe-negative strains previously reported in Europe (12, 26, 33) was confirmed by our study, which shows that the frequency of Accuprobe-negative *M. kansasii* strains is high in France.

Epidemiologically related strains. Among the 63 *M. kansasii* isolates included in our study, we defined six sets of strains which were possibly epidemiologically related strains.

Set 1 included clinical isolates from 14 patients attending the same hospital where 5 patients from a single medical unit were infected with *M. kansasii* over a short period. Laboratory contamination or a nosocomial outbreak could therefore be suspected. However, PFGE studies revealed a high level of heterogeneity within this set: the 14 strains belonged to three subspecies and seven different subgroup types (Tables 1 and 2). Thus, the strains were unrelated, rejecting the hypotheses of laboratory contamination or dissemination of an epidemic strain. Only two patients hospitalized in the same medical unit were infected with strains with identical genomic patterns, as determined with the different molecular markers. However, epidemiological investigation of these patients did not identify a possible common source of infection not available to the other patients. That these two strains are identical cannot be strictly concluded: their profile identity may be due to the lack of polymorphism of the PFGE and AFLP analysis subgroup.

The fingerprinting of set 2 showed identical patterns for strains from patients from a single family living in the same household and also for a water isolate recovered from their home. Moreover, the other clinical and tap water isolates from set 2 belonged to another single PFGE and AFLP analysis subgroup. This indicates that the source of infection for the family was independent of the hospital and was linked to their home. In a previous study, such cases of infection among members of a family have been reported, but no mycobacteria could be detected from the home water supply (15).

The molecular markers showed that strains isolated from tap water at a single hospital (sets 3 to 6) were not all identical and that polyclonal colonization was observed in different hospitals (Table 2).

The presence of *M. kansasii* in hospital tap water may lead to a false-positive diagnosis of *M. kansasii* infection, especially if *M. kansasii* isolates are repeatedly recovered from patients (13, 19). Conventional techniques for mycobacterial isolation are usually sensitive enough, but the development of a PCR technique would simplify the tap water analysis. The K1-K2 PCR test is a good candidate for the development of a specific test for the detection of *M. kansasii* from water samples.

Conclusion. This study demonstrates that *M. kansasii* contains five subspecies. The subspecies were identified by independent molecular markers, some revealing polymorphisms that allowed further strain characterization. The markers contributed to identifying the epidemiological relationships between strains isolated from patients hospitalized in the same medical unit and from a single family. Strain typing revealed that polyclonal colonization of tap water could occur. Because strain typing showed high similarities for clinical and water isolates, this suggests that water may be the vehicle of transmission of *M. kansasii* to humans.

ACKNOWLEDGMENTS

We thank S. Dubrou from Laboratoire d'Hygiène de la Ville de Paris for supplying us with water isolates.

This study was financially supported in part by Sidaction. M.P. is the recipient of Association Nationale de Recherches sur le SIDA. G.P. received financial support from Centre Hospitalier Universitaire Vaudois, Ciba-Geigy-Jubiläums-Stiftung, and Merck-Sharp and Dohme-Gibret SA.

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