JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 1989, p. 2823–2826
0095-1137/89/122823-04$02.00/0

Vol. 27, No. 12

DNA Polymorphism in *Mycobacterium paratuberculosis*, "Wood Pigeon Mycobacteria," and Related Mycobacteria Analyzed by Field Inversion Gel Electrophoresis

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Received 30 May 1989/Accepted 12 September 1989

***Mycobacterium paratuberculosis* strains, mycobacteria from patients suffering from Crohn's disease, "wood pigeon mycobacteria," and representatives of *Mycobacterium avium-Mycobacterium intracellulare* were compared by restriction endonuclease *Dra*I digestion and field inversion gel electrophoresis. Characteristic profiles were seen for *M. paratuberculosis*, including isolates from patients suffering from Crohn's disease, for wood pigeon mycobacteria, and for *M. avium-M. intracellulare* serotypes 2, 16, 18, and 19. Two *M. paratuberculosis* strains used for vaccine production (St 18 and 316 F) presented patterns different from those of the other *M. paratuberculosis* strains. Strains St 18 yielded a pattern identical to that of the *M. avium* type strain serotype 2, whereas 316 F gave a unique pattern. The method developed in this study represents a useful taxonomic tool for the identification and classification of mycobacteria.**

DNA-DNA hybridization studies showed that *Mycobacterium avium*, *Mycobacterium paratuberculosis* (including isolates from patients with Crohn's disease), and "wood pigeon mycobacteria" belonged to a single genomic group (13, 23, 29, 30) not including *Mycobacterium intracellulare* (1, 2). A 383-base-pair segment of the gene coding for the 65-kilodalton mycobacterial antigen, consisting of a probe useful for the differentiation of tubercle bacilli from *M. avium* and from *Mycobacterium fortuitum*, revealed no nucleotide sequence differences between *M. paratuberculosis* and *M. avium* (12). Nonetheless, it is of clinical and veterinary importance to define subspecific differentiation in this group, as *M. avium* is a potential pathogen for animals and humans, especially in patients with acquired immunodeficiency syndrome (21, 22), and is frequently encountered in nature whereas *M. paratuberculosis* and wood pigeon mycobacteria have been isolated only from infected tissues (20, 26). Moreover, *M. paratuberculosis* may represent an etiologic agent of Crohn's disease (7).

The most striking bacteriological characteristic of *M. paratuberculosis* is mycobactin dependence. However, *M. avium-M. intracellulare* (MAI) isolates and wood pigeon mycobacteria may show a variable mycobactin dependence, often pronounced on primary isolation and attenuated on further subculturing. Assignment of a mycobactin-dependent strain to a specific taxon depends on the source of isolation and associated pathogenicity. Other criteria such as microbiological and biochemical tests, fatty acid composition, peptidoglycolipid content, and immunodiffusion analysis have not established a reliable correlation between phenotype and pathogenicity (3, 4, 10, 18, 24, 26).

Restriction endonuclease digestion profiles of the DNA from *M. avium* and *M. paratuberculosis* strains showed stringent conservation of genetic composition among strains of *M. paratuberculosis* and high heterogeneity within *M. avium* species (9, 27, 28). However, these electrophoretic analyses require the use of restriction enzymes with highly frequent cleavage sites. Patterns thus obtained may be difficult to interpret, as usually more than 50 closely spaced bands are produced. Hybridization with specific radioactively labeled probes results in simple patterns and allows analysis of restriction fragment length polymorphism. By using as probes either an rDNA gene of *Escherichia coli* or a cloned DNA fragment from an isolate obtained from a patient with Crohn's disease, Chiodini et al. and McFadden et al. confirmed the homogeneity of *M. paratuberculosis* and showed that isolates from patients suffering from Crohn's disease were strictly identical to *M. paratuberculosis* but distinct from *M. avium* (8, 15, 17). The difference between *M. paratuberculosis* and *M. avium* was mainly due to a repetitive sequence present in *M. paratuberculosis* but absent in MAI isolates (11).

In the present study, we have applied a modification of the technique originally described by Schwartz et al. (25), namely field inversion gel electrophoresis (6), to a fine-structure analysis of the restriction patterns of the DNA of *M. paratuberculosis*, wood-pigeon mycobacteria, and MAI isolates. As mycobacteria present a high GC% range, between 65 and 70%, we selected the *Dra*I restriction enzyme with a cleavage site AAATTT in order to get few DNA fragments. The method developed here is capable of the resolution of high-molecular-weight DNA fragments of mycobacteria and shows characteristic whole DNA patterns with few bands which can be used for strain identification without requiring the use of DNA probes.

MATERIALS AND METHODS

Bacterial strains. Table 1 lists the sources of the mycobacterial strains included in this study. Cultures were grown at 37°C in 7H9 Middlebrook liquid medium and were supplemented with 0.05% Tween 80 and 2 μg of mycobactin J per ml (Rhône-Mérieux, France). Four-week-old cultures (three-week-old cultures, for MAI isolates) were harvested

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TABLE 1. List of the strains used in the study

Organism	Source	Transmission[a]
M. paratuberculosis		
ATCC 19698	Type strain	CIPT
147-89	Goat	LCRV
316 F	Vaccine	Weybridge
St 18	Vaccine	CVM
7912	Cow	LCRV
3418	Cow	LCRV
1077	Sheep	CVM
2103	Goat	LCRV
Wood pigeon mycobacteria		
V1-72	Deer	SVSL
3135	Wood pigeon	LCRV
6861	Wood pigeon	LCRV
6409	Wood pigeon	LCRV
M. avium-M. intracellulare		
ATCC 25291 (serotype 2)	Type strain	CIPT
ATCC 13950 (serotype 16)	Type strain	CIPT
O'Connor 4990 (serotype 18)		NJH
W-552 (serotype 19)		NJH
Crohn's disease isolates		
CD 2569	Human	CDI
CD Lyon	Human	EVL

[a] Abbreviations: LCRV, Laboratoire Central de Recherches Vétérinaires, Maisons-Alfort, France; CVM, College of Veterinary Medicine, Ames, Iowa; SVSL, State Veterinary Serum Laboratory, Copenhagen, Denmark; CIPT, Collection Institut Pasteur Tuberculose, Paris, France; NJH, National Jewish Hospital, Denver, Colo.; CDI, Centraal Diergeneeskundig Instituut, Lelystad, The Netherlands; EVL, Ecole Vétérinaire de Lyon, Lyon, France.

by centrifugation at 8,000 × *g*, and bacterial pellets were stored frozen at −20°C.

DNA preparation. For this purpose, fresh cultures were prepared. Cells were suspended in 40 ml of fresh medium at an optical density at 650 nm of 0.05. The cultures were incubated to an optical density at 650 nm of 0.2, centrifuged at 7,000 × *g*, and washed with 50 mM EDTA (pH 8). Cell pellets were suspended in 1 ml of a prelysing solution consisting of 6 ml of 50 mM EDTA (pH 8), 6 ml of 10 mM EDTA (pH 8), 0.1 M sodium citrate, 150 μl of β-mercaptoethanol, and 5 mg of zymolyase (Seikagaku Kogyo, Tokyo, Japan). Cell suspensions were then mixed with an equal volume of 1% low-melting-point agarose (Bio-Rad Laboratories, Richmond, Calif.) prepared in 125 mM EDTA (pH 8) that had been melted and cooled at 45°C. The mixture was dispensed immediately in a slot former containing 60-μl spaces. Agarose blocks were transferred to tubes with 0.5 M EDTA containing 7.5% β-mercaptoethanol and were incubated overnight at 37°C. Agarose blocks were extensively washed with TE (10 mM Tris, 1 mM EDTA, pH 8) and incubated in 10 mM Tris–1 mM EDTA with 1 mg of lysozyme per ml for 5 h at 37°C. The solution was changed to 0.5 M EDTA containing 1% sodium lauroyl sarcosine (Sigma Chemical Co., St. Louis, Mo.) and 2 mg of proteinase K (Boehringer Gmbh, Mannheim, Federal Republic of Germany) per ml, allowed to stand for 48 h at 55°C, and washed with TE. Proteinase K was inactivated by washing the inserts twice for 30 min at 55°C in TE plus 0.04 mg of phenylmethylsulfonyl fluoride per ml (Bio-Rad Laboratories) and three times for 30 min each at room temperature in TE alone. After this, agarose blocks could be stored at 4°C in 0.5 M EDTA. They were extensively washed with TE before restriction enzyme digestion.

Restriction enzyme digestion. Blocks containing about 10 μg of DNA were digested with 50 U of *Dra*I (Bio-Rad Laboratories) in a total volume of 120 μl containing 75 μl of distilled water, 20 μl of bovine serum albumin (Pharmacia, Inc., Piscataway, N.J.), and 20 μl of appropriate buffer as recommended by the manufacturer.

Pulsed-field gel electrophoresis. Blocks were loaded into a 1% agarose (Bio-Rad Laboratories) gel, prepared, and run in 0.0587 M Tris (pH 8)–0.0587 M boric acid–0.0013 M EDTA (0.66× TBE). The inserts were then sealed in the slots with 1% low-melting-point agarose in 0.66× TBE. Field inversion gel electrophoresis was carried out with a Dnastar Pulse (Dnastar, Madison, Wis.) apparatus. Forward and reverse pulses were set at 0.33 and 0.11 s at the beginning of the run and 60 and 20 s at the end of the run, respectively, according to a linear ramp of forward and reverse pulses during the 36-h run time. The voltage used was 100 V and produced about 35 mA. The buffer was allowed to recirculate through a heat exchanger to maintain 18°C. Lambda concatemers were used as molecular weight markers. At the end of the run, the gels were stained with ethidium bromide and photographed under UV light.

RESULTS

Figure 1 shows the *Dra*I restriction patterns of the different strains listed in Table 1 compared to those of λ concatemers. As the molecular weight of a single phage λ is about 50 kilobases, the upper limit of resolution equivalent to 14 concatemerized λ phages corresponds roughly to 700 kilobases.

Figure 1 shows that different strains of *M. paratuberculosis* (Fig. 1A, lanes 1, 3, 7, and 9; Fig. 1B, lanes 1, 5, and 6; Fig. 1C, lane 3), including the type strain ATCC 19698 (data not shown), produce similar patterns except for three strains, namely, St 18 and 316 F, both used for vaccine production, and strain 2103. The five other strains tested show identical profiles different from the MAI type strains (Fig. 1B, lanes 2 and 3; Fig. 1C, lanes 1 and 2), as well as *M. intracellulare* serotypes 18 and 19 (Fig. 1A, lanes 4 and 8). In addition, strains isolated from patients suffering from Crohn's disease (Fig. 1A, lane 6; Fig. 1C, lanes 9 and 10) were found to be indistinguishable from the other *M. paratuberculosis* strains isolated from infected animals.

The two *M. paratuberculosis* strains used for vaccine production yielded restriction patterns differing from the other *M. paratuberculosis* strains. It is interesting to note that the St 18 fingerprint is similar to the *M. avium* type strain (serotype 2) (Fig. 1C, lanes 2 and 3). However, the other vaccine strain, *M. paratuberculosis* 316 F (14), yielded a unique pattern.

M. paratuberculosis 2103, isolated from a goat lymph node, was identified as a *M. paratuberculosis* strain on the basis of mycobactin dependence seen on first isolation. However, on subculture the strain lost its mycobactin dependence and became able to grow without the addition of mycobactin. In addition, the strain was rough on first isolation and smooth on subsequent cultures. The pulsed-field pattern did not allow the identification as a *M. paratuberculosis* strain, and the misidentification underlines the difficulties of identification relying on usual microbiological tests.

Wood pigeon mycobacteria produced restriction fingerprints (Fig. 1A, lane 2; Fig. 1C, lanes 4, 5, 7, and 8) which allowed their differentiation from *M. paratuberculosis* strains, including those isolated from patients with Crohn's

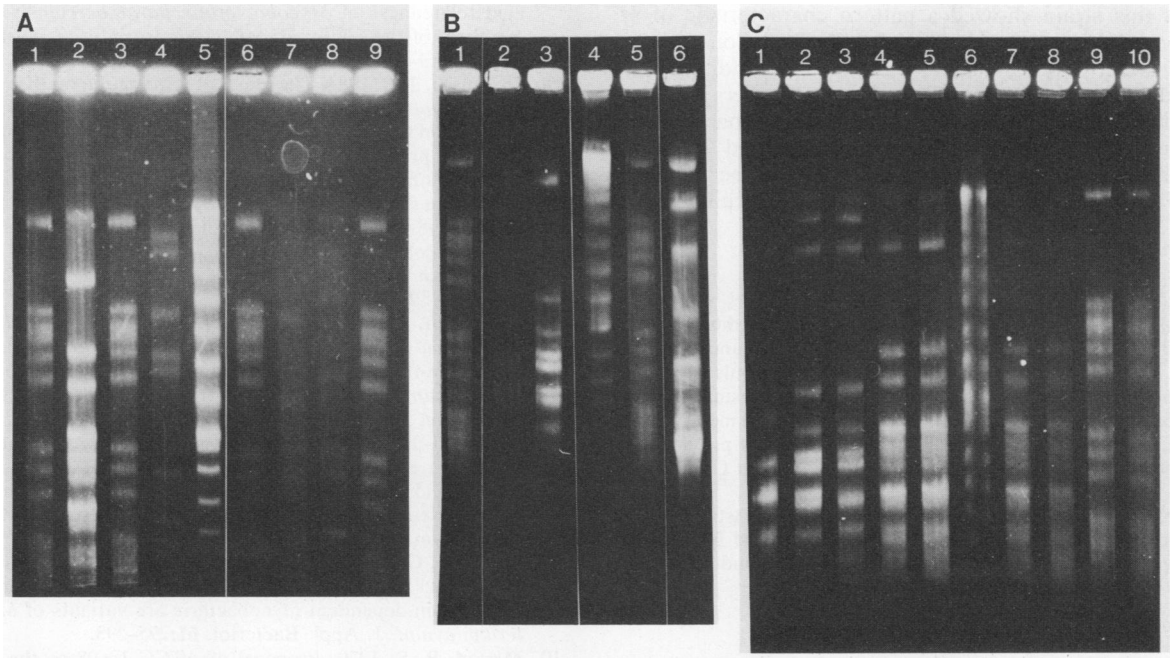

FIG. 1. Pulsed-field gel electrophoresis of mycobacterial DNA digested by *Dra*I. (A) Lanes: 1, *M. paratuberculosis* 7912; 2, wood pigeon strain 6409; 3, *M. paratuberculosis* 1077; 4, *M. avium* serotype 18; 5, λ concatemers; 6, Crohn's disease strain isolated in Lyon; 7, *M. paratuberculosis* 2103; 8, *M. avium* serotype 19; 9, *M. paratuberculosis* 3418. (B) Lanes: 1, *M. paratuberculosis* 147-89; 2, *M. intracellulare* ATCC 13950; 3, *M. avium* ATCC 25291; 4, λ concatemers; 5, *M. paratuberculosis* 1077; 6, *M. paratuberculosis* 316 F. (C) Lanes: 1, *M. intracellulare* ATCC 13950; 2, *M. avium* ATCC 25291; 3, *M. paratuberculosis* St 18; 4, wood pigeon strain 6861; 5, wood pigeon strain 6409; 6, λ concatemers; 7, wood pigeon strain 3135; 8, wood pigeon strain V1-72; 9, Crohn's disease isolate 2569; 10, Crohn's disease strain isolated in Lyon.

disease, and from serovars of MAI. The homogeneity of wood pigeon mycobacteria was obvious, as all strains gave the same pattern.

Serotypes 2, 16, 18, and 19 of MAI gave different patterns. None of them could be related to those obtained with wood pigeon mycobacteria or *M. paratuberculosis* strains.

DISCUSSION

Pulsed-field electrophoretic restriction patterns shown in the present study confirm analyses of restriction fragment length polymorphism (8, 15, 17), as identical profiles were found for *M. paratuberculosis* strains and mycobacteria isolated from patients with Crohn's disease. Furthermore, this technique demonstrated the homogeneity of wood pigeon mycobacteria. Both groups gave definite and peculiar pulsed-field profiles allowing their differentiation from each other and from MAI serotypes. As shown by the study of strain 2103, *M. paratuberculosis* strains could be easily identified by their pulsed-field profiles, which helped to detect possible misidentification according to the results of microbiological and biochemical tests.

Phenotypic methods investigated were not capable of differentiating *M. paratuberculosis* from wood pigeon mycobacteria or both groups from MAI. Fatty acid gas chromatography allowed Damato et al. to detect an unidentified peak in *M. paratuberculosis* extracts which was absent in MAI strains (10). However, Saxegaard et al. showed only minor quantitative differences in the gas chromatograms of *M. paratuberculosis* and MAI and no species-specific peak could be demonstrated (24). MAI, wood pigeon mycobacteria, and *M. paratuberculosis*, which had lost their original mycobactin dependence, were found to be closely related according to the chromatographic properties of their mycobactins (3). Immunodiffusion analysis of *M. paratuberculosis* and wood pigeon mycobacteria showed slight but significant differences (18, 26). However, all strains were found antigenically identical with MAI, as patterns observed corresponded to the two immunodiffusion types demonstrated for MAI (18). Whereas other criteria showed high homogeneity among *M. paratuberculosis* strains, peptidoglycolipid analysis revealed some heterogeneity. Most isolates did not synthesize any peptidoglycolipid, but about 10% of the strains contained the type-specific antigen of *M. avium* serotype 8 (4). Camphausen et al. described a new trehalose-containing lipooligosaccharide from a single *M. paratuberculosis* strain isolated from a patient with Crohn's disease (5). The distribution and occurrence of this glycolipid antigen has to be further evaluated, as it could provide a useful marker for precise identification of *M. paratuberculosis* strains.

In the present study, all the *M. paratuberculosis* strains were found to be identical except the two strains used for vaccine production. Strain St 18 (ATCC 12227) was considered as a working type of *M. paratuberculosis* until Merkal proposed ATCC 19698 as a neotype (19). Merkal's proposal was motivated by the atypical properties of St-18, including rapid and profuse growth, mycobactin production, and absence of pathogenicity for calves. Our study unambiguously shows identity between St 18 and the *M. avium* type strain and thus confirmed previous analyses of restriction fragment patterns by conventional electrophoresis and serotype-specific peptidoglycolipid characterization (4, 16, 28). Pulsed-field electrophoresis revealed that *M. paratuberculosis* strain 316 F (14) differed from the other *M. paratuberculosis* strains,

whereas this strain showed a pattern characteristic of *M. paratuberculosis* when examined by restriction enzyme analysis under only forward electrophoresis conditions (9).

In conclusion, the method described here offers the same sensitivity as DNA probes examining restriction fragment length polymorphisms. However, this method does not require radioactively labeled molecules and can be applied to the study of mycobacterial groups for which specific probes are not available.

ACKNOWLEDGMENTS

We gratefully thank J. Davies and H. L. David for their constant interest during this study and critical reading of the manuscript. We thank B. Canard and C. Petit for helpful advice in pulsed-field gel electrophoresis conditions. We are grateful to J. Haagsma, Centraal Diergeneeskundig Instituut, Lelystad, The Netherlands, and F. Perard, Ecole Vétérinaire de Lyon, Lyon, France, for providing *M. paratuberculosis* strains from patients suffering from Crohn's disease.

This investigation received financial support from Institut Pasteur and the United Nations Development Program-World Bank-World Health Organization Special Program for Research and Training in Tropical Diseases.

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