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Epidemiologic Investigation by Macrorestriction Analysis and by Using Monoclonal Antibodies of Nosocomial Pneumonia Caused by *Legionella pneumophila* Serogroup 10†

P. CHRISTIAN LÜCK,[1]* JÜRGEN H. HELBIG,[1] ULLRICH GÜNTER,[2] MICHAEL ASSMANN,[2] RALF BLAU,[1] HEIDEMARIE KOCH,[3] AND MARIA KLEPP[3]

Institut für Medizinische Mikrobiologie und Hygiene, Universitätsklinikum, TU Dresden, D-01307 Dresden,[1] Kreiskrankenhaus Riesa, Innere Klinik, D-01589 Riesa,[2] and Fachgebiet Krankenhaushygiene, Landesuntersuchungsanstalt für das Gesundheits- und Veterinärwesen, Bereich Leipzig, D-04107 Leipzig,[3] Germany

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A 67-year-old woman was hospitalized with an acute pneumonia of the left lower lobe. *Legionella pneumophila* serogroup 10 was cultured from two sputum specimens taken on days 18 and 20 and was also detected by direct immunofluorescence assay by using a commercially available species-specific monoclonal antibody as well as serogroup 10-specific monoclonal antibodies. Antigenuria was detected in enzyme-linked immunosorbent assays by using serogroup 10-specific polyclonal and monoclonal antibodies. In the indirect immunofluorescence test rising antibody titers against serogroups 1, 4, 5, 8, 9, 10, 14, and 15 were found in serum, with the highest titers found against serogroups 8, 9, and 10. *L. pneumophila* serogroups 10 and 6 and a strain that reacted with serogroup 4 and 14 antisera were cultured from both central and peripheral hot water systems of the hospital. Macrorestriction analyses of the genomic DNAs by pulsed-field gel electrophoresis showed that the isolate from the patient was identical to the serogroup 10 strains from the hospital hot water system. In contrast, the genomic DNAs of 16 unrelated *L. pneumophila* serogroup 10 strains showed 12 different restriction patterns. Monoclonal antibody subtyping revealed only minor differences in *L. pneumophila* serogroup 10 strains isolated from different sources. In conclusion, macrorestriction analysis is a valuable tool for studying the molecular epidemiology of *L. pneumophila* serogroup 10.

Legionellae can be found in both natural water systems (rivers, lakes, soils) and artificial water systems (hot water supplies, cooling towers, dental units) (6, 8, 9, 17). In aquatic habitats they live in close association with free-living amoebae and ciliated protozoa (17). Legionellae multiply within the vacuoles of the amoebae, especially at temperatures of between 30 and 45°C. Infected amoebae or the legionellae released from amoebae spread from contaminated aquatic environments to susceptible persons and in this way may cause human illness (17).

Legionella pneumophila is now recognized as an important cause of nosocomial infections. Among hospitalized patients, those with impaired host defenses are at an increased risk of acquiring legionellosis. Up to now, 15 serogroups of *L. pneumophila* have been defined by using absorbed polyclonal rabbit antisera (1, 2). Serogroup 1 is still the most common clinical and environmental isolate; this is followed by serogroup 6 (8, 17). Other serogroups account for 10 to 20% of clinical isolates and 20 to 60% of environmental isolates. We describe here a case of nosocomial legionellosis caused by *L. pneumophila* serogroup 10. In addition, we report on the usefulness of macrorestriction analysis by pulsed-field gel electrophoresis (PFGE) for detecting the causative strain in the hospital water supply.

MATERIALS AND METHODS

Case report. A 67-year-old woman was admitted to the psychiatric clinic (hospital A) for depression on 21 September 1992. She was treated with tri- and tetracyclic thymoleptic drugs and dexamethasone. Except for a 5-day holiday over Christmas, she stayed in hospital A for 4 months before she developed pneumonia on 20 January 1993. The patient was febrile (38.8°C) on admission to the internal medicine ward of hospital B. Laboratory data and radiological findings were consistent with those reported for *Legionella* pneumonia (13). The patient was put on cefotiam therapy (4 g/day). On day 10 the therapy was changed to roxithromycin (300 mg/day) because of a clinical suspicion of *Legionella* pneumonia. During this therapy the infiltrations regressed slowly but continuously. Bronchoscopy performed on day 22 revealed oropharyngeal flora on sheep's blood and chocolate agars as well as a negative culture for tuberculosis.

Microbiological methods for *Legionella* species. The immunofluorescence assay (IFA) technique with Formalin-killed bacteria was used for the *Legionella* species (13). Antigens for the test were prepared from *L. pneumophila* serogroups 1 to 14, *L. pneumophila* Lansing-3 (serogroup 15), *L. micdadei*, *L. bozemanii* serogroup 1, *L. dumoffii*, *L. jordanis*, and *L. longbeachae* serogroups 1 and 2. For the detection of antibodies to these antigens in the patient's serum, polyvalent antibody conjugate to human immunoglobulins (Institut für Immunpräparate und Nährmedien, Berlin, Germany) was used.

Sputum samples were cultured on selective buffered charcoal-yeast extract (BCYE) agar supplemented with 0.1% α-ketoglutarate (Sigma Corp., Munich, Germany), 0.3% glycine (Serva, Heidelberg, Germany), 1 mg of vancomycin (Lilly,

* Corresponding author. Mailing address: Institut für Medizinische Mikrobiologie und Hygiene, Universitätsklinikum, TU Dresden, Dürerstrasse 24, D-01307 Dresden, Germany. Phone: 0049-351-4579 362. Fax: 0049-351-4573286.

† This paper is dedicated in memoriam to Karsten Seidel.

TABLE 1. *L. pneumophila* serogroup 10 strains analyzed by macrorestriction analysis by PFGE of genomic DNAs and MAbs

Strain	Origin of strain	Macrorestriction analysis pattern		Reactivity against MAbs[a]				
		*Sfi*I	*Not*I	11-9, 6-1, 13-2, 17-2, 17-3	5-1, 13-1	5-2	18-2	27-1
P 41-93	Patients and water isolates, hospital A[b]	1	1	++	0	0	0	0
Leiden-1	ATCC 43283, patient, The Netherlands	2	2	++	0	0	0	0
W 38	Water, hospital B, Riesa, Germany	3	3	++	0	0	0	0
St 176	Dental unit, Dresden, Germany	4	4	++	0	0	0	0
S 685	Hospital water, Lübeck, Germany	5	1	++	0	0	0	0
W 443	Municipal water, Dresden, Germany	6	4	++	0	0	0	0
116239-86	Municipal water, Vienna, Austria	7	5	++	0	0	0	0
W 293-3	Hospital water, Pirna, Germany	8	6	++	0	0	0	0
W 323-1	Hospital water, Greifswald, Germany	9	7	++	0	0	0	0
Leipzig 15	Municipal water, Leipzig, Germany	10	8	++	0	+	0	+
Leipzig 52	Municipal water, Leipzig, Germany	1	1	++	0	0	0	0
Leipzig L32	Water, hotel A, Leipzig, Germany	1	1	++	0	0	0	0
Leipzig L35	Water, hotel B, Leipzig, Germany	11	7	++	0	0	0	0
Chemnitz-5	Hospital water, Chemnitz, Germany	2	2	++	0	0	0	0
Betz 1-2	Patient, hospital, Ulm, Germany	12	9	++	0	0	+	0
Schubl-1	Municipal water, Greifswald, Germany	10	8	++	0	+	0	+

[a] Specificities of MAbs: MAb 11-9, specific for *L. pneumophila* serogroups 2 to 6, 8 to 10, and 12 to 15; MAb 6-1, specific for *L. pneumophila* serogroups 8 and 10; MAb 13-2, specific for *L. pneumophila* serogroups 4, 5 (subtype Dallas), 8, and 10; MAb 17-2, specific for *L. pneumophila* serogroup 10; MAb 17-3 specific for *L. pneumophila* serogroups 5 (subtype Dallas) and 10; MAb 5-1, specific for *L. pneumophila* serogroup 4; MAb 13-1, specific for *L. pneumophila* serogroup 8; MAb 5-2, specific for *L. pneumophila* serogroup 5 (subtype Dallas); MAb 18-2, specific for *L. pneumophila* serogroups 4, 5 (subtype Cambridge), and 10; MAb 27-1, specific for *L. pneumophila* serogroup 5. ++, good fluorescence in the indirect IFA and an optical density of >0.6 in the ELISA; +, weak or no fluorescence in the indirect IFA and an optical density ranging from 0.15 to 0.6 in the ELISA; 0, negative in the indirect IFA and the ELISA.

[b] Ten environmental strains showed identical macrorestriction analysis results and MAb reactivities.

Giessen, Germany) per liter, 80,000 U of polymyxin B (Pfizer, Karlsruhe, Germany) per liter, and 50,000 U of amphotericin B (Squipp-van Heiden, Munich, Germany) per liter after heat treatment for 3 min at 60°C (13).

Direct IFAs were performed with a commercially available monoclonal antibody (MAb) against the major outer membrane protein of the species *L. pneumophila* (Fresenius, Oberursel, Germany) (13). After the isolation of *L. pneumophila* serogroup 10 from two sputum samples, immunofluorescence antigen detection was repeated by using two MAbs from our laboratory. MAb 17-2 reacted with all serogroup 10 strains ($n = 28$) tested. MAb 11-9 recognized a lipopolysaccharide (LPS) epitope on all strains of serogroups 2 to 6, 8 to 10, and 12 to 15.

Urinary antigen detection assays were performed with monoclonal and polyclonal enzyme-linked immunosorbent assays (ELISAs) for serogroup 1 as described previously (3). Serogroup 10-specific ELISAs used a polyclonal serogroup 10 antiserum from a rabbit as the first antibody. For detection the serogroup 10-specific MAb 17-2 and the multireactive MAb 11-9 were used. Urine samples were tested before and after heating for 5 min.

Environmental surveillance of hospital water supply in hospital A. Water samples were collected from the central water heater and storage tank and from the showerheads and hot water faucets in the patient's ward. Water samples were collected in sterile bottles and were immediately taken to the laboratory. Nonconcentrated water samples (0.5 and 0.1 ml) were plated on the selective BCYE agar used for the sputum sample cultures (8). On day 5 *Legionella* CFU was estimated by counting the colonies.

Serological typing by using polyclonal antibodies and MAbs. Absorbed serogroup-specific rabbit sera for all 14 serogroups of *L. pneumophila*, *L. pneumophila* Lansing-3 (serogroup 15), and 16 other *Legionella* species prepared in our laboratory and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit globulin (Institut für Immunpräparate und Nährmedien) were used in the IFA as described previously (8).

MAbs made to react with *L. pneumophila* strains were prepared in our laboratory by previously described protocols (3, 6). The reactivities of our MAbs were determined by using American Type Culture Collection strains of *L. pneumophila* serogroups 1 to 15 in the IFA and ELISA. The same techniques were used for typing the *L. pneumophila* serogroup 10 strains included in the present study. The IFA was performed by using an FITC conjugate to mouse immunoglobulins (Institut für Immunpräparate und Nährmedien) (8). In the ELISA, microtiter plates were coated with 50 μl of IFA antigen and were air-dried overnight. After incubation with the MAbs, which were applied as 1:10-diluted cell culture supernatants, bound antibodies were detected by using an anti-mouse (polyvalent) horseradish peroxidase conjugate (Sigma).

The specificities of our MAbs used for typing in the present study are shown in Table 1. Serogroup 6 strains were subtyped with MAbs from our laboratory (6).

Macrorestriction analysis of genomic DNAs. The genomic DNAs of *L. pneumophila* strains were prepared as described previously (6). The restriction enzymes *Sfi*I and *Not*I were obtained from New England Biolabs (Schwahlbach, Germany). Cleavage was carried out for 15 h at 50°C for *Sfi*I and at 37°C for *Not*I by following the manufacturer's instructions. The plugs containing cleaved DNA were loaded onto 1% agarose (GTG-agarose; Biometra, Göttingen, Germany) in 0.5× Tris-borate-EDTA buffer (pH 8.3). PFGE was performed by using Rotaphor° equipment (Biometra, Göttingen, Germany), and runs were for 30 h at 13°C. Runs were carried out by using the following parameters: angles, 100 to 125° with logarithmic ramping; voltage, 200 to 160 V with linear ramping; and pulse times, 50 to 2 s with logarithmic ramping for *Sfi*I and 100 to 5 s with logarithmic ramping for *Not*I. *Saccharomyces cerevisiae* WAY 5-4A chromosomes and bacteriophage lambda concatemers (Biometra) were used as DNA size markers. PFGE gels were stained with ethidium bromide and photographed under UV light.

Unrelated *L. pneumophila* serogroup 10 strains. Fifteen

unrelated *L. pneumophila* serogroup 10 strains were investigated for comparison (Table 1). These strains were either isolated in our laboratory or sent to us by colleagues. All but strain Betz 1-2 were isolated from water specimens collected in Germany or Austria. Strain Betz 1-2 was cultured from a sputum sample taken from a 65-year-old man suffering from pneumonia. The American Type Culture Collection strain Leiden-1 (ATCC 43283) was originally isolated during an outbreak of nosocomial legionellosis in The Netherlands (9).

RESULTS

Microbiological results in a case of *L. pneumophila* serogroup 10 infection. *Legionella*-like colonies were isolated after 4 days of incubation from two sputum samples from the case patient obtained on days 18 and 20 of the illness. Altogether, 18 colonies were picked and serotyped as serogroup 10 by using absorbed rabbit antisera and MAbs. IFAs with the species (major outer membrane protein)-specific MAb, the serogroup 10 (LPS)-specific MAb 17-2, and MAb 11-9, which recognizes an LPS epitope present on all strains of serogroups 2 to 6, 8 to 10, and 12 to 15, were positive for both sputum samples. One sputum specimen collected on day 19 was negative by IFAs and culture.

Urinary antigen was detected by using serogroup 10-specific ELISAs, but it was not found in our serogroup 1-specific assays. Both the serogroup 10-specific and cross-reacting MAbs used in the IFAs of sputum were able to detect urinary antigen in two urine samples collected on days 18 and 19. A third urine specimen (day 20) was positive in the ELISA with the cross-reacting MAb 11-9.

An acute-phase serological sample obtained on day 6 was negative for serogroup 1 and revealed nonsignificant titers for serogroups 5, 8, 10, and 14. Serum samples collected later showed significant rises in titer for serogroups 1, 4, 5, 8, 9, 10, 12, 13, 14, and 15, with the highest titers (1,024/2,048) being against serogroups 4, 5, 8, 10, and 14.

Environmental studies. Legionellae were isolated from the hot water tap in the patient's room, the hot water faucet and showerhead in the bathroom of the patient's ward, as well as the central heating boilers, the cold water inlet to one of them, and the main pipe connecting the central heating station to the wards.

Using absorbed rabbit antisera for serogroups 1 to 15, three different strains were identified. The serogroup 10 strain was the prevalent one in all water samples taken from peripheral sites. Of 103 colonies tested, 70 (68%) belonged to this serogroup. Thirteen strains were typed as serogroup 6, monoclonal subtype Dresden. Twenty colonies did not react brightly with our absorbed antisera for serogroups 1 to 15 but were positive when unabsorbed antisera were used. The highest titers were found against serogroups 4 and 14. This strain is thus recorded as either a new serogroup or a serovariant of serogroups 4 or 14.

Legionella counts were between 10 and 300 CFU/ml. Except for one cold water sample (12°C), the temperatures of all other water samples were between 30 and 46°C.

Three weeks after the temperature in the central storage tanks was raised to 60°C, the *Legionella* organism count was found to be reduced, but all three *Legionella* strains were still present in the water system.

Serological subtyping of *L. pneumophila* serogroup 10 strains. The results of subtyping of serogroup 10 strains with MAbs are given in Table 1. All serogroup 10 strains obtained from the patient and related environmental sources as well as 15 unrelated strains showed the same reaction patterns when

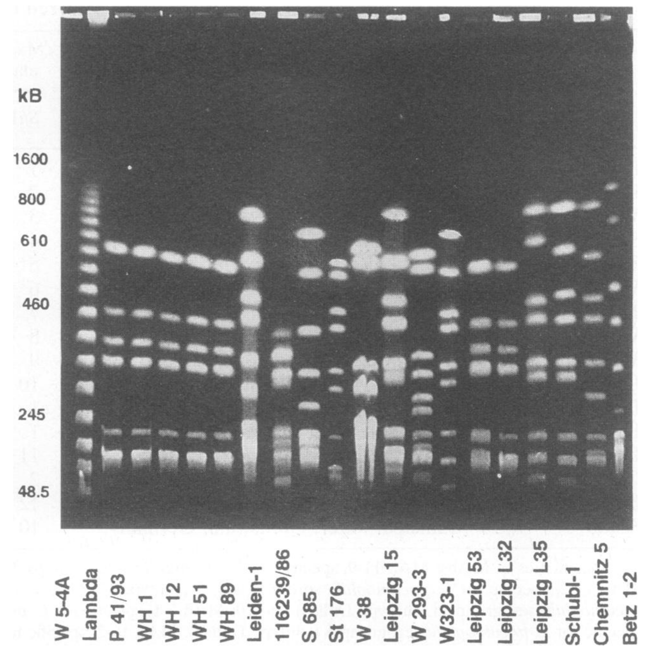

FIG. 1. PFGE of *Sfi*I-cleaved DNAs of the *L. pneumophila* serogroup 10 strains listed in Table 1. DNA sizes (in kilobases) are indicated to the left of the gel.

we used MAbs that recognized the major epitopes detectable in the IFA. Cross-reactive epitopes associated with strains of serogroups 4, 5, 8, and 10 were found on the surfaces of a few serogroup 10 strains. In terms of reactivity, they were attributed as minor antigenic determinants, producing optical densities of from 0.15 to 0.6 in the ELISA. No significant reactivity was found when these MAbs were used in the IFA. These results showed that *L. pneumophila* serogroup 10 strains are homogeneous in their antigenic compositions, with small differences in minor epitopes.

Macrorestriction analysis. Macrorestriction analysis with the *Sfi*I and *Not*I profiles of the genomic DNAs showed that the patient's strains and 10 arbitrarily selected environmental isolates of *L. pneumophila* serogroup 10 from different sites of the hospital hot water system were identical. A few of the restriction patterns of these strains are given in Fig. 1 and 2.

The disease-causing strain and 15 unrelated strains of *L. pneumophila* serogroup 10 showed 12 different restriction patterns of genomic DNAs when the *Sfi*I enzyme was used and 9 restriction patterns when the *Not*I enzyme was used. Two strains (Leipzig 52 and Leipzig L32) isolated from two water samples collected in a hotel and a private home in Leipzig, Germany, 60 km away from hospital A, showed the same restriction patterns with both enzymes in the macrorestriction analysis as the disease-causing strain in hospital A. The same *Not*I pattern was noted in a strain (S 685) isolated from a hospital water system in Lübeck, Germany, but the *Sfi*I pattern was completely different from those of strains Leipzig 52, Leipzig L32, and the strains from hospital A. In addition, we found two pairs of serogroup 10 strains, strains Leiden-1 and Chemnitz-5 and strains Schubl-1 and Leipzig 15; each of them showed identical DNA cleavage patterns by using both *Sfi*I and *Not*I enzymes. The *Not*I patterns of strains W 443 and St 176 and those of strains Leipzig 15 and W 323-1 were identical, but they were different when the *Sfi*I enzyme was used.

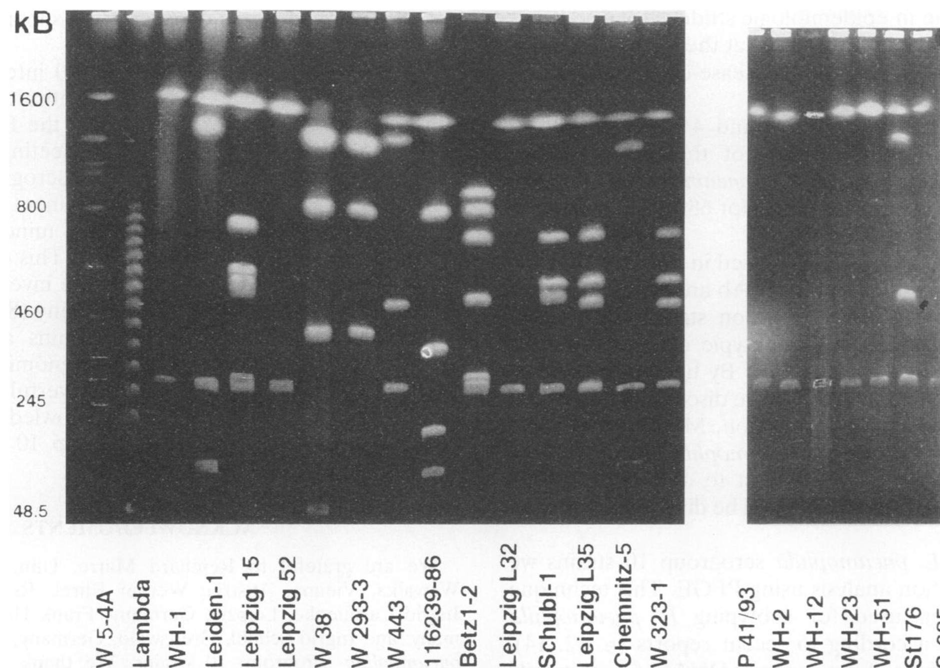

FIG. 2. PFGE of *Not*I-cleaved DNAs of the *L. pneumophila* serogroup 10 strains listed in Table 1. DNA sizes (in kilobases) are indicated to the left of the gel.

DISCUSSION

The clinical status of the patient described here provided unequivocal evidence of a hospital-acquired *L. pneumophila* serogroup 10 infection. The primary illness in our patient was depression. She was treated with thymoleptic drugs and corticosteroids, a known risk factor for acquiring legionellosis (17). The clinical course was typical for Legionnaires' disease (13). Because of a clinical suspicion of *Legionella* pneumonia the patient was given roxithromycin therapy for 20 days. During this therapy she slowly improved.

The microbiological diagnosis was based on the isolation of *L. pneumophila* serogroup 10 from two sputum samples, positive direct IFA results for sputum specimens, positive results in the assays for urinary antigen, and seroconversion. Only *L. pneumophila* serogroup 10 (18 colonies tested) was grown from two subsequent sputum samples. The serogroup of the isolated strains was confirmed in the IFA with both absorbed polyclonal antibodies and MAbs. It is noteworthy that the causative agent was cultured after 8 and 10 days of roxithromycin therapy. This pointed out that attempts to cultivate legionellae from patient specimens are worthwhile, even when the patient has received *Legionella*-directed therapy. It might be speculated that the strain survived in macrophages with impaired microbicidal function following corticosteroid treatment and was still cultivable. Our results underline the fact that erythromycin, which acts bacteriostatically, should be administered for at least 3 weeks to eliminate legionellae (17). For epidemiologic studies it is important to obtain *Legionella* strains from patients. The comparison of these strains with environmental isolates provides evidence of or disproves the transmission of the strains from a suspected source of infection to humans.

The species-specific MAb, which recognizes the major outer membrane protein, the serogroup 10-specific MAb 17-2, and the multireactive MAb 11-9, were successfully used for the detection of *L. pneumophila* serogroup 10 in the two sputum samples from which the strain was grown.

It was possible to detect urinary antigen by using serogroup 10-specific assays with a polyclonal serogroup 10 antibody as the first antibody. In this case both serogroup 10-specific and cross-reactive MAbs could be used to detect urinary antigen. The monoclonal and polyclonal serogroup 1-specific assays used in our laboratory since 1987 failed to do so. With regard to the polyclonal serogroup 1-specific ELISA, this observation is in contrast to that in the report of Kohler et al. (5), who found *Legionella* antigen in the urine of patients with culture-proven infections caused by serogroups 1, 4, and 10. It might be speculated that the sensitivity of our assay was too low for our patient with the serogroup 10 infection. On the other hand, we reported the usefulness of the same polyclonal serogroup 1-specific assay in a case of *L. pneumophila* serogroup 3 infection verified by isolation of the causative strain (7). Only limited information is available concerning the cross-reactivities of antigens detected in urine specimens. Our results point to the necessity of using broad-spectrum assays for detecting urinary antigen. Tang et al. (16) reported the usefulness of such an ELISA for diagnosing legionellosis caused by different serogroups and species.

An evaluation of the indirect IFA for antibody detection with antigens other than *L. pneumophila* serogroup 1 has been limited by the rareness of culture-confirmed cases of infection caused by other serogroups. In our case the antibody response against serogroup 1 was not significant until day 20. In contrast, in serum samples collected on days 6 and 13, presumptive titers of 128 and rising titers to 512, respectively, were found against serogroup 8 and 10 strains. To improve the serological diagnosis of legionellosis it is therefore recommended that multiple antigens be used in the IFA. Serogroups 4, 5, 8, and 10, which are known to cross-react with each other (9), gave the highest titers in our assay. Our results again confirm that serological

data are of little value in epidemiologic studies because it was impossible to state with any certainty that the antibodies in the patient's sera were induced by the disease-causing strain, *L. pneumophila* serogroup 10.

L. pneumophila serogroups 10, 6, and 4/14 were isolated from central and peripheral outlets of the hospital water system. The disease-causing strain, *L. pneumophila* serogroup 10, was the most prevalent, accounting for 68% of the colonies tested.

In the IFA all serogroup 10 strains used in the present study reacted with a serogroup 10-specific MAb and with multireactive MAbs recognizing LPS epitopes on strains of different serogroups. Considering these phenotypic characterizations, all serogroup 10 strains were identical. By the more sensitive ELISA technique, small differences were disclosed when cross-reactive MAbs were used. In conclusion, MAb typing is not useful for the subtyping of *L. pneumophila* serogroup 10 isolates. These findings are in contrast to observations concerning serogroup 1 strains, which could be divided into several MAb subtypes (4).

For subtyping of *L. pneumophila* serogroup 10 strains we applied macrorestriction analysis using PFGE. This technique has successfully been used for subtyping *L. pneumophila* serogroups 1 and 6, according to recent reports (6, 12, 14). Macrorestriction analysis of the genomic DNAs of 1 arbitrarily selected serogroup 10 strain from the patient as well as 10 strains from the hot water supply system revealed identical restriction patterns for both *Sfi*I and *Not*I enzymes (Fig. 1 and 2, respectively). We are thus certain that the strain was transferred from the hot water supply system to the patient. Our results provide further evidence that hot water systems are the main source of *Legionella* infection, especially in immunocompromised patients (9–12, 14). It could not be ascertained where the patient acquired her infection, since the serogroup 10 strain was isolated in the patient's room, in the bathroom (showerhead and faucet), the ward's physiotherapeutic cabinet, as well as other wards of the hospital.

Cooling towers and air-conditioning systems, which have been reported previously to be sources of infection (17), were not in operation in hospital A.

Genome analysis revealed that the serogroup 10 strains isolated from 16 different locations showed 12 different *Sfi*I and 9 different *Not*I cleavage patterns. A comparison of the *Sfi*I and *Not*I patterns indicated that both enzymes are useful in subtyping *L. pneumophila* serogroup 10 strains. The results obtained by these two techniques were in close agreement. However, the use of the restriction enzyme *Sfi*I allowed the subdivision of *Not*I patterns. This is not surprising since *Not*I produced fewer bands than *Sfi*I in the macrorestriction analysis. Interestingly, two strains (Leipzig 52 and Leipzig L32) isolated from the water systems of two different buildings in Leipzig, 60 km away from hospital A, were identical to the disease-causing strain in hospital A. Two sets of *L. pneumophila* serogroup 10 strains, strains Leiden-1 and Chemnitz-5 and strains Schubl-1 and Leipzig 35, isolated in locations at distances of 1,500 and 750 km from each other, respectively, were shown to be identical to each other by using both enzymes as well as by MAb typing. These results underline the observation made by Selander et al. (15), viz., that although *Legionella pneumophila* strains are genetically heterogeneous, a distribution of clones occurs throughout the world. Therefore, the investigation of *Legionella* outbreaks should include subtyping data as well as epidemiologic information.

A serogroup 10 strain (W 38) isolated from the hospital water supply system in hospital B showed a completely different restriction pattern. A nosocomial superinfection acquired in hospital B could thus be excluded.

The true incidence of serogroup 10 infections has not yet been defined. In 1992, 33 culture-confirmed cases caused by serogroups 2 to 14 were reported by the European Working Group on *Legionella* infections (8th Meeting, Vienna, 10 to 12 May 1993). Of these, six (18%) were serogroup 10 infections. Thus, serogroup 10 is one of the prominent serogroups among clinical isolates in Europe, bearing in mind that serogroup 1 accounts for 70 to 80% of infections. This observation agrees very well with our findings made while investigating hot water supply systems in southeastern Germany (8).

L. pneumophila serogroup 10 strains are phenotypically uniform but heterogeneous in their genomic structures. Thus, macrorestriction analysis is a very powerful tool for subtyping strains of this serogroup. To our knowledge this is the first report on the subtyping of serogroup 10 strains by macrorestriction analysis.

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