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Clinical and environmental distribution of *Legionella pneumophila* in a university hospital in Italy: efficacy of ultraviolet disinfection

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Summary The molecular epidemiology of *Legionella pneumophila* in the 'V. Monaldi' University Hospital was studied. Seven cases of nosocomial Legionnaires' disease were diagnosed between 1999 and 2003. Two clinical legionella strains obtained from two patients in the adult cardiac surgery unit (CSU) and 30 environmental legionella strains from the paediatric and adult CSUs, neonatal intensive care unit (NICU) and the cardiorespiratory intensive care unit (CR-ICU) were serotyped and genotyped. *L. pneumophila* serogroup 1/Philadelphia with an identical pulsed-field gel electrophoresis (PFGE) profile A was isolated from two patients in the adult CSU, and from three and one water samples taken in the adult CSU and the paediatric CSU, respectively, from 2001 to 2002. Furthermore, *L. pneumophila* serogroup 3 with an identical PFGE profile B was identified in 20 environmental strains from all wards, *L. pneumophila* serogroup 3 with PFGE profile C was identified in a single environmental strain from the CR-ICU, and non-*pneumophila Legionella* with identical PFGE profile D was identified in five environmental strains from the adult CSU, paediatric CSU and NICU. Ultraviolet irradiation was effective in disinfection of the hospital water supplies in the adult and paediatric CSUs contaminated by *L. pneumophila* clone associated with nosocomial Legionnaires' disease. In conclusion, these data demonstrate that two cases of nosocomial legionellosis were caused by

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the persistence of a single clone of *L. pneumophila* serogroup 1/Philadelphia in the hospital environment, and that disinfection by ultraviolet irradiation may represent an effective measure to prevent nosocomial Legionnaires' disease.

Introduction

Legionella is a facultative intracellular pathogen known to cause both community- and hospital-acquired pneumonia.[1-4] The genus *Legionella pneumophila* accounts for 90% of cases of legionellosis,[1-3] and about 85% are due to serogroup 1;[6-10] other *Legionella* spp. are rarely pathogenic in humans.[10,11]

In recent years, nosocomial Legionnaires' disease has been on the increase.[1-5] Although cases of nosocomial legionella pneumonia are increasingly recognized, there is no general consensus regarding the prevention of legionellosis in hospitals. The Centers for Disease Control and Prevention (CDC) only recommend routine environmental investigation in water samples from high-risk wards housing transplanted patients.[12,13] Legionella infection occurs mainly by inhalation of aerosols generated from water sources.[1,14,15] Various disinfection methods have been used to eradicate legionella from hospital water systems, including hyperchlorination, thermal disinfection and ultraviolet (UV) irradiation.[16,17]

The objectives of the present study were: (1) to investigate the circulation of legionella in different wards of the 'V. Monaldi' University Hospital; (2) to analyse the molecular epidemiology of clinical and environmental legionella isolates; and (3) to validate the efficacy of a disinfection method for eradication of legionella from the hospital water system.

Materials and methods

Setting

The hospital is a tertiary care teaching hospital with 36 wards and 601 beds housed in an H-shaping building. There are five floors in each limb and three floors in the central block. The adult cardiac surgery unit (CSU) and the paediatric CSU are situated on the third and fifth floors of the same arm (north–east) of the building, respectively. The adult CSU consists of 18 rooms; 16 two-bed rooms and two rooms of six and 12 patients. The paediatric CSU consists of three rooms; two four-bed rooms and one room of 10 patients. The cardiorespiratory intensive care unit (CR-ICU) and the neonatal intensive care unit (NICU) are located on the first and second floors of the south–east and south–west arms of the building, respectively. The hospital obtains water from a single source supplied by the city and is chlorinated at <0.1 mg/L. A recirculating pressurized system supplies hot and cold water to the wards at 30 and 25 m^3/h, respectively.

Culture methods

The clinical strains studied were obtained from the bronchoalveolar lavage or the bronchial aspirate of two patients in the adult CSU in 2000 and 2001. Strains were isolated by direct culture on to buffered charcoal yeast extract agar (BCYE). Environmental isolates were obtained from multiple sites in patients' rooms as follows. For each outlet, 1 L of hot water was collected in a sterile bottle containing 1 mL of a 10 mg/mL solution of sodium thiosulphate. The water was allowed to flow for 3 min before collecting another 1 L from the same tap. The water temperature and residual free chlorine were determined immediately after collection. Samples were concentrated by filtration through cellulose acetate membrane filters (Millipore S.p.A., Milan, Italy, 0.22 μm pore size) and resuspended into 10 mL of the filtrate. Aliquots (150 μL) of the suspension were plated on to BCYE with legionella GVPC and MWY selective supplements (Oxoid, Basingstoke, UK). Plates were incubated in 2.5% CO_2 for five days at 37 °C and examined daily for evidence of growth. Gram-negative typical colonies requiring L-cysteine for growth were harvested, centrifuged at 3000 rpm for 20 min and resuspended in sterile distilled water prior to serological identification.

Urinary antigen detection

The Biotest legionella urine antigen enzyme-immunoassay, that recognized all *L. pneumophila* serogroups as well as other *Legionella* spp., was used for the in vitro detection of legionella urinary

antigen in patients with pneumonia (Biotest, Landsteiner, Germany).

Definitions

The diagnosis of *L. pneumophila* pneumonia was based on the following: isolation of *L. pneumophila* from respiratory samples or detection of urinary antigen. Cases of nosocomial Legionnaires' disease were classified as definite or suspected according to CDC guidelines.[12]

Serogrouping and monoclonal subgrouping of clinical and environmental isolates

Legionella isolates were serotyped into two groups (serotype 1 or serotype 2–14) by the legionella latex test (Oxoid, Basingstoke, UK) and into the other serogroups by the legionella serological test (Denka Seiken, Oakthorpe, UK). Identification of the subgroup *L. pneumophila* Philadelphia 1 was performed by a filter immunodetection assay, using the monoclonal antibody 2F10 to *L. pneumophila* LPS Philadelphia 1 strain (Biodesign, Saco, Maine, USA) as the primary antibody and anti-mouse IgGs conjugated to horseradish peroxidase as the secondary antibody. Protein–antibody complexes were revealed using the enhanced chemiluminescence detection system (Amersham Biosciences, Cologno Monzese, Milano, Italy). *L. pneumophila* Philadelphia 1 strain (ATCC 33152) was used as the positive control.

Molecular typing by pulsed-field gel electrophoresis (PFGE)

The preparation of genomic DNA of legionella isolates and DNA restriction digestion with *Sfi*I (New England Biolabs, Beverly, MA, USA) were performed as described previously.[10] PFGE was run in CHEF-DR II apparatus (BioRad Laboratories, Hercules, CA, USA) for 20 h at 12 °C with 5.3–49.9 s of linear ramping at 200 V. The gels were stained with ethidium bromide (1 μg/mL) and photographed. Interpretation of chromosomal DNA restriction patterns was based on the criteria of Tenover *et al.*[18] Strains showing more than three fragment variations were assumed to represent major PFGE patterns, while one to three fragment differences were considered to represent PFGE pattern subtypes.

Disinfection procedures

Thermal shock treatment for emergency disinfection of hot-water distribution systems was performed according to the American Society of Plumbing Engineers.[19] The water temperature was maintained at 80 °C for 3 h for flushing all outlets, taps and showerheads. During the treatment period, the water temperature at distal points was required to reach 65 °C.

For UV disinfection, two independent UV devices (E-TF AP20, Enertek, Caserta, Italy) were installed into a bypass valve at the origin of the hot-water supply to the adult CSU and the paediatric CSU. The system was designed to treat flows between 4 and 50 m^3/h with a maximum pressure of 7 bar. The light source consisted of 12 monochromatic low-pressure UV-C lamps (254 nm) complying with EN 61199. The fluency rate of the fluorescent lamps was 33 000 $\mu W \cdot s/cm^2$. For weaker decontamination of water, hydrogen peroxide (Biopol-H-30) at a 50% dilution was applied for 6–8 h before the start of UV treatment. Hydrogen peroxide treatment was repeated every six months.

Results

Legionella spp. circulation

A cluster of cases of *L. pneumophila* pneumonia was observed between 1999 and 2001 (Table I). Four

Table I Legionella in 'V. Monaldi' hospital during the study period

Ward	Clinical isolates			Urinary antigen positive tests					Environmental survey[a]		
	Years			Years					Sampling period[a]		
	1999	2002	2001	2000	2001	2002	2003	2004	11/2001	05/2002	12/2002
Adult CSU	1	1	1	1	2	2	1	-	3/8	3/8	4/8
Paediatric CSU	-	-	-	-	-	-	-	-	3/5	2/5	2/5
NICU	-	-	-	-	-	-	-	-	2/3	3/3	2/3
CR-ICU	1	-	-	-	-	1	1	-	2/5	2/5	2/5

CSU, cardiac surgery unit; NICU, neonatal intensive care unit; CR-ICU, cardiorespiratory intensive care unit.
[a] Number of positive sites over total sites analysed from November 2001 to December 2002. Month and year is indicated for each time point.

patients from the adult CSU were hospitalized for more than 10 days, and were considered to be cases of nosocomial Legionnaires' disease. The patient from the CR-ICU was hospitalized for 24 h at the time of *L. pneumophila* isolation, and was considered to be a case of community-acquired Legionnaires' disease.

Additional cases of nosocomial Legionnaires' disease were further diagnosed during 2002 and 2003 on the basis of the legionella urinary antigen test (Table I). Three patients from the adult CSU were considered to be definite cases of nosocomial Legionnaires' disease, while two patients from the CR-ICU were considered to be cases of community-acquired Legionnaires' disease. No positive legionella urinary antigen tests were reported from any of the wards during 2004 (Table I).

Environmental tests for *Legionella* spp were performed from November 2001 to December 2002 in the adult CSU, and in other wards in which transplanted or immunocompromised patients were hospitalized. Thirty of 63 water samples collected from 21 sites in the adult CSU, paediatric CSU, NICU and CR-ICU were positive for *Legionella* spp., with two to four different sites being contaminated in all wards examined at each sampling period (Table I). The 30 positive samples contained between 10^2 and 10^4 colony-forming units (cfu)/L.

Serotype and genotype analysis of clinical and environmental legionella isolates

To evaluate the relationship between clinical and environmental legionella isolates, the distribution of species and serogroups for all strains was determined (Table II). The two clinical strains isolated from the two patients in the adult CSU in 2000 and 2001 were identified as *L. pneumophila* serogroup 1, *L. pneumophila* serogroup 3 was the most prevalent environmental isolate (21/30 strains), and *L. pneumophila* serogroup 1 was identified in four of 30 isolates. Five of 30 environmental isolates were classified as non-*pneumophila Legionella* spp. *L. pneumophila* serogroup 3 strains and non-*pneumophila Legionella* spp. were isolated in all wards analysed, while three and one *L. pneumophila* serogroup 1 strains were isolated in the adult CSU and the paediatric CSU, respectively (Table II).

To investigate the relationship between clinical and environmental strains, antigenic subtyping was performed on *L. pneumophila* serogroup 1 isolates and genomic typing was performed on all legionella isolates. Antigenic subtyping showed that all clinical and environmental *L. pneumophila* serogroup 1 isolates reacted with monoclonal antibody 2F10 to *L. pneumophila* LPS Philadelphia 1 strain. Genotype analysis of all legionella isolates by *Sfi*I digestion and PFGE analysis identified four major PFGE patterns, named A–D, that differed in migration of at least four DNA fragments (Figure 1). The two clinical and four environmental *L. pneumophila* serogroup 1/Philadelphia strains showed an identical PFGE profile A (Figure 1, lanes 1–5 and Table II). Twenty environmental *L. pneumophila* serogroup 3 strains showed PFGE profile B (Figure 1, lanes 6–8 and Table II), and one environmental *L. pneumophila* serogroup 3 strain showed PFGE profile C (Figure 1, lane 9 and Table II). All environmental non-*pneumophila Legionella* isolates showed an identical PFGE profile D (Figure 1, lane 10 and Table II). The concentration of *L. pneumophila* serogroup 1/Philadelphia with

Table II Serotype and genotype analysis of clinical and environmental legionella isolates

Type of isolate and number	Ward	Species	Serotype	PFGE type
Clinical (2)	Adult CSU	*L. pneumophila*	1/Philadelphia	A
Environmental (3)	Adult CSU	*L. pneumophila*	1/Philadelphia	A
Environmental (5)	Adult CSU	*L. pneumophila*	3	B
Environmental (2)	Adult CSU	*Legionella* spp.	-	D
Environmental (1)	Paediatric CSU	*L. pneumophila*	1/Philadelphia	A
Environmental (4)	Paediatric CSU	*L. pneumophila*	3	B
Environmental (2)	Paediatric CSU	*Legionella* spp.	-	D
Environmental (6)	NICU	*L. pneumophila*	3	B
Environmental (1)	NICU	*Legionella* spp.	-	D
Environmental (5)	CR-ICU	*L. pneumophila*	3	B
Environmental (1)	CR-ICU	*L. pneumophila*	3	C

CSU, cardiac surgery unit; NICU, neonatal intensive care unit; CR-ICU, cardiorespiratory intensive care unit; PFGE, pulsed-field gel electrophoresis.

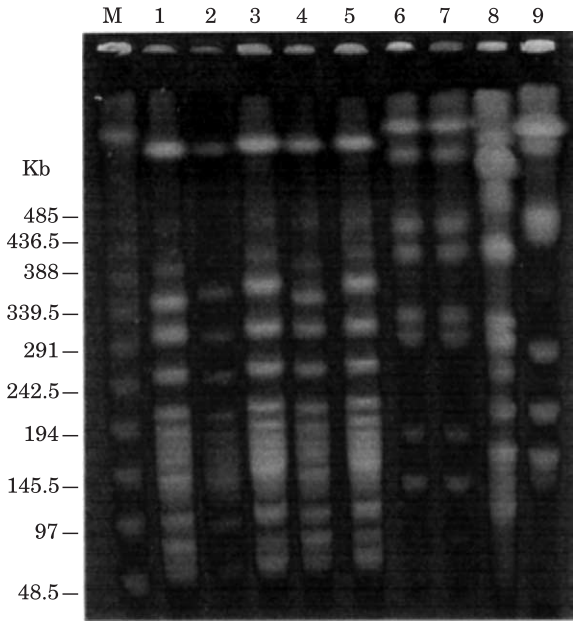

Figure 1 Pulsed-field gel electrophoresis profiles of clinical and environmental legionella isolates from the university hospital 'V. Monaldi'. Lanes 1, 2, clinical strains from two patients in the adult cardiac surgery unit (CSU); lanes 3, 4, environmental *L. pneumophila* serogroup 1 strains from the adult CSU; lane 5, environmental *L. pneumophila* serogroup 1 strain from the paediatric CSU; lanes 6 and 7, environmental *L. pneumophila* serogroup 3 strains from the adult and paediatric CSUs, respectively; lane 8, environmental *L. pneumophila* serogroup 3 strain from the cardiorespiratory intensive care unit; lane 9, environmental legionella strain from the adult CSU; lane M, multimers of phage lambda DNA (48.5 kb) molecular mass marker. Sizes in kilobases (kb) of lambda DNA molecular mass markers are indicated on the left of the panel.

identical PFGE profile A isolated from water samples in the adult CSU ranged from 5×10^3 to 1×10^4 cfu/L.

Disinfection of the contaminated hospital water supply

A number of control measures were adopted to disinfect hospital water in the adult and paediatric CSUs. Coarse filters and strainers were cleaned or replaced, and infrequently used showers and taps were removed. In October 2002, a 3-h superheating treatment of the hot-water distribution system was performed, and the temperature of the water in distal points reached 65 °C. On completion of the disinfection procedures, water samples were collected and examined for legionella contamination immediately and at one month. As legionella were re-isolated one month after thermal shock treatment, a UV disinfection procedure was installed.

UV lamp systems were inserted in the hot-water pipes supplying the adult and paediatric CSUs from January to December 2003. The mean flow of water in the circuit was 25 mc/h. Hydrogen peroxide (50%) was injected before the start of UV treatment and repeated every six months. Cultures were performed from samples collected before and after continuous UV treatment from eight and five distal points in the adult CSU and paediatric CSU, respectively. As shown in Figure 2, water samples from both wards were contaminated by *L. pneumophila* serogroup 1/Philadelphia PFGE type A clone, *L. pneumophila* serogroup 3 PFGE type B clone and non-*pneumophila Legionella* spp. PFGE type D clone before UV irradiation. UV irradiation in the adult CSU abolished contamination from *L. pneumophila* serogroup 1/Philadelphia PFGE type A clone and non-*pneumophila Legionella* spp. PFGE type D clone, and decreased the concentration of *L. pneumophila* serogroup 3 PFGE type B clone by 20-fold (from 2.5×10^4 to 1.2×10^3 cfu/L). No additional effect was observed following hydrogen peroxide treatment at six months [Figure 2(A)]. The effect of UV treatment was more pronounced in the paediatric CSU, where no distal points showed contamination by any legionella [Figure 2(B)].

Discussion

Healthcare-associated Legionnaires' disease represents an important public health problem. In North America, the prevalence of nosocomial pneumonias caused by *Legionella* spp. has been reported from individual hospitals to be from zero to 14%.[1,13] In Europe, between 2000 and 2002, the overall proportion of cases linked to hospital infections was 8.6%, with France, Italy and Spain reporting 73% of the total nosocomial cases.[3,4,13]

Molecular typing of two clinical isolates and 30 environmental strains from water samples in different hospital wards demonstrated the circulation and the persistence of three prevalent clones in the hospital environment. In fact, a single clone of *L. pneumophila* serogroup 1/Philadelphia with identical PFGE profile A persisted in the adult CSU for at least three years. *L. pneumophila* serogroup 3 with identical PFGE profile B was isolated in all wards and non-*pneumophila Legionella* isolates with identical PFGE profile D was isolated in the adult CSU, the paediatric CSU and the NICU. These data are in agreement with previous reports which show that clones of *Legionella* spp. can persist in the hospital environment for several years and can

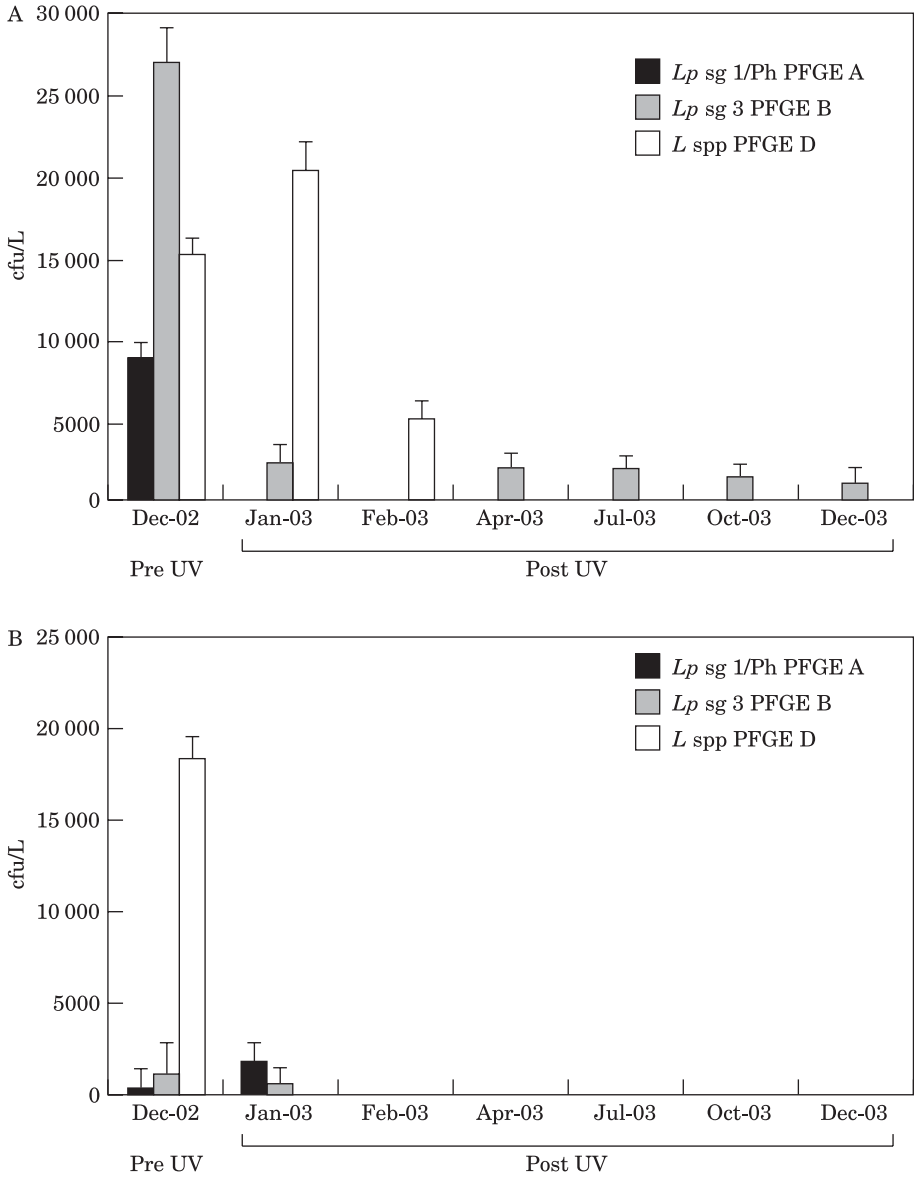

Figure 2 Effect of ultraviolet (UV) irradiation on legionella contamination of water in (A) the adult cardiac surgery unit (CSU) and (B) the paediatric CSU. cfu, colony-forming units; PFGE, pulsed-field gel electrophoresis. Values are the means with standard deviations from concentrations of different legionella clones at all sites analysed in the ward.

be responsible for outbreaks or sporadic infections.[2–11]

Although *L. pneumophila* serogroup 1/Philadelphia with PFGE profile A was not the most prevalent clone isolated in the hospital environment, it was isolated in hot-water samples from the same ward during 2001 and 2002 at a concentration ranging from 5×10^3 to $>10^4$ cfu/L. This is sufficient to cause one or more cases of infection per year.[14] One may conclude that the infecting legionella strains originated in the water supply and that the same clone may have been responsible for the subsequent nosocomial legionella infections that occurred in the ward during 2002 and 2003. This agrees with previous studies, which found *L. pneumophila* serogroup 1 in 64% of nosocomial Legionnaires' disease cases in Europe[2] and in 95% of total clinical isolates in France.[7] Subgroup Philadelphia has been frequently identified in clinical strains[2] and has been shown to contain several genes that confer the ability to survive in the mammalian host.[6]

The persistence of *L. pneumophila* serogroup 3 and non-*pneumophila Legionella* clones does not appear to have contributed to nosocomial legionellosis. Although the urinary antigen test was able to detect all *L. pneumophila* serogroups as well as other *Legionella* spp., no positive urinary antigen

test was found in patients from the adult CSU, NICU and CR-ICU wards during 2004, when *L. pneumophila* serogroup 3 and non-*pneumophila Legionella* clones were present in the environment.

Nosocomial Legionnaires' disease in the authors' institution was caused by legionella contamination of the water system, and two disinfection procedures were undertaken to eradicate it from the water supply in the adult CSU and paediatric CSU (*L. pneumophila* serogroup 1/Philadelphia PFGE type A) that was associated with nosocomial legionellosis. No cases of nosocomial legionellosis occurred in other hospital wards. Thermal shock at 80 °C for 3 h was not effective in decreasing or eliminating legionella. Other authors have shown that superheating and flushing of contaminated water is only temporarily efficacious in reducing bacterial contamination.[16] However, the present data are in contrast to a recent report indicating that increasing the hot-water temperature to 65 °C for 48 h is the most effective control measure for legionella contamination.[20] This discrepancy may be due to the different times and conditions of temperature increase adopted.

UV irradiation was effective in the control of contamination in the adult and paediatric CSUs almost immediately after application, even when the water had high levels of bacteria. However, UV irradiation and hydrogen peroxide disinfection was only partially effective in the adult CSU, where contamination at 1.2×10^3 cfu/L of *L. pneumophila* serogroup 3 was still present at a single site. This may be due to differences in the pipework, as the distribution system of the adult CSU supplies more than 18 rooms, while that of the paediatric CSU supplies three rooms. Nevertheless, UV irradiation and hydrogen peroxide treatment was effective in the eradication of *L. pneumophila* serogroup 1/Philadelphia clone of PFGE type A from the water systems of both wards. Several reports have shown that UV irradiation is an alternative method for disinfection of water systems, and is more effective than chlorination, heating or other methods of ionization.[16,17] The technical characteristics of the UV system adopted in this study, i.e. the elevated flow of water (25 m^3/h) into the circuit and the use of Teflon pipes that tolerate high pressure and are well penetrated by UV light, may have been important in inhibiting the formation of sediments and biofilms.

In conclusion, at least two cases of nosocomial legionella infections in the authors' institution were caused by the persistence in the environment of a single clone of *L. pneumophila* serogroup 1/Philadelphia. Disinfection by UV irradiation was effective in controlling legionella in the hospital water system.

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