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Use of Amplified Fragment Length Polymorphism in Molecular Typing of *Legionella pneumophila* and Application to Epidemiological Studies

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A novel method for molecular typing of organisms, amplified fragment length polymorphism analysis, was tested for its suitability in epidemiological studies in medical microbiology. Amplified fragment length polymorphism analysis, originally developed for typing crop plants, consists of a simple restriction-ligation reaction and a subsequent PCR amplification. In a single-step reaction, the genomic DNA is digested and the restriction fragments are ligated to specially constructed adapters. PCR amplification of such tagged restriction fragments with primers complementary to the adapters allows the detection of restriction fragment length polymorphisms upon resolution on agarose gels. The method is fast, efficient, and reproducible for typing strains of *Legionella pneumophila* isolated from both humans and the environment. The accuracy of the method was tested by comparison with standard restriction fragment length polymorphism typing performed with both a ribosomal and a genomic probe.

Several techniques for molecular typing of microorganisms have been developed in recent years. Among the best known are ribotyping, restriction fragment length polymorphism (RFLP) analysis, multilocus enzyme electrophoresis, and random amplified polymorphic DNA (RAPD) analysis. We present here a recent DNA typing method potentially suitable for epidemiological studies in medical microbiology: the amplified fragment length polymorphism (AFLP) method. The PCR-based method, originally developed in plant breeding and patented in 1992 (European patent application 0534858A1), allows the detection of RFLPs directly on agarose gels, eliminating the need for Southern blotting hybridization manipulations.

Description of AFLP methodology. The method consists of a simple restriction-ligation reaction followed by PCR amplification (see flow chart in Fig. 1). In a one-step reaction, the genomic DNA is digested with a specific restriction enzyme and specially constructed adapters are ligated to the restriction fragments that are generated. The adapters have three major features: they allow sticky-end ligation compatible with the endonuclease used for digesting the target DNA, eliminate the restriction sites after ligation, and create a template sequence for subsequent PCR amplification. Restriction fragments prepared by this method, referred to as "tagged fragments," are likely to be amplified in a standard PCR with primers complementary to the adapters. Length polymorphisms of PCR products can be easily resolved on agarose or polyacrylamide gels and can be detected by staining with ethidium bromide or silver. The different primers used for PCR amplification of the tagged fragments define the fragments that are to be amplified. Primers fully complementary to the adapters (nonselective primers) will amplify indiscriminately all tagged fragments, whereas primers with one or more nucleotides extending beyond the adapters at the 3′ end (selective primers) are expected to selectively amplify only part of the tagged fragment population.

AFLP analysis was carried out on a collection of strains belonging to a species of clinical interest: *Legionella pneumophila*, the main causative agent of legionellosis. The results obtained were compared with available information generated by standard RFLP analysis performed with both a ribosomal and a genomic probe.

MATERIALS AND METHODS

Bacterial strains. The set of 28 strains of *L. pneumophila* used in the study was from our collection, which includes clinical and environmental strains from widely different parts of Switzerland (Table 1). The strains used to generate Fig. 2 were epidemic isolates associated with nosocomial and community-acquired cases of legionellosis. The set of 18 strains used to generate Fig. 3 represents a random collection of isolates from different sources. The individual isolates were cultivated on αBCYE agar plates at 37°C for 3 days (4).

DNA extraction. Genomic DNA was extracted from cultures of *L. pneumophila* as described by Ausubel et al. (1). Briefly, bacterial cells cultured on αBCYE agar were resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) and lysed at 37°C for 1 h with proteinase K (50 μg/ml; Boehringer GmbH, Mannheim, Germany) and 1% sodium dodecyl sulfate. Polysaccharides and other contaminants were removed by a 10-min incubation at 65°C with 1% hexadecyl-trimethylammonium bromide in 0.7 M NaCl. After chloroform and phenol-chloroform extractions, DNA was precipitated in isopropanol, dried, and resuspended in TE buffer.

Restriction-ligation reaction. The restriction-ligation reaction was performed at 37°C for 3 h in a total volume of 20 μl. It consisted of approximately 1 to 2 μg of genomic DNA, 0.2 μg of each adapter-oligonucleotide (LG1, 5′-CTCGTA GACTGCGTACATGCA-3′, and LG2, 5′-TGTACGCAGTCTAC-3′; Fig. 1), 20 U of *Pst*I (Boehringer), 1 U of T4 DNA ligase (Boehringer), and ligase buffer (10 mM Tris [pH 7.5], 10 mM magnesium acetate, 50 mM potassium acetate, 2 mM dithiothreitol, and 0.5 mM ATP). The tagged DNA fragments were then precipitated by the addition of salt (ammonium acetate to a final concentration of 2.5 M in 100 μl) and 100 μl of chilled absolute ethanol. In order to selectively remove unused adapters, the incubation in ethanol did not exceed 5 min at room temperature and centrifugation was carried out at 4°C (12,000 × *g*, 10 min). After a washing step in 70% ethanol, the DNA was resuspended in TE buffer and was used as template for the PCR. The resuspended DNA was stored at −20°C and diluted with distilled water at the proper concentration just before use.

PCR. PCR was performed in a standard reaction mixture of 50 μl overlaid with paraffin oil (Merck, Darmstadt, Germany). The reaction mixture consisted of 1 ng of template DNA, 150 ng of each primer, 1 U of *Taq* polymerase (AmpliTaq; Perkin-Elmer Cetus, Norwalk, Conn.), 20 mM Tris-HCl (pH 8.3), 100 mM KCl, 5 mM $MgCl_2$, 100 mM (each) deoxynucleoside triphosphate, and 0.02% (wt/vol) gelatin. The mixture was subjected to 33 amplification cycles, as follows: 94°C for

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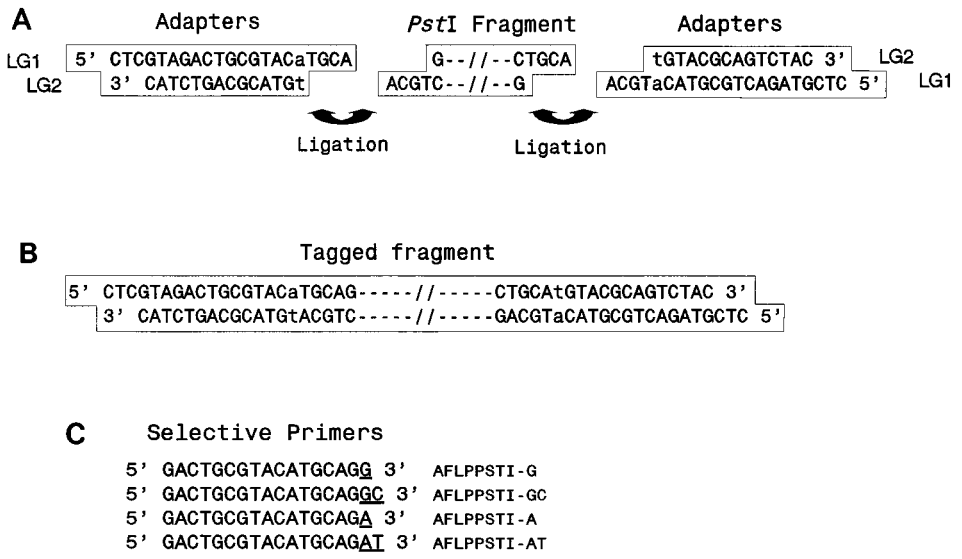

FIG. 1. Schematic flow chart of the AFLP procedure. (A) Adapters are designed to be complementary to each other and to allow sticky-end ligation with *Pst*I restriction fragments. (B) Tagged fragments represent the DNA template in the following PCR reaction. (C) Selective primers used in the final amplification step. Nucleotides in lowercase letters represent the "locking nucleotide" in the adapters responsible for the elimination of the *Pst*I restriction site (5′-CTGCA/G-3′) upon ligation. Underlined nucleotides are responsible for selectivity among AFLP primers.

1 min, 60°C for 1 min, and 72°C for 2.5 min. The amplified products were separated by standard horizontal gel electrophoresis on a 1.5% agarose gel in TBE buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA) and were stained with ethidium bromide (0.5 μg/ml).

Selection of PCR primers. Several primers were used for selective amplification of the tagged DNA fragments generated in the restriction-ligation reaction.

TABLE 1. Origins of strains

Strain code[a]	Source	Geographic origin
26	Patient, bronchial aspirate (case 1)	Canton of Ticino
324	Patient, bronchial aspirate (case 2)	Canton of Ticino
323	Patient, bronchial aspirate (case 3)	Canton of Ticino
134	Hot spring water (case 1)	Canton of St. Gallen
159	Water systems of hotel (case 1)	Canton of St. Gallen
160	Water systems of hotel (case 1)	Canton of St. Gallen
191	Water systems of hospital C (case 2)	Canton of Ticino
180	Water systems of hospital C (case 3)	Canton of Ticino
184	Water systems of hospital C (case 3)	Canton of Ticino
200	Water systems of clinic E (case 3)	Canton of Ticino
317	Reference strain NCTC 11230	United States
318	Reference strain NCTC 11232	United States
300	Patient, bronchial aspirate	Canton of Vaud
302	Patient, bronchial aspirate	Canton of Vaud
305	Patient, bronchial aspirate	Canton of Vaud
306	Patient, bronchial aspirate	Canton of Vaud
307	Patient, bronchial aspirate	Canton of Vaud
312	Patient, bronchial aspirate	Canton of Genève
319	Patient, bronchial aspirate	Canton of Genève
320	Patient, bronchial aspirate	Canton of Ticino
321	Patient, bronchial aspirate	Canton of Ticino
192	River Vedeggio	Canton of Ticino
193	River Bolletta	Canton of Ticino
17	Water systems of hospital A	Canton of Ticino
22	Water systems of hospital B	Canton of Ticino
80	Water systems of clinic D	Canton of Ticino
106	Water systems of hospital B	Canton of Ticino
158	Water systems of hot spring	Canton of St. Gallen

[a] The first 10 strains were involved in the three cases of legionellosis described in Fig. 2; the last 18 strains were used to generate Fig. 3.

Reported here, as model examples, are the following selective primers: AFLPP stI-G, 5′-GACTGCGTACATGCAG*G*-3′; AFLPPstI-GC, 5′-GACTGCGTA CATGCAG*GC*-3′; AFLPPstI-A, 5′-GACTGCGTACATGCAG*A*-3′; and AFLP PstI-AT, 5′-GACTGCGTACATGCAG*AT*-3′ (see also Fig. 1 and Fig. 3). Furthermore, the adapter oligonucleotide LG1 can be used as a nonselective primer for amplifying indiscriminately every tagged fragment.

Ribotyping and RFLP. Ribotyping and RFLP of *L. pneumophila* were performed as described previously (7). Briefly, DNA samples were concomitantly digested with restriction endonucleases *Pst*I and *Eco*RI (Boehringer), the digested products were separated by electrophoresis on a 0.8% agarose gel in TBE, and the DNA was transferred to a nylon membrane and probed with digoxigenin-labelled plasmids. The probes were as follows: for ribotyping, the plasmid pKK3535 (a pBR322-derived plasmid carrying the *rrnB* rRNA operon of *Escherichia coli* [3]); for RFLP analysis, a genomic *Eco*RI DNA fragment of *L. pneumophila* carried by pVG8, a pKNot plasmid (6).

RESULTS AND DISCUSSION

The potential use of the AFLP method for molecular typing of microorganisms was tested on a collection of *L. pneumophila* strains recovered from both the environment and symptomatic patients. As shown in Fig. 3 and discussed later, the method detects sufficient diversity among natural isolates.

In a previous study (7), transmission of particular strains from the environment to patients through contaminated water was assessed by ribotyping, an established technique in epidemiological surveillance of nosocomial outbreaks (2, 8). The typing information generated by ribotyping and RFLP analysis based on a genomic probe constructed in our laboratory (subsequently referred to as "RFLP"; unpublished data) was taken as a reference data set for AFLP evaluation. Ten strains involved in three cases of legionellosis among symptomatic patients and isolated from the patients and their living environments were subjected to AFLP, ribotyping, and RFLP analyses (Fig. 2). By comparing the profiles of strains isolated from patients with those from their living environments, it was possible to unequivocally assess the transmission of the pathogen by all three methods. In the first clinical case, the patient was infected during a thermal bath cure in the canton of St. Gallen. Among the strains isolated from the thermal bath establishment (strains 134, 159, and 160), strains 134 and 159 showed profiles identical to that of strain 26, which was isolated from

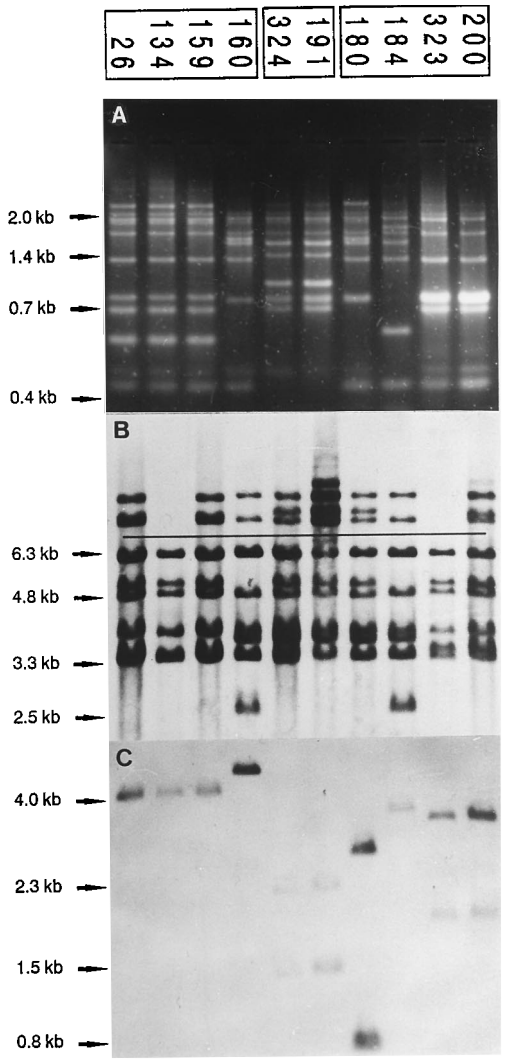

FIG. 2. AFLP (A), ribotyping (B), and RFLP (C) analyses of *L. pneumophila* strains involved in three cases of legionnaires' disease. Strains represent both the patients and their living environments. AFLP analysis was performed by using the selective primer AFLPPstI-G. Case 1 included patient strain 26 and environmental strains 134, 159, and 160; case 2 included patient strain 324 and environmental strain 191; and case 3 included patient strain 323 and environmental strains 180, 184, and 200. The positions of DNA size standards are shown on the left of each gel. Bands above the line in ribotyping profiles represent partial digests and should not be considered in comparisons of strains.

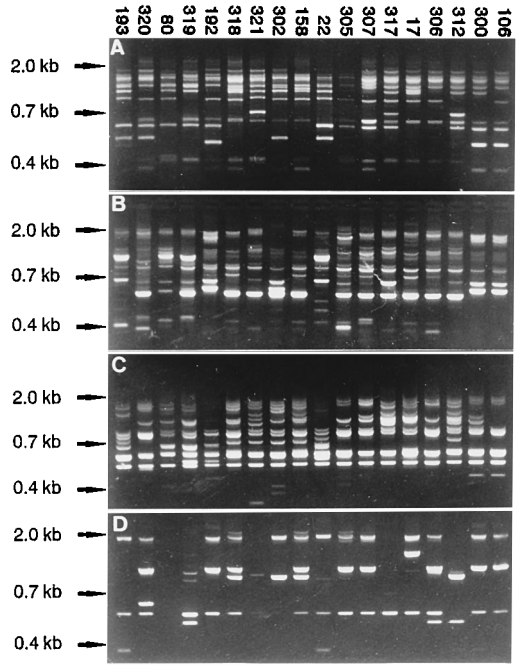

FIG. 3. AFLP analysis performed on a set of 18 isolates randomly chosen from our collection showing genetic diversity in *L. pneumophila*. The analysis was performed with four different selective primers: AFLPPstI-G (A), AFLPPstI-GC (B), AFLPPstI-A (C), and AFLPPstI-AT (D). The positions of DNA size standards are shown on the left of each gel.

the patient. In the second clinical case, the patient (characterized by strain 324) was infected during a stay in hospital C (characterized by strain 191); both strains presented identical typing profiles. In the third clinical case, the patient was transferred from clinic E to hospital C with an interstitial pneumopathy; a few days later a strain of *Legionella* was isolated from the patient (strain 323). In this patient, the infection was acquired in the clinic (strain 200), prior to hospitalization, and not in the hospital (strains 180 and 184).

The AFLP method presents several advantages when it is compared with other typing methods. It allows the detection of RFLPs directly on agarose gels by using the PCR technology. Tedious manipulations, such as gel transfer on solid membrane supports and probe hybridization, are not needed for AFLP analysis, making this method easier to perform than the traditional Southern blotting hybridization used for RFLP studies. AFLP analysis also presents obvious technical advantages with respect to multilocus enzyme electrophoresis and DNA-DNA hybridization techniques, in particular, avoidance of delicate native enzyme stainings following starch gel electrophoresis or time-consuming manipulations involved in DNA extraction, labelling, and hybridization. The flexibility of AFLP manipulations is indeed comparable to that of RAPD technology. RAPD analysis is more versatile than AFLP analysis with respect to primer screening, because for AFLP analysis, a new DNA matrix is required for each adapter-enzyme system to be screened. However, the reliability of AFLP analysis is expected to be greater, provided that the PCR system is accurately designed under optimal conditions for primer annealing. The amount of DNA required for AFLP analysis is small: 1 to 2 μg of purified genomic DNA. Furthermore, the DNA template produced in the restriction-ligation reaction can be used for hundreds of PCR amplifications, allowing screening of several selective primers and making AFLP analysis a convenient method when speed and simplicity are required. In our hands, AFLP profiles could be reproduced with fidelity starting from independent cultures of the same strain (data not shown). Finally, PCR performed with intact genomic DNA instead of restricted-ligated DNA generated, at best, barely visible smears after gel separation of the amplification products.

In order to generate informative profiles, a reasonable number of polymorphic bands is required. To achieve this objective, the choice of a suitable restriction enzyme is crucial in AFLP analysis. The endonuclease used should be adapted to the GC content and the size of the organism's genome to be tested. In the present study, AFLP analysis with *Pst*I as the restriction enzyme (GC-rich consensus sequence 5′-CTGC AG-3′) was appropriate for typing *L. pneumophila* but not for typing *Borrelia burgdorferi* sensu lato. While multiple banding profiles could be generated with the *Legionella* strains, less

informative patterns were obtained with *Borrelia* strains (data not shown). This might be due to the small size (about 10^6 bp) and low GC content (about 27%) of the *Borrelia* genome (5).

Another important point in AFLP analysis is the choice of an appropriate primer for PCR amplification. Two categories of primers can be distinguished: selective ones, extending into the restriction fragment, and nonselective ones, not extending beyond the adapter. Whereas selective primers amplify only a portion of the tagged fragment population, nonselective primers amplify the whole population. AFLP profiles differ according to the selective primer used (Fig. 3). In Fig. 3, the degree of discrimination of AFLP among a random selection of *L. pneumophila* strains is illustrated in an analysis of a collection of 18 isolates.

Further resolution of genetic diversity may be achieved by digesting AFLP reaction mixtures with restriction enzymes. This might allow a further distinction of strains presenting identical AFLP profiles.

To our knowledge, this is the first application of AFLP analysis in the field of clinical microbiology. Analysis of more pathogens by this technology should confirm its usefulness in broad practice.

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