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NOTES

DNA Polymorphisms in Strains of *Legionella pneumophila* Serogroups 3 and 4 Detected by Macrorestriction Analysis and Their Use for Epidemiological Investigation of Nosocomial Legionellosis†

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Genomic DNAs of clinical and environmental isolates of *Legionella pneumophila* belonging to serogroups 3 and 4 were analyzed by macrorestriction analysis by pulsed-field gel electrophoresis. The restriction enzymes *Sfi*I and *Not*I allowed easy visual separation of epidemiologically unrelated serogroup 3 strains. Three unrelated serogroup 3 strains that were isolated from different locations were identical by this genome mapping technique. Five unrelated serogroup 4 strains were separable by this technique. The electrophoretic patterns obtained after *Sfi*I or *Not*I cleavage of the DNA of strains isolated from four patients with hospital-acquired legionellosis were identical to the patterns of strains isolated from the hot water supply systems of the buildings in which the patients were hospitalized. In conclusion, macrorestriction analysis is a valuable tool for epidemiological studies of infections caused by *L. pneumophila* serogroups 3 and 4.

Legionella pneumophila accounts for a high percentage of cases of nosocomial pneumonia. Patients with impaired host defenses are at increased risk for acquiring legionellosis. Fifteen serogroups of *L. pneumophila* have been defined by using absorbed polyclonal rabbit antisera (1). Among clinical and environmental strains, serogroup 1 is the most common (14, 17). Other serogroups account for 10 to 20% of clinical isolates and 20 to 60% of environmental isolates (8, 11, 17). In epidemiological investigations, molecular subtyping techniques such as monoclonal antibody typing (3, 5, 6, 16), restriction enzyme analysis (16), hybridization-based restriction fragment length polymorphism analysis (3, 13, 16), multilocus enzyme electrophoresis (1), and arbitrarily primed PCR (3, 16) have been used to subtype *L. pneumophila* serogroup 1 strains. However, only a few studies in which subtyping techniques were applied to other serogroups of *L. pneumophila* were performed (4, 13, 15). We used macrorestriction analysis by pulsed-field gel electrophoresis to subtype *L. pneumophila* strains belonging to serogroups 3 and 4, and the usefulness of this technique for detecting the causative strains in the hospital water supply systems in four cases of nosocomial legionellosis is reported here.

In this study, which lasted from July 1986 to August 1987, we investigated *Legionella* strains isolated from patients and strains isolated from the hospital hot water system (environmental strains). All patients had clinical and roentgenological evidence of pneumonia combined with severe underlying diseases (Table 1). Patients' strains were isolated from sputum samples or tracheal secretions on selective buffered charcoal-yeast extract agar (Medium Wadowsky Yee; Unipath, Wessel, Germany) (2, 7). Briefly, clinical samples were homogenized, diluted 1:2 to 1:4 in 1% KCl solution and heated for 5 min at 60°C (2, 7). Patients' isolates were serotyped as serogroup 3 (two patients) and serogroup 4 (two patients). For serotyping, absorbed serogroup-specific rabbit sera for all 14 serogroups of *L. pneumophila*, strain Lansing-3 (serogroup 15), and 16 other species of *Legionella* prepared in our laboratory and fluorescein isothiocyanate-conjugated antibodies to serogroups 1 to 8 (Centers for Disease Control and Prevention, Atlanta, Ga.) were used (7, 11). When more than one colony was grown from a clinical sample, all colonies were identified as belonging to the same serogroup. Thus, we did not observe any case of multiple infection caused by strains belonging to different serogroups. Pooled antigens of *L. pneumophila* serogroups 1 to 8 were used in the indirect immunofluorescence assay for the detection of antibodies in patients' sera. From three patients, acute-phase serum samples were available. All were negative in the indirect immunofluorescence assay. Patient 2 showed a nonsignificant titer of 64 on day 9 of his illness. This serum sample was not retested against monovalent antigens. All cases of infection occurred sporadically during the 14-month study period (Table 1). Despite intensive searching, no cases have been observed since 1987.

Fifty-seven environmental samples were collected from central heaters ($n = 20$) and peripheral outlets of the water systems ($n = 37$) in 11 clinics (buildings) of the university in Heidelberg. All facilities are separate buildings with separate hot water supply systems that are supplied by the same cold

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TABLE 1. Clinical, epidemiological, and microbiological data for patients

Patient	Age	Sex	Underlying disease(s) or condition(s)	Cytostatic therapy	Radiation therapy	*L. pneumophila* serogroup(s)		Date of infection	Patient outcome
						Isolated from patients	Prevalent in hospital water supply		
1	76	Male	Adenocarcinoma of the lung, diabetes mellitus, and chronic emphysematous bronchitis	No	Yes	3	1, 3 (clinic H)	March 1987	Recovery
2	64	Male	Glioblastoma multiform and craniotomy	No	No	3	1, 3, 4 (clinic E)	July 1987	Recovery
3	21	Female	Metastasis of leiomyosarcoma	Yes	No	4	1, 3, 4 (clinic E)	October 1986	Recovery
4	48	Male	T-cell lymphoma	Yes	No	4	1, 3, 4 (clinic E)	August 1987	Death

water inlet. The water specimens were concentrated by filtration through polycarbonate filters (pore size, 0.2 μm; Nuclepore, Heidelberg, Germany) or plated unconcentrated on the selective buffered charcoal-yeast extract agar plates (7). Suspected colonies were picked and subcultured on buffered charcoal-yeast extract agar without antibiotics and on sheep blood agar. Colonies that grew on the former agar but not on the latter were serotyped. By using absorbed rabbit antisera for serogroups 1 to 15, strains of *L. pneumophila* serogroups 1, 3, 4, and 5 were identified. The occurrence of serogroups in the individual water systems was not significantly different between central and peripheral samples or among the 11 clinics studied.

Cases of legionellosis occurred in two buildings. This probably reflects the increased susceptibility of some of the patients rather than an elevated hygienic risk for all of the patients hospitalized in the two buildings. In each case, the disease-causing strain was prevalent in the water system, accounting for the majority of the colonies picked and serotyped. In building H, three of four peripheral samples and one central sample yielded *Legionella* isolates. In building E, all water specimens (nine water specimens collected from peripheral outlets and one specimen from the central heater) contained *Legionella* isolates. A short summary of the environmental surveillance of the hospital water system, i.e., the prevalence of the different serogroups in the clinics, is given in Table 1. In all cases, environmental isolates obtained from the patients' wards were selected for pulsed-field analysis.

Epidemiologically unrelated *L. pneumophila* strains of serogroups 3 and 4 were investigated for comparison. These strains were either isolated in our laboratory or sent to us by colleagues. Serogroup 3 strains included Bloomington-2 (ATCC 33155), Riesa-1, Rostock-2, K 190/88, K 103/88, W 30/3, St 247, Leipzig-50, Leipzig-64, P 421/88, and P 815/91. Unrelated serogroup 4 strains used were Los Angeles-1 (ATCC 33156), S 667, Seidel-4, and Jena-1. All strains except P 421/88 and P 815/91 were isolated from water samples in Germany or Austria. Strains P 421/88 and P 815/91 were cultured from patients with legionellosis in Dresden.

The genomic DNA of *L. pneumophila* strains was prepared as described recently (10). Restriction enzymes *Sfi*I and *Not*I were used as recommended by the manufacturer (New England Biolabs, Schwahlbach, Germany). Pulsed-field gel electrophoresis was performed on 1% agarose (GTG-agarose; Biometra, Göttingen, Germany) in 0.5× Tris-borate-EDTA buffer (pH = 8.3) with Rotaphor equipment (Biometra) as described recently (10). Yeast chromosomes (*Saccharomyces cerevisiae* WAY 5-4A) and lambda concatemers (Biometra) were used as DNA size markers. Pulsed-field gel electrophoresis gels were stained with ethidium bromide and photographed under UV light.

Analysis of serogroup 3 strains by macrorestriction analysis showed that the strain that caused disease and 11 unrelated strains showed 10 different restriction patterns of the genomic DNA when *Sfi*I and *Not*I were used (Fig. 1 and 2). Three strains (Bloomington-2, Leipzig-64, and P 815/92) isolated from a patient in the United States, from a water sample collected in a private home in Leipzig, and from a patient in Dresden, respectively, were indistinguishable by macrorestriction analysis. These results showed again that defined *Legionella* clones can be isolated from unrelated sources throughout the world as reported by Selander et al. (14).

Five unrelated serogroup 4 strains showed five different DNA cleavage patterns with both *Sfi*I (Fig. 3) and *Not*I (data not shown). Strains of this serogroup were rarely isolated in

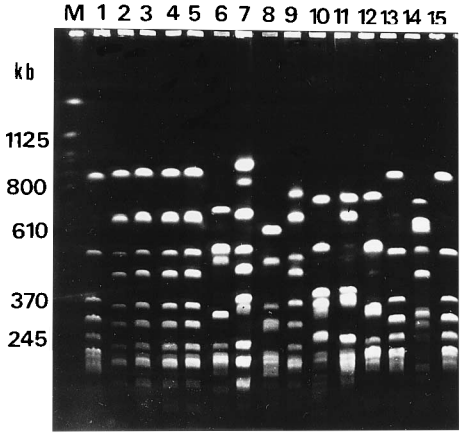

FIG. 1. Macrorestriction analysis of *Sfi*I-cleaved DNAs of *L. pneumophila* serogroup 3 strains. DNA sizes are indicated on the left. Lanes: M, yeast chromosomal DNA standard; 1, Bloomington-2 (ATCC 33155); 2, patient's isolate (case 1); 3, environmental isolate (case 1); 4, patient's isolate (case 2); 5, environmental isolate (case 2); 6, Riesa-1; 7, Rostock-2; 8, K 190/88; 9, K 103/88; 10, W 30/3; 11, St 247; 12, Leipzig-50; 13, Leipzig-64; 14, P 421/88; 15, P 815/91.

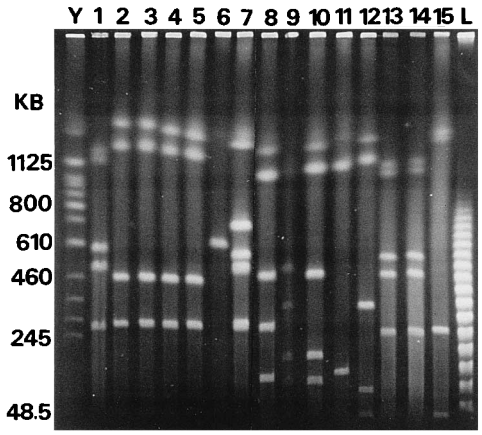

FIG. 2. Macrorestriction analysis of *Not*I-cleaved DNAs of *L. pneumophila* serogroup 3 strains. DNA sizes are indicated on the left. Lanes: Y, yeast chromosomal DNA standard; 1, Bloomington-2 (ATCC 33155); 2, patient's isolate (case 1); 3, environmental isolate (case 1); 4, patient's isolate (case 2); 5, environmental isolate (case 2); 6, Riesa-1; 7, Rostock-2; 8, K 103/88; 9, W 30/3; 10, St 247; 11, Leipzig-50; 12, P 421/88; 13, Leipzig-64; 14, P 815/91; 15, K 190/88; L, lambda concatemers.

Germany (11). Therefore, the number of strains investigated in this study was rather small. Nevertheless, it became obvious that strains of serogroup 4 displayed considerable DNA polymorphism. The *Sfi*I and *Not*I profiles of the genomic DNAs revealed that in all cases the patient's strain and selected environmental isolates of the same serogroups from the hospital hot water system were identical (Fig. 1 through 3). Thus, our results provide strong evidence that the hot water system was the source of infection in our immunocompromised patients. Cooling towers and air conditioning systems, which have been mentioned in other reports as sources of infection (17), were not in operation in the clinics involved in this study. Other possible sources of infection around the hospital or in the private homes of the patients were not included in this study.

In our study, two patients (patients 3 and 4) had strong epidemiological evidence of a hospital-acquired *L. pneumophila* infection, since they stayed in the clinics for more than 10 days before they became infected (8). For the other two patients, the nosocomial origin of the infection was not clearly established, since these patients became infected after staying in the hospital for less than 10 days.

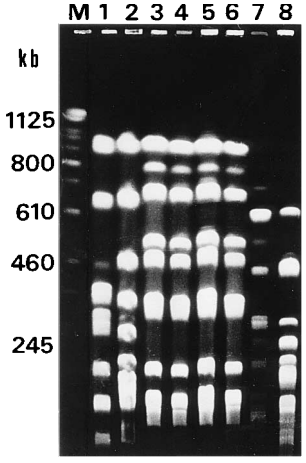

FIG. 3. Macrorestriction analysis of *Sfi*I-cleaved DNAs of *L. pneumophila* serogroup 4 strains. DNA sizes are indicated on the left. Lanes: M, yeast chromosomal DNA standard; 1, S667; 2, Seidel-4; 3, patient's isolate (case 3); 4, environmental isolate (case 4); 5, patient's isolate (case 4); 6, environmental isolate (case 4); 7, Los Angeles-1 (ATCC 33156); 8, Jena-1.

In contrast to serogroup 1 strains, which can be subtyped with monoclonal antibodies (6), *L. pneumophila* serogroup 3 and 4 strains were found to be uniform in antigenic surface composition. Therefore, other techniques should be applied for subtyping. Previous studies by Harrison et al. (4) and Tram et al. (15) suggested that strains of serogroup 3 were heterogeneous in their genome as determined by hybridization-based restriction fragment length polymorphism typing. Concerning serogroup 4, no investigations have been described so far. For subtyping of strains belonging to both serogroups, we used macrorestriction analysis by pulsed-field gel electrophoresis of chromosomal DNAs restricted with the rare-cutting enzymes *Sfi*I and *Not*I. This technique had been successfully used for subtyping *L. pneumophila* serogroups 1, 6, and 10, according to recent reports (5, 9, 10, 12, 13).

In general, both macrorestriction analysis and restriction fragment length polymorphism analysis were found to be suitable for subtyping unrelated *L. pneumophila* strains (3, 12, 13, 15, 16). A comparison of these techniques showed that hybridization-based restriction fragment length polymorphism types were subdivided by macrorestriction analysis (references 13 and 16 and our unpublished results). Thus it seems that macrorestriction analysis is the most discriminating technique developed so far for subtyping *Legionella* strains. Additionally, this method is easily performed, reliable, and easy to interpret.

In conclusion, macrorestriction analysis is a very powerful tool for assessing epidemiological relationships of *L. pneumophila* serogroup 3 and 4 strains during epidemiological investigations.

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