

## Comparison of *Legionella pneumophila* Isolates by Arbitrarily Primed PCR and Pulsed-Field Gel Electrophoresis: Analysis from Seven Epidemic Investigations

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Received 5 May 1995/Returned for modification 29 June 1995/Accepted 23 August 1995

**Arbitrarily primed PCR (AP-PCR) and pulsed-field gel electrophoresis (PFGE) subtyping were applied to clinical and environmental isolates from seven unrelated outbreaks of Legionnaires' disease. The patterns observed with each method matched patient isolates and the epidemiologically linked source of disease for each of the seven outbreaks. PFGE allowed more discrimination among various isolates, although AP-PCR usually gave comparable results. With both methods, certain patterns appeared to predominate in the comparison of the seven outbreaks. Of five clinical isolates not associated with the outbreaks, three gave profiles distinct from those observed in the outbreaks by both methods. This suggests that there are at least two predominant subtypes of *Legionella pneumophila* serogroup 1 associated with outbreaks. Investigations of outbreaks of legionellosis should employ either PFGE or AP-PCR in addition to monoclonal antibody analysis.**

Legionellae are human pathogens causing pneumonia and are commonly found in water environments. There are presently 39 *Legionella* species representing 54 serogroups (2). *Legionella pneumophila* serogroup 1 is the major cause of legionellosis, accounting for 72% of cases (4, 17). There are at least 10 subtypes of *L. pneumophila* serogroup 1 on the basis of monoclonal antibody (MAB) analysis (12). Since these bacteria are widespread in water environments and there is such diversity within this genus, it is very common to isolate more than one *Legionella* strain from environmental sources tested during investigations of legionellosis. Without appropriate subtyping techniques, it may be extremely difficult to identify the environmental source for transmitting the disease during outbreaks of Legionnaires' disease.

A variety of subtyping techniques have been used to identify and characterize *Legionella* strains. These techniques include MAB analysis (12), restriction enzyme analysis, ribotyping (11), plasmid analyses (19), pulsed-field gel electrophoresis (PFGE) (24), repetitive element PCR (7), and arbitrarily primed PCR (AP-PCR) (30).

AP-PCR amplifies genomic DNA by using a single nonspecific primer, a low annealing temperature, and a high magnesium concentration to generate a collection of amplicons. The amplicons are then analyzed by agarose gel electrophoresis. Because of the low stringency inherent in this procedure, the patterns generated by AP-PCR are potentially variable. Consistency in performing the procedure is imperative. In our laboratory, the AP-PCR patterns for *L. pneumophila* serogroup 1 are reproducible. *Staphylococcus* spp. (26), *Enterobacter cloacae* (10), and *Helicobacter pylori* (1) have also been successfully characterized by AP-PCR. We recently compared AP-PCR with ribotyping and MAB analysis and found that the degrees of discrimination among strains of *L. pneumophila* serogroup 1 by these methods were comparable (8).

PFGE compares the electrophoretic mobilities of large frag-

ments of genomic DNA after digestion with infrequently cutting restriction enzymes. Many different investigators have used PFGE successfully to subtype *L. pneumophila* (15, 24, 25), as well as *Staphylococcus* spp. (14), *Klebsiella pneumoniae* (21), *Campylobacter hyointestinalis* (22), and *Enterococcus faecalis* (9).

We applied AP-PCR and PFGE to isolates collected from patients and environmental sites during investigations of seven unrelated outbreaks of Legionnaires' disease for which corresponding patient and environmental isolates as well as MAB subtyping information were available (3, 4, 13, 16, 27, 29). These outbreaks occurred between 1987 and 1993 and in all regions of the United States. Fourteen isolates collected from sporadic clinical cases and nonimplicated environmental sites during the investigation of one of these outbreaks were also tested (29).

### MATERIALS AND METHODS

PFGE was compared with AP-PCR in a blinded study. Isolates from implicated and nonimplicated environmental sources as well as epidemic-related and non-epidemic-related clinical isolates were assigned code numbers at random. PFGE was then performed on these isolates, and the patterns were compared with the AP-PCR patterns.

**Bacterial strains.** *L. pneumophila* was isolated from environmental samples according to established protocols of The Centers for Disease Control and Prevention (6). Clinical isolates were also recovered according to established Centers for Disease Control and Prevention protocols (5). All cultures were maintained on buffered charcoal yeast extract (BCYE) agar or frozen at  $-70^{\circ}\text{C}$  in defibrinated sheep blood after initial isolation. Cultures were grown for 72 h on BCYE agar at  $35^{\circ}\text{C}$  in 2.5%  $\text{CO}_2$ .

**MAB subtyping analysis.** All isolates were formalinized and then subjected to MAB analysis by using a rapid dot blot procedure (23) and a panel of MABs to *L. pneumophila* serogroup 1 (12).

**AP-PCRs.** Total genomic DNA was isolated from the bacterial cells as previously described (8), with the following modification: an aliquot containing  $2.8 \times 10^8$  bacteria was boiled in 1.0 ml of a suspension of 10% Chelex (Bio-Rad Laboratories, Hercules, Calif.) in 10 mM Tris (pH 8.0)–0.1 mM EDTA–0.1% sodium azide for 10 min. The AP-PCRs were prepared and amplified as previously described (8). Amplification products were analyzed by electrophoresis in 1.4% agarose gels and then by staining with ethidium bromide.

Pattern designations were assigned by using dominant and moderate intensity bands. Because of their variable nature, faint bands were not used for scoring. An

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TABLE 1. Summary of AP-PCR and PFGE subtyping of isolates<sup>a</sup>

| AP-PCR | Subtype |         | Source         | Outbreak (reference) | No. of isolates tested |
|--------|---------|---------|----------------|----------------------|------------------------|
|        | PFGE    | MAB     |                |                      |                        |
| 1      | A2      | 1,2,5,6 | Environment    | 1 (29)               | 5                      |
| 1      | A2      | 1,2,5,6 | Patient        | 1                    |                        |
| 1      | A       | 1,2,3   | Environment    | 2 (13)               | 4                      |
| 1      | A       | 1,2,3   | Patient        | 2                    |                        |
| 1      | A       | 1,2,5,6 | Environment    | 6 (27)               | 3                      |
| 1      | A       | 1,2,5,6 | Patient        | 6                    |                        |
| 1      | J       | 1,2,5,6 | Philadelphia 1 |                      |                        |
| 2      | B       | 1,2,5,6 | Environment    | 4 (3)                | 4                      |
| 2      | B       | 1,2,5,6 | Patient        | 4                    |                        |
| 2      | B       | 1,6     | Environment    | 5 <sup>b</sup>       | 3                      |
| 2      | B       | 1,6     | Patient        | 5                    |                        |
| 2      | C2      | 1,2,5,6 | Environment    | 3 (4)                | 4                      |
| 2      | C       | 1,2,5,6 | Environment    | 3                    |                        |
| 2      | C       | 1,2,5,6 | Patient        | 3                    |                        |
| 2      | C       | 1,2,5,6 | Patient        | 3                    |                        |
| 3      | D       | 1,2,5,7 | Environment    | 7 (16)               | 4                      |
| 3      | D       | 1,2,5,7 | Patient        | 7                    |                        |

<sup>a</sup> Isolates are from patients involved in seven outbreaks of Legionnaires' disease (patient) or the epidemiologically linked environmental sites (environment).

<sup>b</sup> No reference available.

occasional increase in the intensity of faint bands did not affect the interpretation of the pattern. Patterns that differed slightly from previously observed patterns were confirmed by a second amplification of the template.

**PFGE.** Bacteria were grown on BCYE agar (BBL, Becton Dickinson Microbiology Systems, Cockeysville, Md.) at 37°C in 5% CO<sub>2</sub> for 72 h. Bacterial cells were washed and resuspended in TES buffer (10 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA), and the turbidity was adjusted to optical density at 600 nm of 2.0. DNA was subsequently prepared as previously described (18, 20). The plugs were proteolyzed and washed as previously described (18). Restriction digestion of chromosomal DNA was performed with 20 U of *Sfi* I (New England Biolabs, Inc., Beverly, Mass.) for 4 h at the appropriate temperature. The plugs were loaded into a 1% PFGE-certified agarose gel prepared and run in 0.5× Tris-borate-EDTA buffer. PFGE was carried out by using the contour-clamped homogenous electric field system (CHEF-DRII; Bio-Rad) at 14°C and 200 V, with increasing switch times from 1 to 35 s, for 30 h. Bacteriophage lambda concatamers (48.5 kb; New England Biolabs) were used as molecular weight standards. After ethidium bromide staining, the gels were photographed with a UV light source.

A pattern designation (letters A to J) was assigned if the electrophoretic profile differed by more than three bands from a previously observed pattern. Subpatterns (e.g., A2 and C2) were defined by differences of three bands or less.

## RESULTS

Analysis of 43 isolates resulted in six different AP-PCR patterns (Tables 1 and 2). Three of these patterns were associated with strains from outbreaks of Legionnaires' disease (Table 1). The remaining three were unique patterns of isolates from sporadic cases of Legionnaires' disease (Table 2). PFGE analysis of these 43 strains resulted in 10 patterns and 4 subpatterns. A pattern designation (letters A to J) was assigned when the electrophoretic profile differed by more than three bands from a previously observed pattern. Subpatterns (e.g., A2 and C2) were defined by differences of three bands or less. Patterns A and C each have two subpatterns (designated A and A2 and C and C2, respectively). Pattern E was determined to have three distinct subpatterns (E, E2, and E3).

All isolates from the seven outbreaks fell into one of three AP-PCR patterns (Table 1). The patient isolates and the corresponding epidemiologically implicated environmental isolates from outbreaks 1, 2, and 6 gave the same AP-PCR pattern, which was designated AP-PCR pattern 1 (Fig. 1A). Philadelphia 1, the type strain for MAb subtype 1,2,5,6, also gave AP-PCR pattern 1 (Fig. 1A). The patient isolates and the

corresponding epidemiologically associated environmental isolates from outbreaks 3, 4, and 5 gave a second AP-PCR pattern, which was designated AP-PCR pattern 2 (Fig. 1A). The patient isolates and two isolates from the epidemiologically implicated environmental site from outbreak 7 gave AP-PCR pattern 3 (Fig. 1A).

All isolates from the seven outbreaks fell into one of four PFGE patterns or a subpattern (Table 1). The epidemiologically linked isolates from outbreaks 2 and 6 gave the same PFGE pattern when the restriction enzyme *Sfi* I was used. This pattern was designated PFGE pattern A (Fig. 1B). All epidemiologically linked isolates from outbreak 1 gave PFGE pattern A2 (Fig. 1B). Pattern A2 differed from pattern A by three bands. All AP-PCR pattern 1 isolates belonged to PFGE pattern A or A2 (Table 1). Philadelphia 1 gave PFGE pattern J (Fig. 1B). Isolates from outbreaks 4 and 5 both gave PFGE pattern B (Fig. 1B). Isolates from outbreak 3 gave pattern C and a subpattern designated C2 that differed by one band (Fig. 1B). PFGE pattern D was observed for isolates from outbreak 7 (Fig. 1B). PFGE patterns and subpatterns were assigned before the strain identification was revealed.

All other environmental isolates and some of the unrelated clinical isolates collected during the investigation of outbreak 1 fell into AP-PCR pattern 2 (Fig. 2A). These isolates varied in MAb subtype (Table 2), the majority being either MAb subtype 1,6 or subtype 1,2,5,6 (29). Three clinical isolates not related to the outbreak had unique AP-PCR patterns (Fig. 2A).

Of the 14 AP-PCR pattern 2 isolates not associated with an outbreak, 10 gave PFGE pattern F (Fig. 2B). Four of the 14 isolates belonged to a set of related PFGE patterns designated pattern E, E2, and E3. PFGE patterns E and E2 differed by one band, as did E2 and E3. Patterns E and E3 differed by two bands.

## DISCUSSION

AP-PCR and PFGE were each able to identify epidemiologically associated isolates when they were applied to the seven outbreaks. For each outbreak, AP-PCR and PFGE gave profiles that matched those of isolates from patients and from their sources of transmission, which were identified by epidemiologic investigations.

Six AP-PCR patterns were observed among the 43 strains tested. Three of these patterns, predominately patterns 1 and 2, belonged to isolates from the seven outbreaks. The remain-

TABLE 2. Summary of AP-PCR and PFGE subtyping of unrelated clinical and environmental isolates collected during the investigation of outbreak 1 (29)

| Source      | MAb type | AP-PCR type | PFGE type |
|-------------|----------|-------------|-----------|
| Environment | 1,6      | 2           | E         |
| Environment | 1,6      | 2           | E2        |
| Patient     | 1,2,5,6  | 2           | E3        |
| Environment | 1,6      | 2           | F         |
| Environment | 1,2,5,6  | 2           | F         |
| Environment | 1,2,5,6  | 2           | F         |
| Environment | 1,2,5,6  | 2           | F         |
| Environment | 1,6      | 2           | F         |
| Environment | 1,6      | 2           | F         |
| Environment | 1,6      | 2           | F         |
| Patient     | 1,6      | 2           | F         |
| Patient     | 1,2,5,6  | 4           | G         |
| Patient     | 1,2,5,6  | 5           | H         |
| Patient     | 1,2,5,7  | 6           | I         |

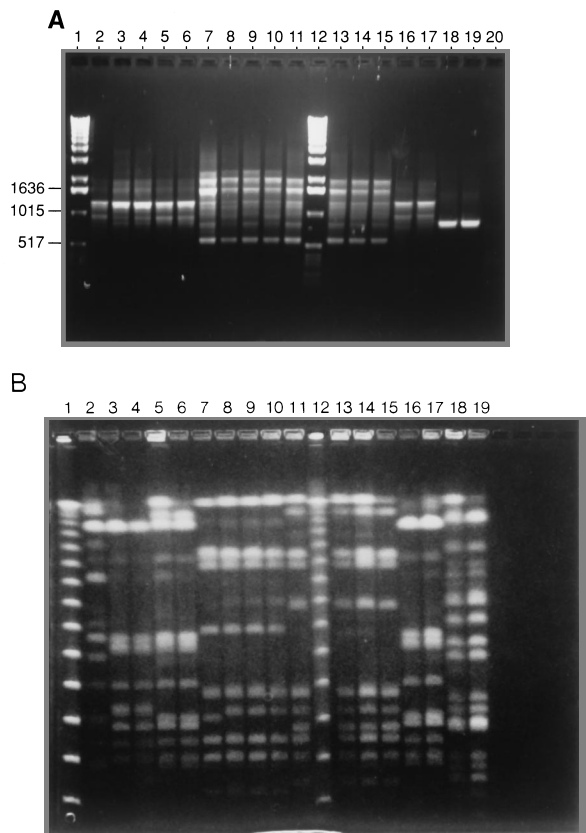


FIG. 1. AP-PCR (A) and PFGE (B) analysis of strains from seven outbreaks. Both gels have the same loading order. Lanes (AP-PCR and PFGE subtypes, respectively, are indicated in parentheses after the isolate identifications): 1, size markers; 2, Philadelphia 1 (1 and J); 3 to 4, environmental and clinical isolates from outbreak 1 (1 and A2); 5 to 6, environmental and clinical isolates from outbreak 2 (1 and A); 7 to 10, environmental and clinical isolates from outbreak 3 (2 and C); 11, environmental isolate from outbreak 4 (2 and B); 12, size markers; 13, clinical isolate for outbreak 4 (2 and B); 14 to 15, environmental and clinical isolates from outbreak 5 (2 and B); 16 to 17, environmental and clinical isolates from outbreak 6 (1 and A); 18 to 19, environmental and clinical isolates from outbreak 7 (3 and D); 20, no DNA control.

ing patterns were unique and not from strains related to outbreaks of Legionnaires' disease. Ten PFGE patterns and four subpatterns were seen.

As previously shown by van Belkum, we found that PFGE offered more discrimination than AP-PCR (28). PFGE divided the two major AP-PCR patterns into five groups. AP-PCR gave the same pattern for outbreaks 1 and 2, even though the isolates were of different monoclonal subtypes. PFGE could distinguish strains from these two outbreaks. PFGE detected a difference between the two environmental isolates of outbreak 3 that neither AP-PCR nor MAb analysis detected (Table 1). The discriminatory power of PFGE varies, depending on the restriction enzyme used. At this time, *Sfi* I appears to be the enzyme of choice for subtyping *Legionella* strains (16).

In certain cases, neither AP-PCR nor PFGE was able to discriminate among strains with differing MAb patterns. For example, strains from outbreaks 2 and 6 were shown to be identical by AP-PCR and PFGE, although the outbreak strains were of two different monoclonal subtypes. Similarly, strains from outbreaks 4 and 5 were also shown to be identical by both techniques but were also of different MAb subtypes. Better discrimination among strains is achieved when MAb analysis is used in combination with either AP-PCR or PFGE. Use of

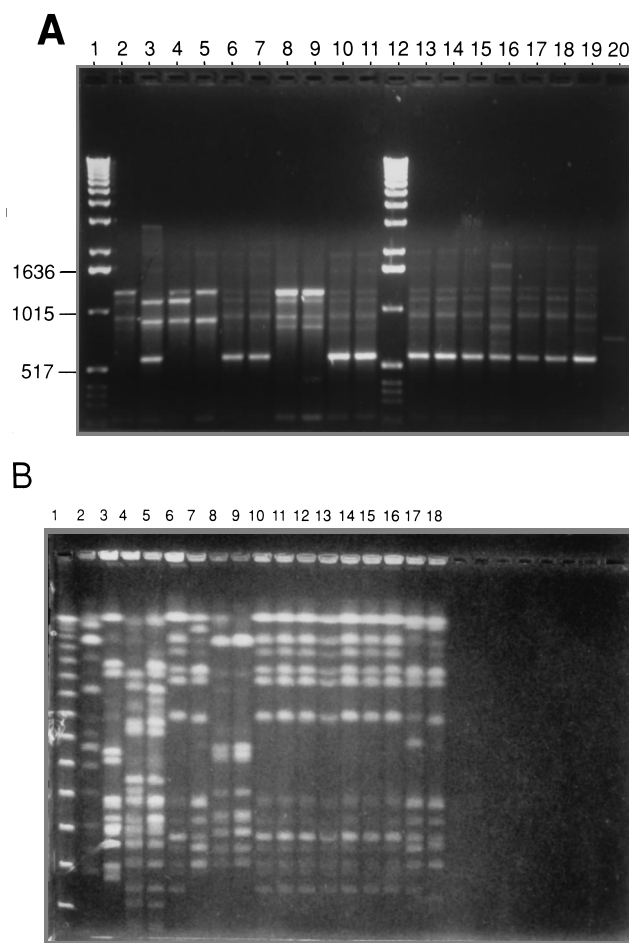


FIG. 2. AP-PCR (A) and PFGE (B) analysis of strains from outbreak 1. Lanes for panel A (AP-PCR type is indicated in parentheses after the isolate identification): 1, size markers; 2, Philadelphia 1 (1); 3 to 7, clinical isolates not related to outbreak (2, 4, 5, and 6); 8, clinical isolate related to outbreak (1); 9, environmental isolate from implicated cooling tower (1); 10 and 11, environmental isolates not related to outbreak (2); 12, size markers; 13 to 19, environmental isolates not related to outbreak (2); 20, no DNA control. Lanes for panel B (PFGE type is indicated in parentheses): 1, size marker; 2, Philadelphia 1 (J); 3 to 7, clinical isolates not related to outbreak (E3, F, G, H, and I); 8, clinical isolate related to outbreak (A2); 9, environmental isolate from implicated cooling tower (A2); 10 to 18, environmental isolates not related to outbreak (E, E2, and F).

MAb typing alone may be inadequate for epidemiologic investigations, since antigenic diversity among strains with related genomic profiles has been observed (25).

The discrimination offered by combined PFGE and MAb analysis is approximately equal to that of combined AP-PCR and MAb analysis. However, the international panel of MAbs is not readily available because of the loss of some of these cell lines. Therefore, until alternative hybridoma cell lines are identified, it is doubtful that this procedure will become more accessible. In the absence of complementary MAb subtyping, PFGE offers better discrimination than AP-PCR. However, PFGE is time-consuming (it takes as long as 3 days to complete) and labor intensive. Extreme care must be used in the isolation of the genomic DNA to prevent shearing. The DNA must be completely digested to allow analysis, and the gels must be run overnight to achieve optimal separation. While AP-PCR is a somewhat less discriminatory subtyping technique for strains associated with outbreaks of legionellosis,

AP-PCR is much less time-consuming and labor intensive. AP-PCR analysis can be performed within 8 to 10 h. The preparation of the DNA template and the AP-PCR mixtures takes approximately 1 h. The agarose gel electrophoresis can be completed within 3 hours. Extreme caution is necessary to prevent contamination of the templates or reaction mixtures. In the future, the discriminatory power of AP-PCR may be enhanced by the use of primers other than the M13 forward primer used.

During outbreak investigations, when MAb subtyping is unavailable, we suggest that AP-PCR be the initial molecular epidemiologic tool used to quickly differentiate clinical case isolates from isolates from environmental reservoirs of devices being evaluated as potential sources of transmission. However, because of the frequency with which some AP-PCR patterns occur, AP-PCR matches should be confirmed by PFGE.

Thirty-three of the 43 strains tested gave AP-PCR patterns 1 or 2. These two patterns span three different MAb subtypes. Similarly, two PFGE patterns were common for isolates of five of the seven outbreaks. As with AP-PCR, these PFGE patterns span three different monoclonal subtypes. This suggests that there are at least two predominate subtypes of *L. pneumophila* serogroup 1. Only a limited number (five) of isolates from sporadic cases of Legionnaires' disease (clinical isolates not associated with outbreaks) were tested. Of these five isolates, three gave unique patterns by AP-PCR and PFGE. This suggests that there are more subtypes of *L. pneumophila* serogroup 1 associated with sporadic cases. Conversely, one or two subtypes may be associated with the majority of outbreaks. Further studies are necessary to support this hypothesis.

In summary, molecular subtyping techniques such as AP-PCR and PFGE have become integral components in epidemiologic investigation of Legionnaires' disease. PFGE offers greater discrimination among *Legionella* isolates than AP-PCR, but AP-PCR is less time-consuming. Finding the same AP-PCR and PFGE patterns among isolates from unrelated outbreaks in this investigation and in those of others (15) reinforces the fact that these techniques must be used in the context of careful epidemiologic fieldwork.

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