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# Genetic Characterization of *Legionella pneumophila* Serogroup 1 Associated with Respiratory Disease in Australia

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**Techniques were developed for genetic characterization of *Legionella pneumophila* serogroup 1 by using restriction fragment length polymorphism analysis. Allozyme analysis provided an index of the discrimination achieved by restriction fragment length polymorphism. Isolates from human cases of legionellosis were examined by both methods, and their profiles were compared with reference strains of *L. pneumophila* serogroup 1 obtained from the American Type Culture Collection. Eighteen distinct clones were evident among the isolates examined. Both methods could be used to trace the source of an outbreak of legionellosis caused by *L. pneumophila* serogroup 1.**

Infections caused by *Legionella* spp. continue to be a public health problem in Australia. Several outbreaks of legionellosis have occurred in the last 5 years, caused mainly by *L. pneumophila* serogroup 1. Recently a cluster of cases of pneumonia caused by *L. longbeachae* serogroup 1 was reported from South Australia (10). All human and most environmental isolates of this organism were shown to be very similar genetically when allozyme analysis and restriction fragment length polymorphism (RFLP) typing were used to compare them (4). We have now developed a similar typing system for *L. pneumophila* and have used it to examine isolates from sporadic cases in South Australia and outbreaks of legionellosis in Eastern Australia.

A total of 21 Australian strains of *L. pneumophila* serogroup 1, all isolated from humans with legionellosis, were available for genetic characterization. These comprised 14 from South Australia (SA1 through SA14) isolated from sporadic cases over several years and not associated with outbreaks and 1 from New South Wales (NSW1) and 6 from Tasmania (TAS1 through TAS6) which were all associated with an outbreak. Eighteen strains of *L. pneumophila* serogroup 1 isolated from environmental sources linked to the Tasmanian outbreak (TAS7 through TAS24) were also examined. A further 10 isolates from the American Type Culture Collection (ATCC) were included as reference strains Allentown (ATCC 43106), Bellingham (ATCC 43111), Benidorm (ATCC 43108), Camperdown (ATCC 43113), France (ATCC 43112), Heysham (ATCC 43107), Knoxville (ATCC 33153), Olda (ATCC 43109), Oxford (ATCC 43110), and Philadelphia (ATCC 33152). The methods used to isolate and identify *Legionella* spp. were those detailed in previous papers (5).

RFLP analysis was performed as detailed previously (10). A genomal bank of *L. pneumophila* serogroup 1 (Philadelphia strain) was constructed in cosmid pHC79 by the method described by Manning et al. (6). Recombinant plasmids were purified from individual clones selected randomly from the bank by the method of Holmes and Quigley (2).

The vector with insert was labeled with digoxigenin (Boehringer GmbH, Mannheim, Germany) or $^{32}P$ by using the manufacturer's protocol. Several clones were tested empirically to find those which gave optimal differentiation of isolates by Southern hybridization. One clone, designated IMVS65, was subsequently chosen as suitable for detailed analysis. Two characterizations were performed on each isolate, one by using the restriction enzyme *Nci*I and another by using double digestion with *Hin*dIII-*Bam*HI.

A second set of RFLP profiles was obtained by Southern hybridization of isolates with *Escherichia coli* 16S and 23S rRNA (Pharmacia LKB Biotechnology, Uppsala, Sweden) end labeled with [$\gamma$-$^{32}P$]dATP.

Filters were hybridized overnight at 42°C in hybridization fluid (50% formamide, 7% sodium dodecyl sulfate, 1% skim milk powder, 5× SSPE [1× SSPE is 0.18 M NaCl–10 mM sodium phosphate–1 mM EDTA], 200 μg of salmon sperm DNA per ml), and labeled recombinant probe DNA or rRNA was added. The next day, filters were washed twice for 10 min at room temperature in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0])–0.1% sodium dodecyl sulfate and then once at 68°C in 1× SSC–0.1% sodium dodecyl sulfate for 45 min and autoradiographed at −70°C.

Allozyme analysis was undertaken on cellulose acetate gels by the methods of Richardson et al. (7). A wide range of enzymes was examined initially to determine suitable genetic markers in *L. pneumophila*. As a result, 37 enzymes, encoding a presumptive 42 loci, were selected as suitable for genetic characterization. Details concerning the enzymes, electrophoretic conditions, and histochemical stains are presented in a previous study on *L. longbeachae* (4). The present study utilized the same enzymes with the exception of enzymes acid phosphatase, glutamate dehydrogenase, glucose-6-phosphate dehydrogenase, glucose-phosphate isomerase, glutathione reductase, phosphaglucomutase, and unidentified kinase (UK [4]), which were not sufficiently active to be useful in *L. pneumophila*.

The isolates fell into 15 electrotypes (ETs) (Table 1). Pairwise comparisons between ETs indicate that the number of allozyme differences ranged between 1 in 42 (ET-1 versus ET-10 and ET-11 versus ET-12) and 23 in 42 (ET-4 versus ET-9), with most ETs being distinguished from all others at numerous loci. The six Tasmanian isolates from humans were obtained during an outbreak of legionellosis and possessed identical ETs and RFLP patterns. For convenience, only one Tasmanian isolate is represented here. In contrast,

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TABLE 1. Clones identified in this study, along with the discriminating ability of each of the molecular characterizations performed

| Clone(s)[a] | ET | RFLP pattern[b] with probe: | | |
|---|---|---|---|---|
| | | rRNA | c65 (*Nci*I) | c65 (*Hin*dIII-*Bam*HI) |
| Olda | ET-1 | A | A | A |
| SA2 | ET-1 | A | A | B |
| SA4, -5, -6, -8, -9, -10 | ET-2 | A | B | C |
| SA3 | ET-2 | A | B | D |
| SA7 | ET-2 | B | C | E |
| SA11, NSW1 | ET-3 | A | D | F |
| SA12, TAS1 to TAS6 | ET-4 | B | E | G |
| Philadelphia | ET-5 | C | F | H |
| Knoxville | ET-6 | D | G | I |
| Bellingham | ET-7 | D | G | J |
| SA13 | ET-8 | A | H | K |
| SA14, Benidorm | ET-9 | A | I | L |
| Camperdown | ET-10 | ND | J | M |
| Heysham | ET-11 | ND | K | N |
| Oxford | ET-12 | ND | L | M |
| SA1 | ET-13 | A | M | O |
| France | ET-14 | ND | N | P |
| Allentown | ET-15 | ND | O | Q |

[a] Australian isolates are designated numerically according to state of origin. Only strains isolated from humans are included in the table; ATCC isolates are given common names.

[b] Each distinct RFLP pattern is designated by a letter (A to Q). c65, cosmid IMVS65. ND, not determined.

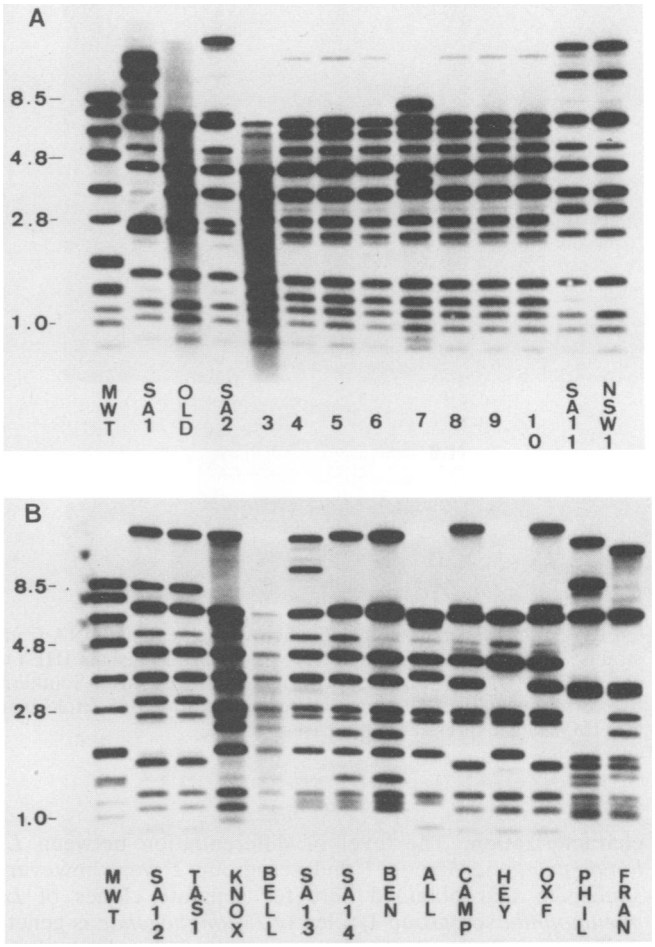


FIG. 1. RFLP profiles obtained when whole genomal DNA from strains of *L. pneumophila* was digested with *Hin*dIII-*Bam*HI and reacted with cosmid IMVS65 labeled with $^{32}P$. (A) Lanes (left to right): molecular size markers (in kilobases), SA1, Olda, SA2 to SA11, NSW1. (B) Lanes (left to right): molecular size markers (in kilobases), SA12, TAS1, Knoxville, Bellingham, SA13, SA14, Benidorm, Allentown, Camperdown, Heysham, Oxford, Philadelphia, France.

the South Australian isolates were isolated sporadically over several years.

RFLP analysis using rRNA as the probe (ribotyping) (1) did not provide as much differentiation among strains as did allozyme analysis (data not shown). However, RFLP typing using the selected cosmid clones was much more successful in this regard (Table 1).

Figures 1A and B are profiles obtained when digests of ATCC reference strains and Australian human isolates of *L. pneumophila* serogroup 1 were reacted in Southern hybridization studies with $^{32}P$-labeled cosmid IMVS65. RFLP profiling using cosmid IMVS65 provided a differentiation of the isolates which was similar to that obtained by allozyme analysis with the following four exceptions. The RFLP patterns for isolates SA3 and SA7 in ET-2 were different from the others when digested with *Nci*I and *Hin*dIII-*Bam*HI (Fig. 1A). SA7 possessed extra bands at 7.5 and 3.8 kb, and SA3 and SA7 did not have the faint band above 9 kb seen in others in ET-2.

Reference strains Bellingham and Knoxville possessed identical RFLP profiles when digested with *Nci*I but differed when digested with *Hin*dIII-*Bam*HI (Fig. 1B). Strains Oxford and Camperdown produced the same profiles when digested with *Hin*dIII-*Bam*HI (Fig. 1B) but differed when digested with *Nci*I (Fig. 2).

Overall, the discriminating abilities of RFLP and allozyme characterization are remarkably similar, suggesting that we have indeed been able to distinguish clones or near clones among the isolates screened. Our results suggest that techniques for RFLP analysis should be developed by using several enzymes and results should be compared with those obtained by another technique, such as allozyme analysis, to ensure the most appropriate combination of restriction enzyme and probe is used to provide the discrimination required.

There were 9 distinct clones present among the Australian isolates, with 6 of the 14 from South Australia belonging to one clone (Table 1). All Australian clones were genetically different from the ATCC strains except for SA14, which was identical to Benidorm, and SA2, which resembled Olda. SA2 was isolated from a patient after renal transplantation.

The 18 environmental isolates examined as part of the investigation into the Tasmanian outbreak were distributed into two groups. Four had RFLP profiles and ETs identical to those of the six strains isolated from humans involved in the outbreak. All four were isolated from an air-conditioning unit epidemiologically associated with the outbreak. All the other environmental strains were identical to the Olda type strain.

The results of this study plus those of Selander et al. (9) and Saunders et al. (8) present a great contrast to those obtained for *L. longbeachae* (4), for which all serogroup 1 isolates gave identical profiles after allozyme and RFLP

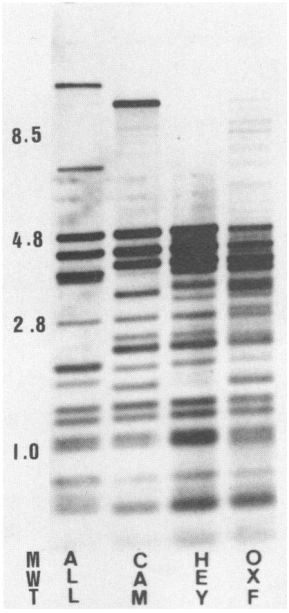


FIG. 2. RFLP profile obtained when whole genomal DNA from strains Allentown (ALL), Camperdown (CAM), Heysham (HEY), and Oxford (OXF) is digested with *Nci*1 and reacted in Southern hybridizations with digoxigenin-labeled cosmid IMVS65. Molecular size (MWT) is expressed in kilobases.

characterization. The level of differentiation between *L. longbeachae* serogroup 1 and serogroup 2 was, however, similar to that obtained here for separate clones of *L. pneumophila* serogroup 1. Clearly *L. longbeachae* is genetically more depauperate than even a small subset of *L. pneumophila*, an observation which is consistent with either a comparatively recent evolutionary origin for *L. longbeachae* or a severe genetic bottleneck some time in its recent past.

When RFLP analysis as described above was used in the laboratory investigation of a second outbreak of legionellosis in New South Wales, a strain identical to Philadelphia was isolated from both the legionellosis case and an environmental source. Other strains isolated as part of the investigation were similar to Olda and another RFLP type not encountered by us previously (data not shown).

In summary, although outbreak tracing using the panel of monoclonal antibodies developed by Joly et al. (3) is likely to be more rapid and cheaper, for those laboratories unable to obtain the monoclonal antibodies, RFLP or allozyme analysis offers a sensitive option with similar specificity. For a diagnostic or reference laboratory with no experience in either technique, RFLP analysis would be simpler to adopt than allozyme analysis and the results would be more easily read if a good discriminatory probe is freely available. Reports demonstrating the efficacy of RFLP typing have appeared previously (8). Both techniques have the advantage over monoclonal antibody panels of requiring less development before use.

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