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Comparison of Arbitrarily Primed Polymerase Chain Reaction, Ribotyping, and Monoclonal Antibody Analysis for Subtyping *Legionella pneumophila* Serogroup 1

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Arbitrarily primed polymerase chain reaction (AP-PCR) was used to characterize *Legionella pneumophila* serogroup 1. Cells from a single colony could be subtyped by AP-PCR within a few hours. The discrimination between strains of *L. pneumophila* serogroup 1 by AP-PCR was equivalent to that by monoclonal antibody analysis and ribotyping. Four strains representing the monoclonal antibody pattern most frequently associated with outbreaks all yielded unique amplicon patterns by AP-PCR.

Legionella pneumophila serogroup 1 is the major etiologic agent of legionellosis and also the serogroup most frequently isolated from the environment (7). A number of molecular techniques have been used for subtyping serogroup 1, including monoclonal antibody (MAb) typing (4), plasmid profile analysis (6), multilocus enzyme electrophoresis (14), analysis of restriction fragment length polymorphisms (RFLP) with or without the use of probes (9, 15), and pulsed-field gel electrophoresis (10). These methods have been compared in several summary or review articles (14, 15). Most have proved useful under certain circumstances, but many are unsuitable for general laboratory use. The Centers for Disease Control and Prevention procedure to discriminate subtypes of *L. pneumophila* serogroup 1 uses a panel of seven MAbs. These MAbs were suggested as an international standard (4); however, these reagents are not readily available, and many laboratories depend on alternate procedures to subtype these bacteria. Currently, 10 subtypes of *L. pneumophila* serogroup 1 have been identified with this panel of MAbs. These 10 type strains were characterized by two additional methods, rRNA probing of RFLP (ribotyping) and arbitrarily primed polymerase chain reaction (AP-PCR).

AP-PCR is based on the amplification of genomic DNA with a single primer of arbitrary nucleotide sequence generating polymorphisms that are detected electrophoretically in agarose gels (16). This assay has been used to characterize *Staphylococcus* spp. (13), *Candida* spp. (5), and *Helicobacter pylori* (1). We applied this technique to the differentiation of *L. pneumophila* serogroup 1.

The *L. pneumophila* serogroup 1 strains studied are listed in Table 1. The strains were stored until needed in defibrinated sheep blood at −70°C and were recovered by subculture on buffered charcoal-yeast extract agar. Inoculated plates were incubated for 48 to 72 h at 37°C in 2.5% CO_2.

For MAb analysis, bacterial cultures were formalinized and examined by a rapid dot blot procedure (8), using the panel of MAbs to *L. pneumophila* serogroup 1 (4).

DNA was prepared as follows. Bacterial cultures were harvested, washed twice and resuspended in STE buffer (100 mM NaCl, 50 mM Tris-HCl, 10 mM sodium EDTA [pH 7.5]), and incubated at room temperature with lysozyme (2 mg/ml) for 1 h. Cells were lysed with sodium dodecyl sulfate (1%, final concentration) at 37°C for 30 min and digested for 30 min with proteinase K (5 mg/ml) at 37°C. The DNA was extracted by a standard phenol-chloroform procedure and resuspended in 100 μl of distilled water.

For rapid DNA extraction, *Legionella* cells were harvested into distilled water and adjusted to an optical density of 2.5 (lambda = 540 nm). One hundred microliters of this suspension was added to 1 ml of DNA extraction reagent (Perkin-Elmer Corp., Alameda, Calif.). The sample was boiled for 10 min and allowed to cool to room temperature before use in AP-PCR.

For ribotyping, chromosomal DNA from strains representing the 10 MAb types of *L. pneumophila* serogroup 1 was reacted with four separate restriction endonucleases, *Eco*RI, *Hin*dIII, *Cla*I, and *Nci*I, under conditions specified by the manufacturer (GIBCO Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Restricted DNA was subjected to electrophoresis on a 1.0% agarose gel in Tris-acetate buffer at 40 V for 16 to 18 h. DNA restriction fragments were transferred to a positively charged nylon membrane (Boehringer Mannheim, Indianapolis, Ind.) by using a pressure blotter (Stratagene, La Jolla, Calif.) according to the manufacturer's instructions. The DNA was cross-linked to the membrane by exposure to UV light for 1 min with a Stratalinker (Stratagene).

Ribotyping was performed using plasmid pKK 3535 (a pBR 322-derived plasmid containing the *rrnB* rRNA operon of *Escherichia coli*) as the probe. The plasmid was digested with *Eco*RI, purified, and labeled with digoxigenin-11-dUTP (2). Prehybridization, hybridization with digoxigenin-labeled probe, posthybridization washing, and immunologic detection were performed according to the protocol (Genius kit; Boehringer Mannheim), except that hybridization and washing were performed at 60°C instead of the recommended 68°C. To detect bound probe, the membrane was reacted with Lumi Phos 530 substrate (Boehringer Mannheim) for 30 min at 37°C and then exposed to X-ray film to record the chemiluminescent signal.

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TABLE 1. Summary of subtyping results of *L. pneumophila* serogroup 1 by three different methods

Strain	MAb type	Ribotype	AP-PCR type
Philadelphia 1	1,2,5,6	R1	AP1
Allentown 1	1,2,5	R2	AP2
Benidorm 030E	1,2,5,7	R3	AP3
Knoxville 1	1,2,3	R4	AP4
France 5811	1,2	R5	AP5
OLDA	1,6,7	R6	AP6
Oxford 4032E	1,6	R6	AP6
Heysham 1	1,3,6,7	R7	AP7
Camperdown 1	1	R6	AP6
Bellingham 1	1,4,7	R4	AP8
No. of different types	10	7	8

AP-PCR amplification reactions were performed in volumes of 50 μl containing 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 4 mM $MgCl_2$; 0.01% gelatin; 200 μM (each) dATP, dCTP, dGTP, and dTTP; 1 μM primer (M13 Forward, 21 bp: 5′ TTA TGT AAA ACG ACG GCC AGT 3′); DNA (concentration of purified DNA, 0.1 μg/μl); and 0.25 U of *Taq* DNA polymerase (Perkin-Elmer Cetus). Amplification was performed in a DNA thermal cycler (Perkin-Elmer Cetus) programmed for 45 cycles of 1 min at 94°C, 1 min at 36°C, and 2 min at 72°C. Amplification products were analyzed by electrophoresis in 1.4% agarose gels and detected by staining with ethidium bromide.

Each of the 10 type strains of *L. pneumophila* serogroup 1 has a unique MAb reaction (Table 1). Ribotyping results were as follows: *Hin*dIII differentiated four ribotypes, *Eco*RI and *Cla*I differentiated six ribotypes, and *Nci*I differentiated seven ribotypes, demonstrating that *Nci*I had the highest discriminatory power (Fig. 1). Of the seven ribotypes obtained with *Nci*I, one was shared by three different strains, OLDA, Oxford 4032E, and Camperdown 1; one was shared by Knoxville 1 and Bellingham 1; and each of the remaining ribotypes was associated with a single strain (Table 1).

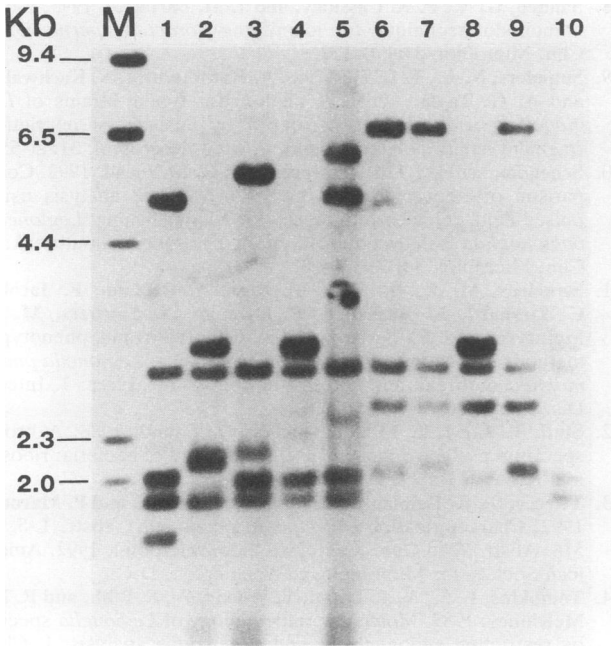

FIG. 1. Ribotyping patterns generated by using the restriction enzyme *Nci*I and hybridization with the *rrnB* ribosomal RNA operon of *E. coli* as a probe. Lanes 1 to 10, Philadelphia 1, Allentown 1, Benidorm 030E, Knoxville 1, France 5811, OLDA, Oxford 4032E, Heysham 1, Camperdown 1, and Bellingham 1. Digoxigenin-labeled DNA molecular weight marker II (Boehringer Mannheim) was used as a molecular weight marker (lane M).

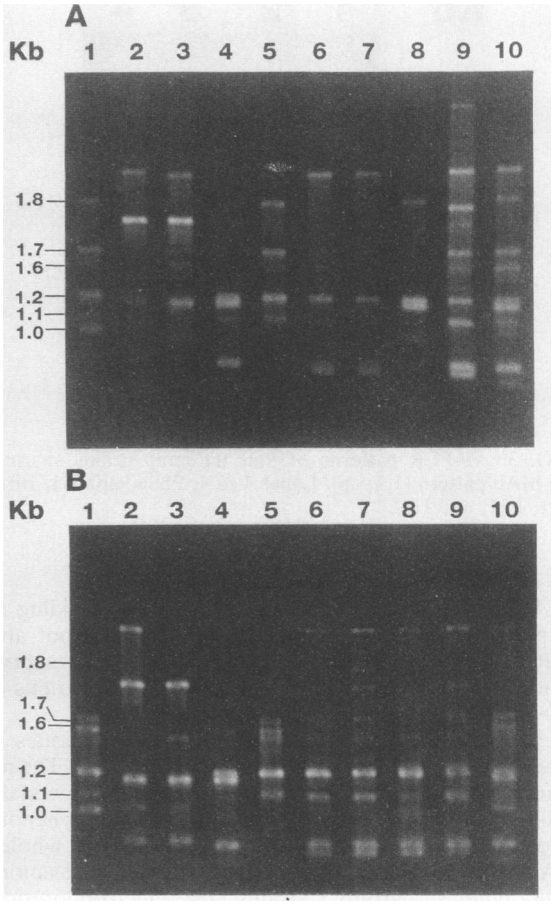

FIG. 2. AP-PCR patterns generated by using purified DNA (A) or DNA extracted by the rapid method (B). Lanes 1 to 10 (in both gels), Philadelphia 1, Allentown 1, Benidorm 030E, Knoxville 1, France 5811, OLDA, Oxford 4032E, Heysham 1, Camperdown 1, and Bellingham 1. Lambda DNA digested with *Nci*I was used as a molecular weight marker.

Eight different AP-PCR types were identified within the 10 type strains (Table 1). Although patterns for individual strains differed, an equivalent degree of discrimination was generated by using purified DNA or DNA extracted by the rapid method (Fig. 2). Knoxville 1 and Bellingham 1 shared the same ribotyping pattern but showed different AP-PCR types. Olda, Oxford 4032E, and Camperdown 1 shared similar AP-PCR and ribotyping patterns.

To determine whether AP-PCR might discriminate between isolates of the same MAb pattern, we tested four strains of the MAb pattern (1,2,5,6) most frequently associated with outbreaks of legionellosis (Philadelphia 1, Birmingham 1 [Centers for Disease Control], L11 [Spain], and L19 [Spain]). All four strains showed distinct AP-PCR patterns (Fig. 3).

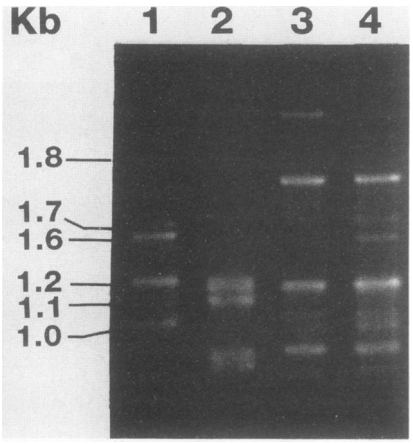

FIG. 3. AP-PCR patterns of four different strains sharing the same MAb pattern (1,2,5,6). Lanes 1 to 4, Philadelphia 1, Birmingham 1, L11, and L19.

MAb subtyping is a rapid method for subdividing serogroup 1 strains. However, the technique does not always result in sufficient discrimination, and a second subtyping method is often necessary to identify epidemic strains of *L. pneumophila* serogroup 1 (11).

Recent developments in DNA analysis techniques have reduced the dependence on detecting phenotypes. The major advantage of genotypic analysis lies in the relative stability of the bacterial genotype versus that of the phenotype. Although restriction endonuclease analysis of whole-cell DNA has demonstrated the ability to discriminate among *L. pneumophila* serogroup 1 strains (15), electrophoretic patterns of the DNA digest are often difficult to interpret. Small-fragment restriction endonuclease analysis (3), RFLP analysis using pulsed-field gel electrophoresis (10), or RFLP analysis by means of probes (9) allows easier interpretation than standard RFLP analysis. Ribotyping employs RNA of *E. coli* or an equivalent (plasmid pKK 3535) as a probe to detect rRNA operon (*rrn*) segments, which are highly conserved throughout the eubacteria. It has been successfully applied to several bacteria (12), including *L. pneumophila* serogroup 1 (10). However, all of these procedures require culturing of the bacteria to produce sufficient quantities of DNA, extensive purification of genomic DNA, and additional procedures. The result is a subtyping technique which is labor-intensive and requires several days for completion.

By AP-PCR and the rapid DNA extraction procedure, growth from a single colony could be subtyped within a few hours. The reproducibility of AP-PCR is critical for successful discrimination of strains. Because this procedure uses an arbitrarily chosen primer and low-stringency hybridization conditions, the potential for variation is considerable. This procedure consistently resulted in reproducible amplicon patterns in our laboratory; however, additional testing in other laboratories may be necessary to determine the laboratory-to-laboratory uniformity of these results.

Although differences were observed in banding patterns of amplified DNA obtained from either phenol-chloroform extraction or the rapid extraction procedure, both were reproducible and indicated the same number of subtypes (Fig. 2). Comparing polymorphisms within the 10 subtypes of *L. pneumophila* serogroup 1 by AP-PCR resulted in discrimination between these strains equivalent to that of MAb analysis or ribotyping. The ability of this technique to differentiate MAb pattern 1,2,5,6 strains is valuable, as this subtype is most commonly associated with legionellosis epidemics.

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