JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 1990, p. 242–245
0095-1137/90/020242-04$02.00/0

Vol. 28, No. 2

Molecular Typing of Nosocomial Isolates of *Legionella pneumophila* Serogroup 3

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Received 12 June 1989/Accepted 18 October 1989

In Paris, France, an outbreak of pneumonia due to *Legionella pneumophila* serogroup 3 was observed in Necker (four cases) and Pitié (six cases) hospitals. Neither the 10 clinical isolates nor 5 tap water isolates from Necker Hospital harbored plasmids. Clinical and environmental serogroup 3 isolates and serogroup 3 reference strain Bloomington 2 were analyzed by chromosomal probe fingerprinting. rRNA, 16S and 23S from *Escherichia coli* and a randomly cloned 15-kilobase-pair nucleotide sequence from *L. pneumophila* serogroup 3 were used as probes. All strains tested showed a single pattern after *Hin*dIII digestion of DNA and hybridization with the ^{32}P-end-labeled rRNA probe, whereas three patterns were obtained after hybridization with the ^{32}P-labeled 15-kilobase-pair DNA probe. One pattern was given by all clinical and tap water isolates from Necker Hospital, another one was given by all clinical isolates from Pitié Hospital, and a last one was given by reference strain Bloomington 2. Thus, molecular analysis showed that the two hospital outbreaks of legionellosis were unrelated and could link the outbreak in Necker Hospital to contaminated tap water.

Pneumonia in hospitalized patients is a major concern since the infectious process is severe and often occurs in immunocompromised hosts. Among the various opportunistic gram-negative bacteria, *Legionella pneumophila* seems to play an important role in nosocomial pneumonia. In the United States, the prevalence of hospital-acquired Legionnaires disease has varied greatly, but in some areas this pathogen has been involved in 10 to 20% of nosocomial pneumonia (12). In France, the prevalence is still unknown. Most clinical isolates (61% in the United States, 89% in France) of *L. pneumophila* belong to serogroup 1 (2, 15).

In a recent survey, about 6% of clinical strains of *L. pneumophila* isolated in the Paris area belonged to serogroup 3 (2). In 1986 and 1987 in Paris, we observed a limited outbreak of nosocomial pneumonia due to *L. pneumophila* serogroup 3 at Necker and Pitié hospitals, occurring primarily in immunocompromised hosts. This puzzling emergence of an infrequent serogroup in two hospitals located in the southern area of Paris was an inducement to pursue an epidemiological study of the origin of the clinical strains and of the possible relationship among the clusters of cases observed. This paper reports the molecular typing of the isolates, which suggested an epidemiological link between clinical and water isolates in one hospital.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Fifteen *L. pneumophila* serogroup 3 isolates and reference strain Bloomington 2 were studied. Ten isolates from respiratory tract secretions or lung biopsy of hospitalized patients and five isolates from water specimens were recovered by using the following procedures. Two-liter samples of water were filtered on a 0.2-μm-pore diameter polycarbonate membrane (Nuclepore Corp., Pleasanton, Calif.). After filtration, bacteria on the filters were suspended in 5 ml of filtrate and shaken. Then, 0.2 ml of the suspension, diluted 1:1 and 1:100, was spread on agar. Clinical or water specimens were plated on both buffered charcoal-yeast extract agar supplemented with 0.1% (wt/vol) α-ketoglutaric acid (Difco Laboratories, Detroit, Mich.) and the same agar supplemented with 4 μg of cefamandole, 80 U of polymyxin B, and 80 μg of anisomycin (Oxoid Ltd., Basingstoke, United Kingdom). Agar plates were incubated at 37°C in humidified atmosphere with 5% (vol/vol) carbon dioxide and examined for growth daily for 5 days. Microorganisms were identified as *L. pneumophila* (25) and were found to belong to serogroup 3 on the basis of their reactivity with an antiserum obtained from a rabbit immunized with strain Bloomington 2. Five colonies from each positive culture were typed. All isolates were subcultured once on buffered charcoal-yeast extract agar supplemented with 0.1% (wt/vol) α-ketoglutaric acid and stored frozen at −80°C.

Plasmid DNA. All strains were screened for plasmids by the method of Kado and Liu (9).

DNA preparation and restriction endonuclease digestion. DNA from *Legionella* strains was prepared as described previously (6) and cleaved by restriction endonuclease *Hin*dIII or *Bam*HI (5 U/μg of DNA) by following the instructions of the manufacturer (Amersham International, Amersham, United Kingdom). The digestion was carried out at 37°C for 4 h, and 5 μg of restricted DNA was separated on a 0.8% (wt/vol) agarose (Appligène, Strasbourg, France) gel in Tris-acetate buffer for 16 h at 1.7 V/cm. DNA fragment size marker Raoul I (Appligène) was used for accurate determination of fragment sizes. After completion of electrophoresis, the DNA fragments in the gel were stained by ethidium bromide (1 μg/ml) and photographed under UV light.

Cloning of *L. pneumophila* DNA. Total DNA from an *L. pneumophila* strain (strain P3) isolated from a patient hospi-

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talized at Pitié Hospital was partially cleaved with restriction endonuclease *Bam*HI and ligated into the *Bam*HI site of bacteriophage lambda vector 1059 (GIBCO BRL, Cergy Pontoise, France). Hybridization of vector DNA alone to restriction endonuclease-digested *L. pneumophila* DNA showed that this vector did not contain any sequences homologous to *L. pneumophila* DNA. The recombinant DNA molecules were packaged into phage lambda particles, using a packaging kit (Amersham), and were used to infect *Escherichia coli* K358. Several plaques were selected from which phages were isolated and amplified to approximately 10^{10} particles. Recombinant DNA clones were recovered from each phage culture by phenol extraction and dialysis against Tris-EDTA.

Radioactive labeling of probes. rRNA, 16S and 23S, from *E. coli* (Boehringer GmbH, Mannheim, Federal Republic of Germany) was end labeled with [γ-^{32}P]ATP (Amersham), using a 5′ DNA terminus labeling kit and T4 polynucleotide kinase (GIBCO BRL), following the manufacturer's instructions. Recombinant phage DNA was labeled with a nick translation kit and [α-^{32}P]dCTP (Amersham), following the manufacturer's instructions.

Southern transfer and hybridization. The transfer of *Legionella* DNA fragments to Hybond-N nylon membrane (Amersham), prehybridization, hybridization with labeled probes at 65°C for 17 h, washing, and autoradiography are described elsewhere (4).

RESULTS

From 1986 to 1987, 10 cases of pneumonia caused by *L. pneumophila* serogroup 3 occurred in two hospitals in Paris. Six cases were observed at Pitié Hospital over a 2-year period in one building. These included two cases (one in January 1986 and the other in February 1986) in patients hospitalized in a surgical intensive care unit and four cases (one in March 1986, one in May 1986, one in January 1987, and one in June 1987) in heart transplant recipients hospitalized in the heart surgery unit for the immediate postoperative period or for treatment of acute heart graft rejection. The four remaining cases occurred over a 3-month period (one in March 1987, two in April 1987, and one in May 1987) at Necker Hospital in renal transplant recipients hospitalized in a unit for immediate posttransplant follow-up. All patients, except those admitted to the intensive care unit at Pitié Hospital, received high doses of immunosuppressive drugs. The diagnosis of Legionnaires disease was demonstrated by the isolation of *L. pneumophila* from transtracheal aspirate, bronchoalveolar lavage, or lung biopsy. All cases, except one observed only 6 days after hospitalization, occurred more than 10 days after the patient's admission to the hospital.

Since the building harboring the renal transplant unit was not fitted with an air-conditioning system, the hot-water supply was strongly suspected to be the reservoir of *Legionella* spp. at Necker Hospital. Moreover, no water treatment system was in place in this building when the outbreak of legionellosis occurred, and hot tap water had a temperature of ~30°C. In May 1987, hot tap water samples were collected over a 10-day period from sink faucets of patient rooms and from the single shower of the renal transplant unit. The titers of *L. pneumophila* serogroup 3 ranged from 10^3 to 10^4 CFU/liter, but in a few specimens *L. pneumophila* serogroup 3 was not detected. Furthermore, *L. pneumophila* serogroup 1 (~10^2 CFU/liter) was associated with *L. pneumophila* serogroup 3 in a few of the samples. Controls of hot water from storage tanks also revealed the presence of *L. pneumophila*. At the beginning of June 1987, the heat exchange apparatus was superchlorinated (50 mg of free residual chlorine per liter) for 24 h. Thereafter, the hot-water supply was continuously chlorinated (5 mg of free residual chlorine per liter) and the hot-water temperature was raised to 50 to 55°C. This resulted in eradication of *Legionella* spp. from hot water, and no new cases of nosocomial legionellosis were observed afterwards at Necker Hospital.

In contrast, at the time of the outbreak of legionellosis at Pitié Hospital, the hot-water supply of the building in which the cases occurred was continuously chlorinated (1 to 2 mg of free residual chlorine per liter) and hot water was maintained at 55°C. Thus, contamination of the hot-water supply was not initially considered. During hospitalization, all patients were treated, for variable periods, with respiratory devices that aerosolized tap water in order to humidify oxygen. Some devices have been randomly investigated a posteriori, and their reservoirs were found not to be contaminated by *Legionella* spp. The water-cooling system serving the air-conditioning in the heart biopsy room was checked once, and no *Legionella* spp. were recovered from this site. The occurrence at Necker Hospital of an outbreak of Legionnaires disease caused by *L. pneumophila* serogroup 3 and resulting from contamination of the water supply by this microorganism initiated a bacterial analysis of hot water at Pitié Hospital. From June to July 1987, 2-liter samples of hot tap water were collected from sink faucets of patient rooms and from the showers of the surgical intensive care unit and the heart surgery unit. No *Legionella* spp. were isolated from any water specimen. Nevertheless, two other cases of legionellosis due to *L. pneumophila* serogroup 3 occurred in heart transplant recipients, one in December 1987 and the other in February 1988; no case was observed thereafter.

Molecular typing of *L. pneumophila* serogroup 3 isolates was performed to demonstrate that all cases of legionellosis were hospital acquired. One isolate from each patient and environmental site was typed. By agarose gel electrophoresis, it was not possible to detect any plasmid in the 10 clinical and 5 environmental strains. These isolates and reference strain Bloomington 2 showed a single rRNA restriction pattern with four fragments of 5.5, 6.0, 10.1, and 12.8 kilobase pairs after *Hin*dIII digestion.

One bacteriophage lambda clone containing a 15-kilobase-pair *Bam*HI insert of *L. pneumophila* strain P3 DNA was chosen for its ability to discriminate, by hybridization, DNA fragments of the 16 strains studied. Hybridization of this ^{32}P-labeled probe with *Hin*dIII-digested DNA revealed three different fingerprinting patterns (Fig. 1): a first pattern with four bands corresponded to the reference strain; a second pattern with four bands was seen in all clinical and environmental isolates from Necker Hospital; and a third pattern with three bands was found in the clinical isolates from Pitié Hospital.

DISCUSSION

Several methods have been proposed for typing strains of *L. pneumophila* belonging to one serogroup. These include monoclonal antibody subtyping for serogroup 1 (3, 8, 10) and alloenzyme, outer membrane protein, and DNA analyses (13, 19, 22). Brown et al. (1) reported that plasmids could be used as epidemiological markers in the investigation of nosocomial Legionnaires disease. Unfortunately, the strains isolated from patients in Necker and Pitié hospitals and from hot water in Necker Hospital did not harbor any plasmids.

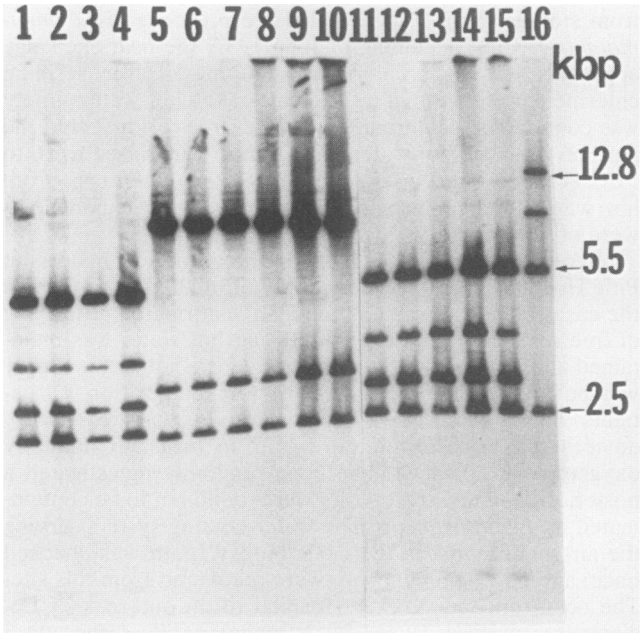

FIG. 1. Autoradiogram of a nylon membrane after transfer of *Hin*dIII restriction fragments of *L. pneumophila* serogroup 3 DNAs and hybridization with a ^{32}P-labeled cloned fragment from *L. pneumophila* serogroup 3. Lanes 1 to 4, Clinical isolates from Necker Hospital; lanes 5 to 10, clinical isolates from Pitié Hospital; lanes 11 to 15, isolates from Necker Hospital water; lane 16, reference strain Bloomington 2. kbp, Kilobase pairs.

Restriction endonuclease study of DNA has been proposed to discriminate among *L. pneumophila* strains lacking plasmids (22, 24). However, electrophoretic patterns of the DNA digests are often difficult to interpret; for this reason, we preferred chromosomal probe fingerprinting analysis.

Determination of rRNA gene restriction pattern was proposed as a general method for identification and typing of bacterial strains (4, 20). This method was useful in typing *Haemophilus influenzae* biogroup aegyptius (7), *Pseudomonas cepacia* (20), and *Providencia stuartii* (14). rRNA restriction patterns can be used in the identification of *Legionella* species (5, 16), and recently Van Ketel and de Wever (23) proposed rRNA gene fingerprinting as a tool in the investigation of nosocomial Legionnaires disease. Unfortunately, this molecular technique did not allow us to discriminate among the strains of *L. pneumophila* serogroup 3 in this study.

Tompkins et al. (21) have demonstrated previously that a random *Salmonella enteritidis* DNA sequence cloned in a cosmid could detect restriction site heterogeneity among *Salmonella* strains. This approach was used to type *L. pneumophila* isolates (17). Our results showed the usefulness of a lambda clone containing an *L. pneumophila* DNA insert. This recombinant lambda clone used as a probe could split a collection of 16 *L. pneumophila* serogroup 3 strains into three types, thus differentiating Necker Hospital isolates from Pitié Hospital isolates. These results confirm the nosocomial character of the Legionnaires disease cases observed and demonstrate that the outbreaks of pneumonia occurring in the two hospitals were unrelated. Furthermore, it supports the hypothesis that tap water was the source of legionellosis at Necker Hospital. This assumption was strengthened by the termination of the outbreak as soon as chlorination of the tap water system of the building in which the cases occurred was initiated. In most cases of Legionnaires disease linked to tap water (11, 13, 18; S. P. Fisher-Hoch, M. G. Smith, and J. S. Colbourne, Letter, Lancet **i**:1073, 1982), the level of contamination was found to range from 10^5 to 19^9 *Legionella* CFU/liter of water. In our study, the level of tap water contamination at Necker Hospital did not exceed 10^4 *Legionella* CFU/liter, and the emergence of legionellosis exclusively in patients recently grafted might be due to the severe immunosuppression of these patients during the early period following kidney graft. Unfortunately, the source of contamination of patients in Pitié Hospital remains unknown.

ACKNOWLEDGMENTS

We gratefully thank J. Fleurette (Centre de Référence des Legionelloses, Faculté de Médecine Alexis Carrel, Lyon, France) for the gift of reference strain Bloomington 2, P. Berche for helpful discussion, and M.-L. Fourneaux for typing the manuscript.

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