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Restriction Fragment Length Polymorphism of rRNA Genes for Molecular Typing of Members of the Family *Legionellaceae*

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Typing of *Legionella pneumophila* remains important in the investigation of outbreaks of Legionnaires' disease and in the control of organisms contaminating hospital water. We found that the discriminatory power of a nonradioactive ribotyping method could be improved by combining results obtained with four restriction enzymes (*Hin*dIII, *Nci*I, *Cla*I, and *Pst*I). Fifty-eight clinical and environmental *L. pneumophila* strains including geographically unrelated as well as epidemiologically connected isolates were investigated. Epidemiologically related strains had the same ribotypes independent of the combinations of enzymes used. Some strains belonging to the same serogroup were assigned to different ribotypes, and some ribotypes contained members of different serogroups, indicating, as others have found, that serogroup and genotype are not always related. The discriminatory power of the method was estimated by calculating an index of discrimination (ID) for individual enzymes and combinations thereof. The combined result with all four enzymes was highly discriminatory (ID = 0.97), but results for three enzymes also yielded ID values acceptable for epidemiological purposes. In addition, the testing of 27 type strains and 6 clinical isolates representing *Legionella* species other than *L. pneumophila* indicated that ribotyping might be of value for species identification within this genus, as previously suggested.

Several members of the family *Legionellaceae* can cause the life-threatening illness known as Legionnaires' disease. In outbreaks of Legionnaires' disease, the typing of clinical and environmental strains is important in locating the source of the infection (31). Various typing methods have also been used in an effort to differentiate between presumed virulent and avirulent *Legionella* strains and to define specific virulence markers (30). The development of standard typing schemes, mainly by use of international panels of monoclonal antibodies (19), has been limited to that of schemes for *Legionella pneumophila* serogroup 1 (SG 1), which is the most frequently isolated etiologic agent of Legionnaires' disease, accounting for more than 50% of cases. Phenotypic characteristics such as monoclonal subtype, however, have been shown to be less stable than originally considered (14, 24, 31). To develop new typing methods independent of phenotypic variation, methods based on the investigation of genomic DNA are an obvious choice. Furthermore, DNA typing has the advantage of not being limited for use with *L. pneumophila* SG 1 but is applicable to any serogroup or *Legionella* species.

In the present study, ribotyping by a nonradioactive procedure (9) was applied to a collection of *L. pneumophila* strains, including geographically unrelated as well as epidemiologically connected isolates from Denmark and abroad, in an effort to estimate and optimize the discriminatory power of the method. Another major goal of the study was to investigate the use of ribotyping for differentiation of *Legionella* species other than *L. pneumophila*, since this is known to be a difficult task.

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MATERIALS AND METHODS

Strains. A total of 91 *Legionella* strains were investigated. Fifty-eight clinical and environmental *L. pneumophila* strains (29 from SG 1 and 29 from serogroups other than SG 1) derived from various places in and outside of Denmark as listed in Tables 1 and 2 were investigated. Four *L. pneumophila* SG 6 strains and seven *L. pneumophila* SG 1 strains, however, were epidemiologically related; i.e., they were corresponding patient and environmental isolates from the cardiopulmonary transplant unit at Rigshospitalet, Copenhagen, Denmark. Species other than *L. pneumophila* that were investigated included 27 type strains and 6 clinical isolates. Type strains were American Type Culture Collection numbers for *L. micdadei*, *L. dumoffii*, *L. gormanii*, *L. bozemanii*, *L. anisa*, *L. birminghamensis*, *L. cherrii*, *L. cincinnatiensis*, *L. erythra*, *L. feeleii*, *L. gratiana*, *L. hackeliae*, *L. israelensis*, *L. jamestowniensis*, *L. jordanis*, *L. longbeachae*, *L. maceachernii*, *L. moravica*, *L. oakridgensis*, *L. parisiensis*, *L. quinlivanii*, *L. rubrilucens*, *L. sainthelensi*, *L. spiritensis*, *L. steigerwaltii*, *L. tucsonensis*, and *L. wadsworthii*. The clinical strains were three *L. micdadei*, one *L. dumoffii*, and two *L. bozemanii* isolates.

Serotyping of *L. pneumophila* strains. Serotyping by direct immunofluorescence with polyclonal serogroup-specific antisera as well as by coagglutination was performed at the Mycoplasma Laboratory, Statens Seruminstitut, Copenhagen, Denmark.

DNA methods. The isolation of DNA was performed with 48-h ACES [*n*-(2-acetamido)-2-aminoethanesulfonic acid] buffer–charcoal–yeast extract–α-ketoglutarate plate cultures and followed a standard procedure (25). The purified DNA was dried and dissolved in 200 μl of TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]). Approximately 1 μg of chromosomal DNA was digested with the restriction enzymes *Hin*dIII, *Nci*I, *Cla*I, and *Pst*I (Boehringer, Mannheim, Germany) following the instructions of the manufacturer.

These four enzymes were chosen from among several others tested on a limited number of strains yielding the clearest patterns and optimal typeability. Furthermore, *Cla*I and *Nci*I have previously been reported to be useful for restriction enzyme analysis of *Legionella* strains (1, 13, 26). Electrophoresis in a 0.7% agarose gel overnight at 50 V in TAE buffer (40 mM Tris-acetate, 1 mM EDTA [pH 8.0]) was followed by Southern blotting by means of rapid vacuum transfer (Bio-Rad, Richmond, Calif.) onto nylon membranes (Zetaprobe; Bio-Rad) with 0.5 M NaOH–0.6 M NaCl as the transfer solution (9). Phage lambda (λ) DNA cut with *Hin*dIII and *Sty*I was used as a molecular size marker.

The digoxigenin-labeled cDNA probe derived from 16S and 23S *Escherichia coli* rRNA was prepared by reverse transcription and random priming with Moloney murine leukemia virus reverse transcriptase (Gibco/BRL, Life Technologies, Copenhagen, Denmark) (5). Prehybridization, hybridization (at 60°C overnight), and development of the nylon membrane blots were done according to the instructions of the manufacturer (DIG DNA Labeling and Detection

TABLE 1. *L. pneumophila* SG 1 strains

Strain	Designation for this study	Source[a]
Lp 1 Philadelphia 1	104	ATCC 33152
Lp 1 Knoxville 1	134	ATCC 33153
Lp 1 HV	106	CI, West Indies, USA
Lp 1 PHLS	108	CI, London, UK
Lp 1 PS	110	CI, Holbæk, Denmark
Lp 1 AA	132	CI, Copenhagen, Denmark
Lp 1 HR	37879	CI, Bordeaux, France
Lp 1 Halifax 1	145	CI, Halifax, Canada
Lp 1 Halifax 2	146	CI, Halifax, Canada
Lp 1 JVM	13909	CI, Herlev, Denmark
Lp 1 RC	13901	CI, Hillerød, Denmark
Lp 1 EØ	1958	CI, Odense, Denmark
Lp 1 BJ	2434	CI, Odense, Denmark
Lp 1 JØH	7177	CI, Århus, Denmark
Lp 1 SAN	12419	CI, Hillerød, Denmark
Lp 1 JPEK	210130	CI, Copenhagen, Denmark
Lp 1 AHL	9020	CI, Næstved, Denmark
Lp 1 EBH	11526	CI, Herning, Denmark
Lp 1 IR	L454	CI, Copenhagen, Denmark
Lp 1 PBC	L437	CI, Copenhagen, Denmark
Lp 1 RH 2408	L469	EI, Copenhagen, Denmark
Lp 1 RH 2408-I	L472	EI, Copenhagen, Denmark
Lp 1 RH 2408-II	L473	EI, Copenhagen, Denmark
Lp 1 RH 2408-III	L474	EI, Copenhagen, Denmark
Lp 1 RH 2408-IV	L475	EI, Copenhagen, Denmark
MM	13573	CI, Odense, Denmark
LS	15634	CI, Køge, Denmark
FBB	13013	CI, Hillerød, Denmark
KR	13631	CI, Copenhagen, Denmark

[a] CI, clinical isolate; EI, environmental isolate; USA, United States of America; UK, United Kingdom.

TABLE 2. *L. pneumophila* strains from serogroups other than SG 1

Strain	Designation for this study	Source[a]
Lp 2 Togus 1	112	ATCC 33154
Lp 3 Bloomington 2	120	ATCC 33155
Lp 3 KEJ	753901	CI, Ålborg, Denmark
Lp 3 GA	13935	CI, Hvidovre, Denmark
Lp 4 Los Angeles 1	131	ATCC 33156
Lp 4 L171/87	140	CI, Berlin, Germany
Lp 4 KTH 601	138	EI, Copenhagen, Denmark
Lp 4 JS	13419	CI, Herlev, Denmark
Lp 5 Dallas 1E 122	122	ATCC 33216
Lp 5 Cambridge 2	175	NCTC 11417
Lp 6 RJ	109	CI, Hvidovre, Denmark
Lp 6 JP	49287	CI, Bornholm, Denmark
Lp 6 FH	139	CI, Frederiksberg, Denmark
Lp 6	14835	CI, Frederiksberg, Denmark
Lp 6 RH 2397	L476	EI, Copenhagen, Denmark
Lp 6 RH 2398	L477	EI, Copenhagen, Denmark
Lp 6 RH 2399	L478	EI, Copenhagen, Denmark
Lp 6 NPM	L404	CI, Copenhagen, Denmark
Lp 7 Chicago 8		ATCC 35096
Lp 7	13889	EI, Copenhagen
Lp 8 Concord 3		ATCC 35096
Lp 9 IN-23-G1-C2		ATCC 35289
Lp 10 Leiden 1		ATCC 43283
Lp 11 797-PA-H		ATCC 43130
Lp 12 570-CO-H		ATCC 43290
Lp 13 Seattle 1		ATCC 43736
Lp 14 1169 MN-H		ATCC 43703
Lp Lansing 3	111	CI, Lansing, Mich., USA
Lp	3303	CI, Roskilde, Denmark

[a] CI, clinical isolate; EI, environmental isolate; USA, United States of America.

Non-Radioactive Applications; Boehringer). The color reaction usually took place for 2 to 3 h and was stopped by washing the membrane in tap water.

The bands were scored visually in relation to the reference types run on each gel, and the apparent fragment molecular weights were calculated for each separate lane. The reproducibility of the method was estimated for each restriction enzyme by observing the day-to-day variation in apparent molecular weights of visualized DNA restriction fragments from 10 strains assigned to the same subtype.

An index of discrimination (ID) for the individual enzymes and for combinations thereof was calculated according to the method of Hunter and Gaston (17). These calculations were based on the ribotyping results from the epidemiologically unrelated strains.

RESULTS

All 58 *L. pneumophila* strains as well as all of the 33 strains other than *L. pneumophila* could be digested with the enzymes used, resulting in a typeability of 100%. The day-to-day maximum variations in molecular weight determinations ranged from 3.4% (*Nci*I) to 9.0% (*Cla*I). For *L. pneumophila* strains, restriction with *Hin*dIII resulted in three to five bands in the range of 3 to 13 kb, with the exception of *L. pneumophila* SG 10, which yielded seven bands. On the basis of the positioning of the bands, 10 different *Hin*dIII ribotypes were defined (H1 to H10) (Table 3). *L. pneumophila* SG 1 was divided into seven clusters, five of which included other serogroups. Three of the *Hin*dIII ribotypes included only one isolate (H3, H9, and H10); the remaining seven types included 2 (H7) to 18 (H5) strains. Epidemiologically related strains (Tables 1 and 2) were grouped within the same ribotype.

Cutting with *Nci*I yielded a further subdivision of types H1 to H7, resulting in 20 combined ribotypes. After subsequent restriction with *Pst*I, the number of composite ribotypes increased to 26. Examples of representative *Pst*I ribotypes are shown in Fig. 1. The use of all four restriction enzymes yielded three more groups, bringing the total number of composite ribotypes to 29, with 1 to 11 isolates in each group. Some individual as well as composite ribotypes contained geographically distant isolates, but for the composite ribotypes, epidemiologically related strains were assigned to the same group. Thus, the largest group of 11 isolates (composite ribotype H6N3C3P1) consisted of the 7 *L. pneumophila* SG 1 environmental and patient strains from Rigshospitalet and 4 apparently unrelated clinical isolates. Two of the four strains, however, were from patients at the same hospital. One ribotype, H8N7C7P7, contained only the epidemiologically related *L. pneumophila* SG 6 strains. Cutting with one of the enzymes, *Hin*dIII, *Nci*I, or *Pst*I, also separated these strains from all others. Calculations of the ID values for individual enzymes and for combinations of enzymes are shown in Table 4. The ID expresses the probability that two epidemiologically unrelated strains will be placed in separate typing groups by the typing method applied, i.e., the closer to 1 (100%), the better the discriminatory power of the method. The ID values for typing with individual enzymes ranged from 0.80 to 0.85, and combining results from two enzymes gave ID values of 0.90 to 0.93. The highest ID value was obtained by combining results from typing with all four enzymes (ID = 0.97), but combining the results from typing with three enzymes allowed us to reach almost the same level (ID = 0.95 to 0.96).

For species other than *L. pneumophila*, a distinct restriction pattern unique to the individual species could be identified (for an example, see Fig. 2) with all four enzymes. The three clinical

TABLE 3. Distribution of *L. pneumophila* strains according to ribotype

*Hin*dIII ribotype	Composite ribotype[a]	Isolate (designation for this study)
H1	H1N1C1P1	Lp 1 Philadelphia (104)
		Lp 1 PHLS (108)
		Lp 12 570 CO-H
	H1N4C1P1	Lp 1 EØ (1958)
	H1N5C1P5	Lp 2 Togus (112)
H2	H2N2C2P2	Lp 1 HV (106)
		Lp 5 Dallas 1E
		Lp Lansing 3
	H2N6C2P2	Lp 4 Los Angeles 1
H3	H3N3C3P3	Lp 1 PS (110)
H4	H4N3C3P1	Lp 1 AA (132)
		Lp 1 Halifax 1 (145)
		Lp 1 Halifax 2 (146)
		Lp 1 JPEK (210130)
		Lp 1 EBH (11526)
	H4N3C3P3	Lp 1 KR (13631)
	H4N4C6P9	Lp 1 BJ (2434)
	H4N5C6P9	Lp (3303)
H5	H5N1C5P4	Lp 1 HR (37879)
		Lp 4 L171/87 (140)
	H5N1C4P4	Lp 7 Chicago 8
	H5N1C8P10	Lp 7 13889
	H5N3C5P6	Lp 4 KTH 601 (138)
	H5N3C7P6	Lp 6 JP (49284)
	H5N4C4P4	Lp 1 Knoxville (134)
		Lp 3 Bloomington (120)
		Lp 3 KEJ (753901)
		Lp 5 Cambridge 2 (175)
	H5N6C5P6	Lp 6 RJ (109)
		Lp 6 FH (139)
		Lp 6 (14835)
	H5N6C5P4	Lp 14 1169 MN-H
	H5N6C7P4	Lp 1 RC (13901)
	H5N6C7P6	Lp 1 MM (13573)
		Lp 4 JS (13419)
		Lp 3 GA
H6	H6N3C3P1	Lp 1 IR (L454)[b]
		Lp 1 PBC (L437)[b]
		Lp 1 RH 2408 (L469)[b]
		Lp 1 RH 2408-I (L472)[b]
		Lp 1 RH 2408-II (L473)[b]
		Lp 1 RH 2408-III (L474)[b]
		Lp 1 RH 2408-IV (L475)[b]
		Lp 1 SAN (12419)
		Lp 1 LS (15634)
		Lp 1 FBB (13013)
		Lp 9 In-23-G1-C2
	H6N3C3P3	Lp 1 JØH (7177)
	H6N3C3P13	Lp 13 Seattle 1
	H6N9C3P14	Lp 1 AHL (9020)
H7	H7N3C9P8	Lp 1 JVM (13909)
	H7N4C11P12	Lp 11 797-PA-H
H8	H8N7C7P7	Lp 6 RH 2397 (L476)[b]
		Lp 6 RH 2398 (L477)[b]
		Lp 6 RH 2399 (L478)[b]
		Lp 6 NPM (L404)[b]
H9	H9N8C3P11	Lp 8 Concord 3
H10	H10N1C10P1	Lp 10 Leiden 1

[a] The letters H, N, C, and P relate to typing with restriction enzymes *Hin*dIII, *Nci*I, *Cla*I, and *Pst*I, respectively.
[b] Epidemiologically related strains.

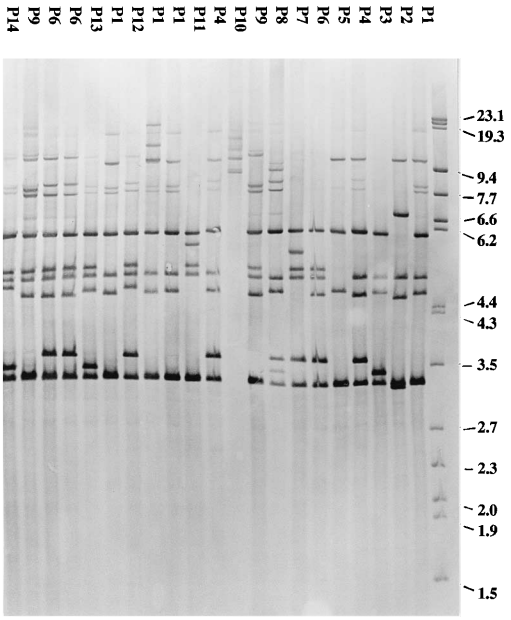

FIG. 1. Examples of *L. pneumophila* ribotypes (P1 to P14) obtained by digestion with *Pst*I. Molecular size markers from λ DNA cut with *Hin*dIII and *Sty*I are given at the right in kilobase pairs.

isolates of *L. micdadei*, two clinical isolates of *L. bozemanii*, and one clinical strain of *L. dumoffii* had restriction patterns identical to those of the respective type strains.

DISCUSSION

Various methods for the typing of members of the *Legionellaceae*, mainly subtyping of *L. pneumophila* SG 1, have been reported in the past and include monoclonal antibody reactivity (19, 21), outer membrane profiles (8, 30), multilocus enzyme electrophoresis (29), antibiotic susceptibility (34), plasmid analysis (18, 30), restriction enzyme analysis with conventional or pulsed-field gel electrophoresis (20, 21), and, more recently, analysis of randomly amplified DNA fragments (10). Ribotyping, originally suggested as a method for species differentiation (11, 12), has been demonstrated to be useful also for epidemiological purposes for several bacteria other than members of the *Legionellaceae* (4, 9) and has the considerable

TABLE 4. ID values calculated with individual enzymes and combinations of enzymes

Enzyme(s)	ID value
*Hin*dIII	0.80
*Nci*I	0.81
*Cla*I	0.85
*Pst*I	0.85
*Hin*dIII + *Nci*I	0.93
*Hin*dIII + *Cla*I	0.91
*Hin*dIII + *Pst*I	0.91
*Nci*I + *Cla*I	0.90
*Nci*I + *Pst*I	0.93
*Cla*I + *Pst*I	0.93
*Hin*dIII + *Nci*I + *Cla*I	0.95
*Hin*dIII + *Nci*I + *Pst*I	0.96
*Hin*dIII + *Cla*I + *Pst*I	0.95
*Nci*I + *Cla*I + *Pst*I	0.95
*Hin*dIII + *Nci*I + *Cla*I + *Pst*I	0.97

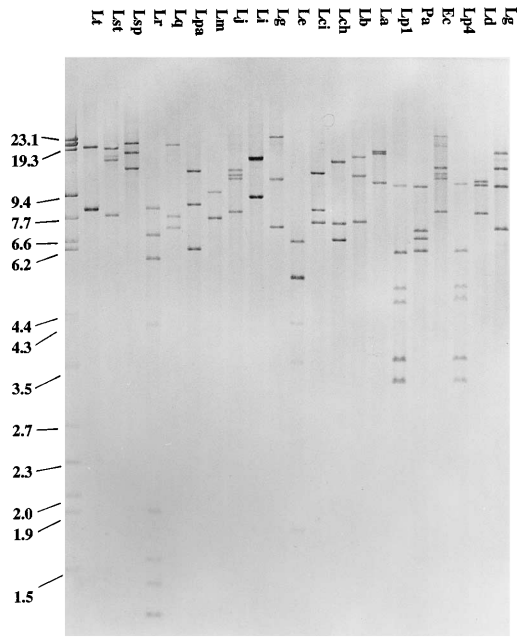

FIG. 2. Examples of ribotypes of *Legionella* species obtained by digestion with *Pst*I. Lg, *L. gormanii* (ATCC 33297); Ld, *L. dumoffii* (ATCC 33279); Lp4, *L. pneumophila* SG 4 (L171/87); Lp1, *L. pneumophila* SG 1 Knoxville 1 (ATCC 33153); La, *L. anisa* (ATCC 35292); Lb, *L. birminghamensis* (ATCC 43702); Lch, *L. cherrii* (ATCC 35252); Lci, *L. cincinnatiensis* (ATCC 43753); Le, *L. erythra* (ATCC 35303); Lg, *L. gratiana* (ATCC 49413); Li, *L. israelensis* (ATCC 43119); Lj, *L. jamestowniensis* (ATCC 35298); Lm, *L. moravica* (ATCC 43877); Lpa, *L. parisiensis* (ATCC 35299); Lq, *L. quinlivanii* (ATCC 43830); Lr, *L. rubrilucens* (ATCC 35304); Lsp, *L. spiritensis* (ATCC 35249); Lst, *L. steigerwaltii* (ATCC 35302); Lt, *L. tucsonensis* (ATCC 49180). Two random clinical isolates of *E. coli* (Ec) and *P. aeruginosa* (Pa) were also tested as controls. The unmarked lane (far left) represents molecular size markers in kilobase pairs (λ DNA cut with *Hin*dIII and *Sty*I).

advantage of being based on a broad-range probe applicable to any bacterial species. Using a nonradioactive approach, we wanted to apply this method to a collection of *L. pneumophila* strains containing geographically separate isolates as well as epidemiologically related strains. Most of the previous work concerning molecular typing methods has focused on small numbers of strains related to a particular outbreak (e.g., see reference 28), which gives an inaccurate picture of the discriminatory power of the methods applied.

Our results are in accordance with those of other studies finding that the division of strains into serogroups has little relation to the underlying genetic structures of the strains (13, 15, 29); in our study, there were several examples of strains from the same serogroup being placed in separate ribotypes. Also, several of the ribotyping groups contained strains of more than one *L. pneumophila* serogroup. Interestingly, *L. pneumophila* Lansing 3, which is unreactive with available serogroup-specific antisera, had the same composite subtype, H2C2P2 (with three enzymes), as the SG 5 Dallas 1E and the SG 4 Los Angeles 1 strains. These three strains all belong to the *fraseri* subspecies of *L. pneumophila* (3). Restriction with *Nci*I, however, placed the SG 4 Los Angeles strain in a separate group.

Epidemiologically related strains were assigned to the same single or composite ribotype. One of these, H8N7C7P7, was unique for the corresponding clinical and environmental isolates, suggesting the high discriminatory power of the method. An objective method for estimating this power is obtained by calculating the ID value; the combination of all four enzymes thus yielded an ID value of 0.97. If the proposed cutoff value of 0.95 (2) is used to define a typing method appropriate for epidemiological purposes, then ribotyping with the combination of all four enzymes, or even with three enzymes (ID = 0.95 to 0.96), would fulfill this criterion. An ID value of 0.95 corresponds to a less than 5% probability that two unrelated strains belong to the same typing group. Thus, the combination of two enzymes for typing, or typing with a single enzyme, did not yield sufficiently high discrimination. These findings are similar to those of Blanc et al. (2), who applied ribotyping to a collection of *Pseudomonas aeruginosa* strains and found that a satisfactory discriminatory power could be obtained by combining three or more restriction enzymes and that ribotyping with two or fewer enzymes yielded ID values of less than 0.95. Since, however, an inverse relationship between discriminatory power and reproducibility has been demonstrated (16), the choice of an ID value of 0.90 as acceptable, as proposed by Hunter and Gaston (17), would allow only two enzymes to be used.

A previous study (28) of epidemiologically related *L. pneumophila* strains concluded that both ribotyping and pulsed-field gel electrophoresis were useful for subtyping. Only two restriction enzymes were used for the ribotyping, however, and a broader and more accurate evaluation based upon epidemiologically unrelated isolates was not attempted. Typing based on the restriction patterns of *L. pneumophila* DNA, including rRNA genes, has been extensively studied by Harrison and coworkers (13–15, 26, 27), who also demonstrated a convincing stability of the defined genotypes, e.g., after repeated subculturing. The same authors stated that analysis of restriction fragment patterns should provide a more accurate measure of the ancestral relationships among strains than serological methods such as serotyping. The discussion of whether a phenotypic or genotypic result reflects the true subtype of a bacterial isolate is difficult. Changes in genome structure by mutations or insertions by phages and transposons would be expected to influence the restriction pattern of a single clone, but such phenomena have not been observed in vivo for members of the *Legionellaceae*. Indeed, in vitro transposon mutagenesis of *L. pneumophila* for virulence studies has been extremely difficult (22). Since variations in monoclonal subtypes of *L. pneumophila* strains are known to occur (7, 14, 24, 31), it seems prudent not to rely on phenotypic results alone. Moreover, monoclonal subtyping is in itself insufficient for distinguishing strains of *L. pneumophila* so that the source of infection in an outbreak situation can be identified, and it has the disadvantage of being limited to a few serogroups, with a generally available international panel of antibodies for subtyping existing only for SG 1 (19).

Another part of our investigation concerned the use of ribotyping for species identification. All *Legionella* species had different ribotyping patterns, and for the few nonreference strains, the results suggested that members of the same species (other than *L. pneumophila*) had identical ribotypes. Since the identification of *Legionella* species other than *L. pneumophila*, which can be easily identified with a commercially available monoclonal antibody recognizing all 14 serogroups, is difficult and requires biochemical tests that are not routinely performed in the ordinary clinical microbiology laboratory (23, 33, 35) or specialized techniques such as fatty acid analysis and classical DNA-DNA hybridization, ribotyping could possibly serve a second purpose here apart from subtyping. A more thorough evaluation of this application, to include the recently reported new species (6, 32) and more clinical and environmental strains, is still needed. Computer-assisted reading of

the patterns, in which the patterns are compared with those of known species stored in a data bank, would facilitate these studies and the standardization of ribotyping as a routine method in the clinical microbiology laboratory.

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