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Molecular Fingerprinting of *Legionella* Species by Repetitive Element PCR

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Repetitive element PCR (rep-PCR) uses outward-facing primers to amplify multiple segments of DNA located between conserved repeated sequences interspersed along the bacterial chromosome. Polymorphisms of rep-PCR amplification products can serve as strain-specific molecular fingerprints. Primers directed at the repetitive extragenic palindromic element were used to characterize isolates of *Legionella pneumophila* and other *Legionella* species. Substantial variation was seen among the rep-PCR fingerprints of different *Legionella* species and serogroups. More limited, but distinct, polymorphisms of the rep-PCR fingerprint were evident among epidemiologically unrelated isolates of *L. pneumophila* serogroup 1. Previously characterized *Legionella* isolates from nosocomial outbreaks were correctly clustered by this method. These results suggest the presence of repetitive extragenic palindromic-like elements within the genomes of members of the family *Legionellaceae* that can be used to discriminate between strains within a serogroup of *L. pneumophila* and between different *Legionella* species. rep-PCR appears to be a useful technique for the molecular fingerprinting of *Legionella* species.

Legionella pneumophila and other *Legionella* species are recognized as causes of outbreaks of disease related to exposure to diverse environmental sources (45). The problem of exposure to and infection with *Legionella* species assumes particular importance within hospitals, where patients with chronic pulmonary disease, advancing age, and underlying immunosuppression are at increased risk of legionellosis (13). Outbreaks of nosocomial *Legionella* infection have been described in both general hospital wards and high-dependency intensive care and transplant units (4, 5, 13, 27, 29, 30, 32) and have been traced to sources including air conditioning systems and reticulated water supplies (20). The demonstration of an epidemiologic relationship among *Legionella* isolates from clinical material or environmental sources requires efficient typing or fingerprinting techniques (5, 45).

Methods that have been previously used to characterize legionellae include serotyping (8, 30), monoclonal antibodies (MAbs) (9, 14, 19, 27, 29, 31), isoenzyme analysis (5, 29, 31), differential antibiotic susceptibility (40), protein profiling (1, 6, 16, 27), plasmid fingerprinting (1, 18, 27), restriction endonuclease analysis (23, 24, 29, 31, 34–36), ribotyping (12, 24, 32, 35), and pulsed-field electrophoresis (17, 21, 29). Most recently, DNA fingerprinting with arbitrarily primed PCRs (AP-PCRs) has been used to study isolates of *L. pneumophila* serogroup 1 (12, 33). When other serogroups of *L. pneumophila* or other *Legionella* species are of concern, most of these methods have limited discriminating capabilities. Therefore, a method with broader applicability is needed. Short, highly conserved repeated bacterial DNA sequences, including the repetitive extragenic palindromic (REP) sequence (11, 26), have recently been utilized in a novel genomic fingerprinting method based on the PCR and known as repetitive element PCR (rep-PCR) (38). In rep-PCR, consensus primers complementary to each end of a repeated sequence are oriented such that PCR amplification of DNA sequences proceeds between adjacent repeated elements. The resulting multiple amplification products have lengths that reflect distance polymorphisms between repeated elements contained within bacterial genomes. Simple agarose gel electrophoresis of the amplification products provides unambiguous strain-specific DNA fingerprints of limited complexity. rep-PCR has already been found useful for the genomic fingerprinting of diverse bacteria, including *Bacillus subtilis* (39), *Rhizobium meliloti* (2), *Citrobacter diversus* (44), *Streptococcus pneumoniae* (37), and *Enterobacter aerogenes* (10). The occurrence of two cases of *L. pneumophila* infection within 1 week in the cardiac transplantation unit of a Houston hospital led us to investigate the utility of rep-PCR as a genomic fingerprinting technique for *L. pneumophila* and other *Legionella* species. Specifically, we wanted to investigate whether the technique could reliably discriminate between strains within a serogroup of *L. pneumophila* and between different *Legionella* species and whether rep-PCR fingerprinting is useful for matching outbreak-related environmental and patient isolates that have been previously matched by both epidemiologic and other typing methods.

MATERIALS AND METHODS

Strains. Local clinical isolates of *Legionella* species were recovered and identified by standard techniques (41, 42). Identifications were verified with direct fluorescent antibody conjugates or by referral to the Centers for Disease Control. These organisms were isolated from two patients who had undergone orthotopic heart transplantation at the same Hous-

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TABLE 1. Details of *Legionella* isolates examined in this study

Legionella species and serogroup	Strain designation	Source[a]	Alternative designation	Description
L. pneumophila				
Serogroup 1	1042054	SLEH		Case 1 isolate
	1166593	SLEH		Case 2 isolate
	Philadelphia 1	CDC	ATCC 33152	Type strain
	2	PVAMC	ATCC 33152	Type strain
	3	PVAMC	ATCC 33153	Clinical isolate
	784	PVAMC		Clinical isolate
	808	PVAMC		Clinical isolate
	814	PVAMC		Environmental isolate
	1075	PVAMC		Clinical isolate
	1080	PVAMC		Environmental isolate
	1086	PVAMC		Environmental isolate
	1089	PVAMC		Clinical isolate
	1107	PVAMC		Clinical isolate
Serogroup 2	4	PVAMC	ATCC 33154	Type strain
Serogroup 3	5	PVAMC	ATCC 33155	Type strain
Serogroup 4	6	PVAMC	ATCC 33156	Type strain
Serogroup 4	Los Angeles 1	CDC	ATCC 33156	Type strain
Serogroup 9	CHD-L8	HDHHS		Clinical isolate
Serogroup 12	630	PVAMC		Clinical isolate
Serogroup 12	643	PVAMC		Environmental isolate
Serogroups 1–6	92-28503	AW		Environmental isolate
L. bozemanii	CHD-L2	HDHHS		Clinical isolate
Serogroup 1	WIGA	CDC	ATCC 33217	Type strain
L. dumoffii	25	PVAMC	ATCC 33279	Type strain
L. feeleii	CHD-L1	HDHHS		Clinical isolate
L. longbeachae, serogroup 1	Long Beach 4	CDC	ATCC 33426	Type strain
L. micdadei	11	PVAMC	ATCC 33218	Type strain
L. rubrilucens	680	PVAMC	ATCC 35304	Type strain

[a] Abbreviations: SLEH, St. Luke's Episcopal Hospital, Houston, Tex.; CDC, Centers for Disease Control, Atlanta, Ga.; PVAMC, Veterans Affairs Medical Center, Pittsburgh, Pa.; HDHHS, Houston Department of Health and Human Services, Houston, Tex.; AW, Alice Weissfeld, Microbiology Specialists Inc., Houston, Tex.

ton hospital within 1 week of each other and subsequently developed pneumonia due to *L. pneumophila* serogroup 1. Both patients were housed in the same ward in the postoperative period; however, despite extensive cultures of all water sources in the implicated hospital ward with buffered charcoal-yeast extract agar, no environmental source for these bacteria could be identified. Reference isolates were obtained from the Centers for Disease Control as lyophilized cultures and were obtained from Victor Yu, Veterans Affairs Medical Center, Pittsburgh, on buffered charcoal-yeast extract slopes. A local environmental isolate of *L. pneumophila* reacting with a polyvalent conjugate for serogroups 1 to 6, which was not serotyped further, was provided by Alice Weissfeld, Microbiology Specialists, Inc., Houston, Tex. Relevant epidemiologic information and available typing data pertaining to clinical isolates obtained from the Pittsburgh Veterans Affairs Medical Center were withheld until the results of rep-PCR fingerprinting were established. The isolates studied are shown in Table 1.

Each isolate was inoculated onto buffered charcoal-yeast extract agar and incubated in humidified room air at 35°C for 72 to 96 h. Organisms were scraped from each plate, washed once in 1 M NaCl, and stored as pellets at −80°C prior to DNA extraction.

DNA extraction. Thawed bacterial pellets were washed twice in 1 M NaCl and twice in sterile double-distilled water and then were resuspended in TE (10 mM Tris, 25 mM EDTA [pH 8.0]) and incubated in 0.2-mg/ml lysozyme (Sigma, St. Louis, Mo.)–0.3-mg/ml RNase A (Sigma)–0.6% sodium dodecyl sulfate (Sigma) at 37°C for 60 min. After addition of 1% *N*-lauryl sarcosine (Sigma) and 0.6 mg of proteinase K per ml (Sigma), the lysate was further incubated for 16 h at 37°C. After heating at 65°C for 45 min, the lysate was extracted twice with phenol, extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1 [vol/vol]), and then extracted repeatedly with chloroform until a clear interface was obtained. DNA was precipitated from the aqueous phase with 0.33 M sodium acetate and 2.5 volumes of cold absolute ethanol, dissolved in TE buffer, and quantitated fluorometrically (model TKO-100 minifluorometer; Hoefer Scientific, San Francisco, Calif.).

rep-PCR. Oligonucleotide primers were based on the highly-conserved REP repeated DNA element (11, 26). For this study, the 18-mer degenerate primers REP1R-Dt (3′-CGGNC TACNGCNGCNIII-5′) and REP2-Dt (3′-CATCCGGNCTA TTCNGCN-5′) (N = A, C, G, and T; I = inosine) were used. The general design and synthesis of these primers have been described in detail previously (38, 39). Each 25-μl PCR mixture contained 50 pmol of each primer; 100 ng of template bacterial DNA; 1.25 mM (each) dATP, dCTP, dGTP, and dTTP (Promega Corp., Madison, Wis.); 2 U of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn.); and 10% dimethylsul-

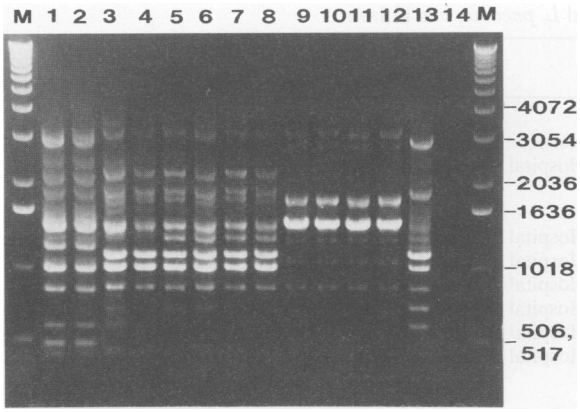

FIG. 1. rep-PCR DNA fingerprints of *L. pneumophila* serogroup 1 isolates 1042054 (lane 1), 1166593 (lane 2), 2 (lane 3), Philadelphia 1 (lane 4), 1075 (lane 5), 1086 (lane 6), 1089 (lane 7), 1107 (lane 8), 784 (lane 9), 808 (lane 10), 814 (lane 11), 1080 (lane 12), and 3 (lane 13). (For strain descriptions, see Table 1.) Lane 14 shows a negative control reaction. Lanes M show DNA reference marker sizes (Gibco BRL) in base pairs.

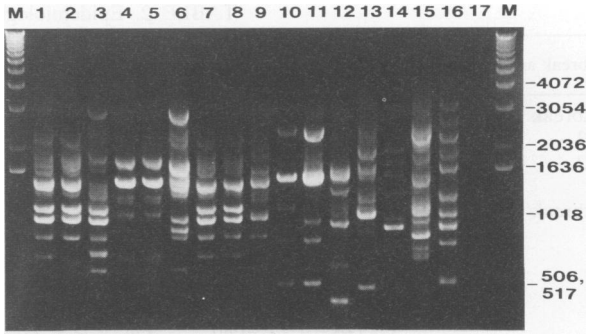

FIG. 2. rep-PCR DNA fingerprints of *L. pneumophila* serovars and other *Legionella* species. Lanes 1 to 9 show the fingerprints of *L. pneumophila* isolates Philadelphia 1 (serogroup 1), 4 (serogroup 2), 5 (serogroup 3), 6 (serogroup 4), Los Angeles 1 (serogroup 4), CHD-L8 (serogroup 9), 630 (serogroup 12), 643 (serogroup 12), and 92-28503 (serogroups 1 to 6), respectively. Additional fingerprints include *L. bozemanii* CHD-L2 (lane 10), *L. bozemanii* WIGA (lane 11), *Legionella feeleii* CHD-L1 (lane 12), *Legionella micdadei* 11 (lane 13), *Legionella longbeachae* Long Beach 4 (lane 14), *Legionella rubrilucens* 680 (lane 15), and *Legionella dumoffii* 25 (lane 16). Lane 17 is a negative control reaction. Lanes M show DNA reference marker sizes in base pairs.

foxide, all in 1× reaction buffer (16.6 mM ammonium sulfate, 67 mM Tris-HCl [pH 8.8 at 25°C], 6.7 mM magnesium chloride, 10 mM β-mercaptoethanol, 6.7 μM EDTA, and 170 μg of bovine serum albumin per ml) (15). PCR was performed in an automated thermal cycler (DNA thermal cycler; Perkin-Elmer Cetus) with an initial denaturation step at 95°C for 7 min followed by 30 cycles of denaturation at 90°C for 30 s, annealing at 40°C for 1 min, and extension at 65°C for 8 min and with a single final extension step at 65°C for 16 min. Seven-microliter samples of the amplification products were electrophoresed in a 1% agarose gel (ultraPure; Gibco BRL, Gaithersburg, Md.) containing 1× TAE (Tris-acetate-EDTA) (22) and 0.5 μg of ethidium bromide per ml and photographed under UV light.

RESULTS

rep-PCR analysis. Molecular typing of *Legionella* isolates by rep-PCR generated multiple amplification products ranging in size from 400 bp to 3.0 kb. Agarose gel electrophoresis of the rep-PCR amplification products provided strain-specific genomic fingerprints for isolates of *L. pneumophila* serogroup 1 (Fig. 1) and legionellae of other serogroups and species (Fig. 2).

Clinical isolates of *L. pneumophila* serogroup 1 identified from two cardiac transplant patients within 1 week at a Houston hospital generated indistinguishable rep-PCR fingerprints (Fig. 1, lanes 1 and 2). These two isolates were also subjected to restriction endonuclease analysis (REA) of genomic DNA with the restriction enzymes *Hin*dIII and *Eco*RI (28). Identical REA fingerprints for the two Houston isolates were obtained after digestion with either enzyme (data not shown). Strains of *L. pneumophila* serogroup 1 ATCC 33152 from two independent sources produced identical rep-PCR fingerprints (Fig. 1, lanes 3 and 4), and these differed by one or more discrete bands from the fingerprints generated by other clinical and reference isolates.

Eight clinical isolates of *L. pneumophila* serogroup 1 obtained from the collection of the Pittsburgh Veterans Affairs Medical Center could be allocated to two distinct genomic groups on the basis of their rep-PCR fingerprints (Fig. 1, lanes 5 to 8 and 9 to 12). Each group encompassed four isolates with indistinguishable fingerprints. A distinct conserved amplification band approximately 800 bp in size was present in the fingerprint of each of the *L. pneumophila* serogroup 1 strains.

Isolates of *L. pneumophila* of serotypes 2, 3, 4, 9, and 12 generated distinctive rep-PCR fingerprints (Fig. 2). Two independent cultures of the type strain of *L. pneumophila* serogroup 4 produced identical fingerprints (Fig. 2, lanes 4 and 5), as did two isolates of *L. pneumophila* serogroup 12 (Fig. 2, lanes 7 and 8). Many clinical and reference strains of *L. pneumophila* of serotypes 1, 2, 3, 9, and 12 shared rep-PCR amplification bands approximately 800 bp and 1.0 kb in size. The type strain of *L. pneumophila* serogroup 4 (Fig. 2, lanes 4 and 5) and one cluster of *L. pneumophila* serogroup 1 isolates (Fig. 1, lanes 9 to 12) did not exhibit these characteristic amplification bands.

Other *Legionella* species also produced diverse rep-PCR fingerprints (Fig. 2), which were distinguished by the absence of the amplification bands characteristic of many *L. pneumophila* serotypes, although a band at about 800 bp was present in all but five (lanes 4, 5, 12, 13, and 14) of the strains. Two unrelated isolates of *Legionella bozemanii* shared several amplification bands of similar size but were easily separated on the basis of their overall rep-PCR patterns (Fig. 2, lanes 10 and 11).

At the completion of rep-PCR fingerprinting, data pertaining to blinded clinical isolates obtained from the Pittsburgh Veterans Affairs Medical Center were analyzed. The relationships between the various epidemiologically linked isolates are indicated in Table 2. Organisms 630 and 643 appeared indistinguishable by rep-PCR (Fig. 2, lanes 7 and 8) and were cultured from the patient and hospital water supply, respectively, in a case of nosocomial *L. pneumophila* serogroup 12 pneumonia. These two isolates were previously found to be identical by REA (28). Similarly, *L. pneumophila* serogroup 1 isolates 808 and 814 were recovered from a patient and the water supply in a nursing home in which the infection developed; both were classified as subtype OLDA by MAbs and were indistinguishable by REA (28) and by rep-PCR fingerprinting (Fig. 1, lanes 10 and 11).

TABLE 2. Epidemiologically related *L. pneumophila* isolates

Outbreak and serogroup	Subtype	Strain	Figure[a]	REP pattern[b]	Source	Previous typing (reference)
Outbreak 1						
12	ND[c]	630	Fig. 2, lane 7	4	Nosocomial pneumonia	Identical by REA (28)
12	ND	643	Fig. 2, lane 8	4	Hospital water supply	
Outbreak 2						
1	Philadelphia	1075	Fig. 1, lane 5	2	Hospital A nosocomial pneumonia[d]	Identical by MAb typing (9)
1	Philadelphia	1089	Fig. 1, lane 7	2	Hospital A nosocomial pneumonia[d]	
1	Philadelphia	1107	Fig. 1, lane 8	2	Hospital A nosocomial pneumonia[d]	
1	Philadelphia	1086	Fig. 1, lane 6	2	Hospital A water supply	
1	OLDA	1080	Fig. 1, lane 12	3	Hospital B water supply	Identical by MAb typing (9)
1	OLDA	784	Fig. 1, lane 9	3	Hospital B nosocomial pneumonia	
Outbreak 3						
1	OLDA	808	Fig. 1, lane 10	3	Nursing home pneumonia	Identical by MAb typing and REA (28)
1	OLDA	814	Fig. 1, lane 11	3	Nursing home water supply	
This study's outbreak						
1	ND	1042054	Fig. 1, lane 1	1	Nosocomial pneumonia	ND
1	ND	1166593	Fig. 1, lane 2	1	Nosocomial pneumonia	ND

[a] Figures 1 and 2 in this article.
[b] REP patterns were arbitrarily assigned a number.
[c] ND, not done.
[d] Three patients were actually hospitalized in hospital B but had been transferred from hospital A shortly before the onset of pneumonia.

Isolates 1075, 1089, and 1107 (Fig. 1, lanes 5, 7, and 8) were derived from three patients in a hospital outbreak of *L. pneumophila* serogroup 1 infection. All were transferred from hospital A to hospital B for cardiac surgery, and each developed postoperative legionellosis while in hospital B. The three clinical isolates were of MAb subtype Philadelphia. Environmental testing of the hospital B water supply recovered *L. pneumophila* serogroup 1 (representative isolate 1080 [Fig. 1, lane 12]), but of subtype OLDA. Nosocomial cases of legionellosis in patients confined exclusively to hospital B (representative isolate 784 [Fig. 1, lane 9]) were caused by isolates of subtype OLDA. Environmental testing in hospital A also recovered *L. pneumophila* serogroup 1, but of subtype Philadelphia (representative isolate 1086 [Fig. 1, lane 6]), suggesting that the three patients had been infected with *Legionella* species at hospital A prior to transfer to hospital B. Thus, the relationships suggested for these isolates by rep-PCR were entirely consistent with the available clinical data and the results of MAb subtyping (9). However, isolates of *L. pneumophila* serogroup 1 subtype OLDA originating from hospital B were not distinguishable by rep-PCR fingerprinting from apparently epidemiologically unrelated *L. pneumophila* isolates 808 and 814 (Fig. 1, lanes 10 and 11) obtained from a nursing home, which were also of serogroup 1 and subtype OLDA. Both hospital B and the nursing home were in Pittsburgh, but they were located across the city from each other. Both institutions were supplied with city water; however, each received its water from a different reservoir.

DISCUSSION

Genomic fingerprinting of *L. pneumophila* and other *Legionella* species by using degenerate primers complementary to the consensus REP sequence provided patterns of amplification products that varied between *Legionella* serogroups and species. rep-PCR was performed without specific knowledge of *Legionella* DNA base sequence information and relied upon the ubiquitous nature of highly conserved REP-like sequences in diverse eubacterial species and distantly related phyla, as recently demonstrated by Versalovic et al. (38) using DNA-DNA hybridization techniques and PCR with primers complementary to the interspersed repeats. The ability to generate reproducible, strain-specific fingerprints by using rep-PCR and primers based on the REP element consensus sequence indicates that REP-like sequences are also present in the genomes of members of the family *Legionellaceae*.

Molecular typing with rep-PCR is a useful technique for the epidemiologic investigation of *Legionella* infections. Independently maintained cultures of several *Legionella* reference isolates displayed indistinguishable rep-PCR fingerprints. Similarly, collections of clinical and environmental isolates of *Legionella* species with established epidemiologic relationships and that had been previously characterized by using MAbs and REA were correctly grouped by the PCR technique. Two local clinical isolates of *L. pneumophila* serogroup 1 recovered from cardiac transplant patients also appeared to be genotypically identical by rep-PCR fingerprinting, supporting the circumstantial evidence for a common source of exposure within the transplant facility.

rep-PCR fingerprints reflect the disposition of repeated REP sequences around the bacterial chromosome (38). In studies of natural populations of *Escherichia coli*, polymorphisms of the repeated REP element have been shown to correlate closely with the results of multilocus enzyme allotyping (3). The finding of substantial similarity among the rep-PCR fingerprints of many *L. pneumophila* serogroup 1 isolates suggests that limited genotypic diversity exists within that serogroup. This is consistent with previous studies demonstrating the clonal nature of *L. pneumophila* serogroup 1 by the multilocus enzyme technique (25).

Indistinguishable rep-PCR fingerprints were generated by clinical and environmental isolates of *L. pneumophila* serogroup 1 originating from two separate institutions (Fig. 1, lanes 9 to 12). Significantly, these isolates were all of monoclonal subtype OLDA. Other investigators have also found that epidemiologically unrelated *Legionella* isolates, including iso-

lates with different serogroup specificities, may appear identical by typing methods such as REA (31, 34), restriction fragment length polymorphisms (23), or multilocus enzyme allotyping (5, 25, 31). This could be due either to inadequate discriminatory power of the particular technique or alternatively may reflect the widespread geographic dissemination of certain *Legionella* clones (25). Nevertheless, we found that other epidemiologically distinct isolates of *L. pneumophila* serogroup 1 included in our study were readily separated by the presence or absence of one or more clear amplification bands within the rep-PCR fingerprint.

Increased variation was observed when the rep-PCR fingerprints of different serogroups of *L. pneumophila* and different *Legionella* species were compared. Of interest, the rep-PCR fingerprint of the type strain of *L. pneumophila* serogroup 4 (Fig. 2, lanes 4 and 5) did not generate several amplification bands characteristic of the fingerprints of other *L. pneumophila* serogroups. This isolate (Los Angeles 1 or ATCC 33156) has previously been shown to differ sufficiently in genotype from typical *L. pneumophila* isolates to be regarded as a separate species (species 1 of reference 25).

AP-PCR is another genomic fingerprinting technique that has been used to subtype isolates of *L. pneumophila* serogroup 1 (12, 33). In one study (33), AP-PCR differentiated between hospital outbreak strains and unrelated isolates of *L. pneumophila* serogroup 1. In another study (12), AP-PCR provided eight distinct fingerprints for 10 *L. pneumophila* serogroup 1 MAb type strains and differentiated between isolates sharing the same MAb pattern. However, AP-PCR patterns varied, depending upon the DNA isolation procedure used (12). Other workers have also observed a critical dependence of AP-PCR fingerprints upon reaction conditions and substrate concentrations (7).

Ribotyping (12, 24, 32, 35), restriction enzyme analysis with pulsed-field gel electrophoresis (17, 21, 24, 29), and restriction fragment length polymorphisms (23) have all been shown to be useful for fingerprinting *L. pneumophila*. These techniques provide discrimination among isolates of all serogroups of *L. pneumophila*, making them more useful than MAb typing when dealing with unknown isolates. Furthermore, these techniques provide greater discriminatory capabilities than MAb typing.

The ability to generate simple and reproducible genomic fingerprints, which vary within and between *Legionella* serogroups and species, indicates that rep-PCR may have applications in epidemiologic analysis and in examination of the genotypic diversity of *Legionella* species. rep-PCR offers the advantages of rapidity, less technical requirements, and relative ease of fingerprint interpretation compared with ribotyping, pulsed-field gel electrophoresis, or restriction fragment length polymorphism analysis. Studies with an increased range and number of well-characterized organisms, as well as direct comparison with these other genomic techniques, should help establish the power of rep-PCR fingerprinting. In addition, the current development of rep-PCR protocols using whole bacterial cells without the requirement for DNA extraction (43) will substantially enhance the clinical applicability of this technique.

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