Comparison of Three Molecular Methods Used for Subtyping of *Legionella pneumophila* Strains Isolated during an Epidemic of Legionellosis in Rome

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In the summer of 2003 a community-acquired outbreak of *Legionella pneumophila* occurred in Rome, Italy. Three molecular typing methods, pulse-field gel electrophoresis, amplified fragment length polymorphism analysis, and sequence-based typing (SBT), were used to establish the clonal correlation among the isolates of the epidemic cluster. By comparison of the methods, SBT was the most rapid and the easiest to perform and provided unambiguous results.

Legionella naturally inhabits protozoa aquatic environments as a parasite. The increased frequency of outbreaks of *Legionella pneumophila* is primarily due to the contamination of artificial aquatic environments. Molecular typing with associated epidemiological investigations can help establish the link between clinical and environmental isolates and identify the source of infection (2, 17, 24). However, the efficiencies of the various typing methods vary, and not all typing methods are standardized (14, 26, 27). The need for extensive comparison among the different molecular typing methods is widely recognized (3, 7). By exploiting the need for molecular characterization of isolates from a recent epidemic of *L. pneumophila* serogroup 1 infection to establish the source of infection, in the present study we have compared three molecular typing methods. The outbreak occurred in the period from August to October 2003 in a neighborhood of Rome, with 15 notified cases, including one death (21). In accordance with epidemiological investigations, microbiological analyses by incubation of α -buffered charcoal yeast extract (Oxoid, United Kingdom) agar plates at 37°C with 2.5% CO₂ showed *L. pneumophila* contamination of water and air samples from the cooling tower of a shopping center in the area. Monoclonal antibody (MAb) typing, performed by indirect immunofluorescence assay with the “Dresden MAb panel” (14), revealed the common subgroup Philadelphia for the single clinical epidemic-related isolate and the nine environmental epidemic-related isolates.

As serotyping is insufficient as a means of ascertaining the actual source of contamination (3, 15), the genetic relatedness between human and environmental strains was determined both by comparison of the genomic profiles by pulse-field gel electrophoresis (PFGE) and amplified fragment length polymorphism analysis (AFLP) analysis and by comparison of the nucleotide sequences of five genes by sequence-based typing (SBT). Data from the epidemic-related isolates were also compared to data from unrelated strains.

PFGE was performed as described by Castellani Pastoris et

al. (5) by using 20 U of NotI (Roche, Italy) for overnight macrorestriction at 37°C of the genomic DNA plugs. *Saccharomyces cerevisiae* chromosomal DNA (Bio-Rad, Hercules, Calif.) was used as a size marker. As shown in Fig. 1, PFGE produced genomic patterns that were the same for the clinical and the environmental isolates from the epidemic, but these patterns were dissimilar from those for the control strains. The NotI enzyme was chosen because of its rare restriction sites in the genome of *Legionella*, in which it produces only a few high-molecular-weight fragments. However, comparison of the genomic profiles showed very thick bands that could be constituted by one or more unresolved fragments. In addition, macrorestriction of only one of the epidemic-related environmental samples produced a faint additional band that was absent from the other isolates. This might constitute a confounding factor in the attribution of clonality. Although the results obtained by PFGE were overall in agreement with those obtained by MAb typing, the isolates of *L. pneumophila* with the same patterns might not come from the same environmental source (18). Therefore, these results, even if they are reliable (16, 22), should be used with caution (7, 25). On the other hand, PFGE is time-consuming, technically difficult, and somewhat cumbersome to perform because of the need to prepare plugs with fresh bacterial cultures, lyse the bacteria, and define the correct parameters for the electrophoretic run.

The second method used in this study was AFLP analysis, currently considered the “gold standard” for the typing of *L. pneumophila* (10, 11). PCR amplification was performed both by using puRe *Taq* Ready-to-Go PCR beads (Amersham BioSciences, United Kingdom) and by using 2.5 U of *Taq* polymerase from a commercial PCR kit (Applied Biosystems) with 75 ng selective primer AFLP-PstI-G (5'-GACTGCGTACAT GCAGG-3'). The MBI Fermentas Ladder Mix (MBI Fermentas, United Kingdom) was used as a molecular size marker. AFLP analysis is technically easier and faster to perform than PFGE and is less expensive. In our study, it confirmed the high similarity of the DNA fingerprints of the cluster-related clinical and environmental isolates (Fig. 2), but AFLP analysis allowed a more reliable comparison than PFGE, because it showed more numerous smaller fragments. Remarkably, as shown in Fig. 2A, the environmental strain unrelated to the epidemic

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FIG. 1. PFGE analysis of genomic DNA from a clinical isolate (sample in lane 1) and environmental isolates (samples in lanes 2 to 10) from the epidemic. The samples in lanes 11 and 12 were unrelated environmental and clinical strains, respectively, and the sample in lane 13 was the *L. pneumophila* ATCC 33152 type strain. Lanes M, molecular weight marker (*S. cerevisiae*).

had a profile very similar to those of the isolates involved in the epidemic, whereas unrelated clinical and type strains had clearly different banding patterns. Figure 2B shows the results of AFLP analysis performed for comparative purposes with *Taq* polymerase from an Applied Biosystems commercial PCR kit. More bands were detected for the environmental isolates from the epidemic, while unrelated strains had a different genomic pattern. More relevant general profiles were different, depending on the *Taq* polymerase used; therefore, comparison of the genome types with those in the AFLP database (www.ewgli.org) could give incorrect results. Moreover, as in PFGE, the analysis of the AFLP banding patterns by visual or computational methods proved to be difficult because band discrimination is subjective.

SBT, proposed by Gaia et al. for *L. pneumophila* serogroup 1 and non-serogroup 1 (12, 13), was also used in this study. We sequenced both strands of the amplicons of five of the six genes (*flaA*, *proA*, *mip*, *asd*, and *pilE*) suggested to be used for sequencing with the primers described by Gaia et al. (13). The sequences were determined by using an ABI PRISM BigDye terminator DNA sequencing kit and were analyzed on a 310 ABI DNA sequencer (Applied BioSystems). The sequenced amplicons of each gene from the isolates from the epidemic showed 100% identity. These sequences were compared with those in the European Working Group for Legionella Infections (EWGLI) SBT database, and for every gene, only the sequences of the isolates from the epidemic all matched the same allelic target with the same allelic number (Table 1). The control strains matched different alleles. The results were always clear, reliable, and reproducible. Although all molecular methods demonstrated the clonal relationship of the epidemic

FIG. 2. AFLP analysis. (A) AFLP performed according to the version 1.2 standard EWGLI AFLP protocol; (B) AFLP performed with *Taq* polymerase from a commercial PCR kit. The genomic patterns of the clinical isolate (sample in lane 1) and environmental isolates (samples in lanes 2 to 10) from the epidemic are shown. The samples in lanes 11 and 12 are unrelated environmental and clinical strains, respectively. The sample in lane 13 is the *L. pneumophila* ATCC 33152 type strain. Lanes M, MBI Fermentas ladder mix.

cluster, SBT was sufficiently rapid and the easiest to perform by using the same PCR cycle and the same sequencing analysis for all genes. Although AFLP analysis and SBT have similar performance times, for each PCR, SBT requires only 50 ng genomic DNA instead of the 1.5 μ g required for AFLP. This means that minipreparations of genomic DNA (1) are sufficient for performance of the SBT assay, which is relevant in typing experiments. Moreover, if we consider that molecular biology laboratories usually have a sequencer, the costs of the equipment required to perform SBT, as well as materials and labor, are similar to those required to perform AFLP analysis, but the results of SBT are unambiguous and reproducible.

SBT has been used successfully to type other bacteria for

TABLE 1. Allelic profiles of the *L. pneumophila* serogroup 1 strains used in this study

Strain ^a	Allele no.				
	<i>flaA</i>	<i>pilE</i>	<i>asd</i>	<i>mip</i>	<i>proA</i>
1	2	3	9	10	1
2	2	3	9	10	1
3	2	3	9	10	1
4	2	3	9	10	1
5	2	3	9	10	1
6	2	3	9	10	1
7	2	3	9	10	1
8	2	3	9	10	1
9	2	3	9	10	1
10	2	3	9	10	1
11	1	4	3	1	1
12	3	4	1	1	1
13	3	4	1	1	9

^a Strains 1 to 10 are of the epidemic cluster; strain 11 and 12 are environmental and clinical-unrelated strains, respectively; strain 13 is the *L. pneumophila* ATCC 33152 type strain.

both evolutionary and epidemiological studies (9, 17, 19). Multilocus sequence typing has been developed to determine the allelic variations of multiple housekeeping genes (8, 20, 23). Recently, some studies have explored the suitability of sequencing single genes to differentiate bacterial strains (4, 6). DNA sequence typing has multiple advantages over fingerprinting-based methods: less subjectivity in interpretation of the results, transferability of the data among laboratories, the portability of the data through web-based databases, and the opportunity for use of the data to probe pathogen evolution and to track the spread of clonal groups. Therefore, the use of the SBT method for robust genotyping with high discriminatory power is warranted, and the method is proposed as the new gold standard for the typing of *L. pneumophila* (12, 13).

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