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Potable water and nosocomial Legionnaires' disease – check water from all rooms in which patient has stayed

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SUMMARY

We studied 7 patients with nosocomial Legionnaires' disease to determine the relationship between isolates of *Legionella pneumophila* recovered from potable water and those recovered from patients. Potable water was cultured from all rooms in which patients had stayed prior to the diagnosis of Legionnaires' disease. The 38 isolates of *L. pneumophila* (31 environmental, 7 patient) were resolved into 9 distinct patterns by pulse-field gel electrophoresis (PFGE), 3 by plasmid content and 2 each with monoclonal antibodies and conventional agarose gel electrophoresis of small fragments of DNA.

Using PFGE it was determined that 4 of the 7 patients were infected with *L. pneumophila* identical to an isolate recovered from the potable water supply in one of the rooms each had occupied prior to the diagnosis of Legionnaires' disease. Patients had resided in a mean of 3·57 rooms before a diagnosis of nosocomial Legionnaires' disease. We conclude that in the setting of contaminated potable water and nosocomial Legionnaires' disease water from all the rooms which the patient has occupied prior to this diagnosis should be cultured. PFGE of large DNA fragments discriminated best among the isolates of *L. pneumophila*.

INTRODUCTION

Legionella pneumophila is an aquatic microorganism that causes both community-acquired and nosocomial pneumonia [1, 2]. Careful epidemiological studies have implicated contaminated potable water as the source of many cases of nosocomial legionellosis [2–4]. We have identified sporadic cases of nosocomial Legionnaires' disease at our hospital since 1981 [2, 5]. During the course of our studies we noted that some patients with nosocomial Legionnaires' disease had stayed in several rooms or units prior to the diagnosis of their nosocomial infection. To determine in which room they acquired Legionella we cultured water

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from all the rooms a patient occupied prior to the diagnosis of nosocomial Legionnaires' disease.

Isolates were examined for plasmid content, and surface antigens were identified using a panel of monoclonal antibodies. Chromosomal DNA was digested using various restriction enzymes and the resulting patterns compared using conventional and pulsed field gas electrophoresis.

METHODS

Case definition

Legionnaires' disease was diagnosed if *Legionella pneumophila* was isolated from the respiratory secretions of a patient with radiographic documented nosocomial pneumonia. The pneumonia was considered to be nosocomially acquired if signs and symptoms of infection developed $\geqslant 72$ h after admission or, in the case of patient number 7, the infection became evident within 10 days of discharge and epidemiologically was considered to have been acquired nosocomially.

Water samples

As soon as a patient was identified as having nosocomial Legionnaire's disease, water samples were collected from all the rooms that he/she had occupied prior to and at the time of the diagnosis.

Water samples were obtained by simultaneously turning on the hot and cold water taps so that the water flowed slowly. Two hundred ml of water was then collected into a sterile bottle containing 0·1 ml of a 10% solution of sodium thiosulfite.

Culture for Legionella pneumophila

Respiratory specimens

Material for culture (sputum, endotracheal secretions, pleural fluid or lung tissue) was inoculated onto 5% sheep blood agar (BA), buffered charcoal yeast extract (BCYE agar) containing alpha-ketoglutarate and two selective media; one, BCYE containing cefamandole, polymyxin B and anisomycin (MPA agar) and the other, BCYE containing polymyxin B, anisomycin and vancomycin (PAV agar) [6]. All plates were incubated at 37 °C in a humidified atmosphere containing 5% carbon dioxide for 7 days and examined daily. Colonies that morphologically resembled Legionella were sub-cultured onto blood and BCYE agar. Those that failed to grow on blood agar and grew on BCYE agar were examined by a direct fluorescent antibody technique [7] employing *Legionella pneumophila* serogroups 1–6 antisera (Mardx, Scotchplains, N.J.). Three to four colonies from each plate were picked for subculture identification. Only one colony was used for typing. Previously we had carried out plasmid typing on up to 10 colonies from the original positive plates and found only one plasmid type each time.

Water

Water samples (50 ml) were centrifuged at 1200 g for 20 min. Most of the supernatant was removed, leaving approximately 10% of the original volume in which the sediment was resuspended. A 0·1 ml aliquot was inoculated onto the surface of the various media and processed as outlined above.

Determination of plasmid contents of legionella isolates

Portions of the growth achieved after 48 h incubation of the isolates on BCYE agar were suspended in 0·5 ml of TE buffer (0·5 M Tris-Hcl, 0·02 M EDTA, pH 8). After pelleting and resuspending in 25 μl of TE, plasmid DNA was extracted from the cells using a modified alkaline SDS procedure [8]. The contents of the extracts were determined by electrophoresis in vertical 0·75% agarose gels followed by ethidium bromide staining. Strains with no detectable plasmids constituted plasmid type O; those carrying a 28 MDa plasmid were type II. Types III and VI were comprised of 96 and 72 MDa and 100 MDa plasmids respectively.

Monoclonal antibody typing

Isolates were typed by Dr Joly, Université Laval, Québec City using a panel of monoclonal antibodies as previously described [9].

Endonuclease restriction analysis of chromosomal DNA

Small fragment DNA (conventional gel electrophoresis)

Chromosomal DNA was recovered from pelleted cells using a modified Roussel–Chabbert procedure [10]. Double digests with *Hpa*I and *Hpa*II were used to differentiate the isolates. Digestion was continued for 8 h at 37 °C in buffers provided by the supplier (Boehringer Mannheim, Dorval, Québec). Restriction fragments were separated in vertical, 0·75% agarose gels and visualized after ethidium bromide staining by ultraviolet irradiation. Resultant distinct patterns were assigned letter codes a, b, c or d.

Large fragment DNA (pulsed-field electrophoresis)

A description of the methods and the selection of restriction endonucleases used for genomic fingerprinting of *L. pneumophila* by PFGE was as previously detailed [11, 12]. The growth from a single plate was used to prepare high molecular weight DNA in agarose plugs. The plugs were digested overnight with 5–10 units of *Bss*HII, *Sal*1 or *Spe*1 as recommended by the manufacturer (Stratagene, Professional Diagnostics Inc., Edmonton, Alberta, Canada). PFGE was performed in 1% agarose gels with a 5 s pulse for 12 h followed by a 10 s pulse for 12 h using a contour clamped homogenous electric field system (Pulsphor Plus, Pharmacia LKB, Uppsala, Sweden). Gels were stained with ethidium bromide and photographed under u.v. illumination. Unique large fragment restriction patterns were given arbitrary numerical designations within each enzyme category.

RESULTS

Seven patients, 4 men and 3 women, with nosocomial pneumonia due to *Legionella pneumphila* serogroup 1 were studied. All but one were receiving immunosuppressive medications including corticosteroids. The one patient who was not immunosuppressed had a complicated course following aortic valve replacement. None of the patients showered while in hospital. However, tap water was used to bed bath these patients. All the patients used tap water to brush their teeth. Tap water was ingested by all except patients 3 and 7. We were unable to determine how much water was ingested and in what rooms it was ingested.

Table 1 gives the results of the culturing of clinical specimens and potable water

Table 1. *Characterization of environmental and patient isolates of* Legionella pneumophila *serogroup 1*

Date/patient	Source of isolate†	Culture result/lab. no.	Plasmid type	MAB	Restriction endonuclease analysis: Small fragments	Restriction endonuclease analysis: Pulsed field-large fragments
Patient 1						
10–12* Sept	11V 232	*L. pneumophila*/4707	II	Olda	b	B1, Sa1, Sp1
Sept 12–17	CVICU	Negative				
Sept 17	6S-020 Rm. sink	*L. pneumophila*/4700	VI	Olda	b	B2, Sa2, Sp2
17 Sept	6S-020 Bathroom sink	*L. pneumophila*/4699	II	Olda	b	B1, Sa1, Sp1
17–27 Sept	6S-282	*L. pneumophila*/4689	III	Olda	d	B2, Sa2, Sp2
	6S-medi prep room	*L. pneumophila*/4705	II	Olda	b	B1, Sa1, Sp1
27–29 Sept	11V-230	*L. pneumophila*/4706	III	Olda	d	B3, Sa2, Sp3‡
29– Sept	SICU-7	*L. pneumophila*/4690	II	Olda	b	B4, Sa3, Sp1
2 Oct	Pt.	*L. pneumophila*/134515	III	Olda	d	B3, Sa2, Sp3
6 Oct	Pt.	*L. pneumophila*/136788	III	Olda	d	B3, Sa2, Sp3
Patient 2						
19 Dec	4S-018	Negative				
20 Dec–2 Jan	8W-227	Negative				
	8S-282	Negative				
2–5 Jan	MICU-4	Negative				
5–15 Jan	4B-007	Negative				
15–19 Jan	8S-288	*L. pneumophila*/4831	III	Olda	d	B3, Sa2, Sp‡
19 Jan–2 Feb	SICU	*L. pneumophila*/4826	II	ND	ND	ND
1 Feb	Pt.	*L. pneumophila*/194977	III	Oxford	d	B3, Sa2, Sp3
Patient 3						
6–11 Dec	8B	*L. pneumophila*/175289	II	Olda	b	B4, Sa3, Sp1
12–13 Dec	8A	*L. pneumophila*/175287	III	Oxford	d	B5, Sa5, Sp3
13–15 Dec	MICU	*L. pneumophila*/175285	VI	Olda	b	B2, Sa2, Sp2
16 Dec	Pt.	*L. pneumophila*/172863	II	Oxford	b	B1, Sa1, Sp1
Patient 4						
21–29 Jan	8B-133	*L. pneumophila*/4838	II	Olda	b	B1, Sa1, Sp1‡
29 Jan–20 Feb	8B-128	*L. pneumophila*/4840	II	Olda	b	B4, Sa4, Sp1
8 Feb	Pt.	*L. pneumophila*/199546	II	Olda	b	B1, Sa1, Sp1

Patient 5						
24 Jan–24 Feb	8B-140-2	ND				
25 Feb	8A-098	Negative				
25 Feb	Pt.	*L. pneumophila*/206897	II	Olda	b	B1, Sa1, Sp1
25 Feb	Pt.	*L. pneumophila*/206897	II	Olda	b	B1, Sa1, Sp1
Patient 6						
28 June–10 July	4B-098	Negative				
10–14 July	CVICU Bed 1	*L. pneumophila*/9366	III	Olda	d	B3, Sa2, Sp3‡
14 July–13 Aug	4B-099	*L. pneumophila*/9362	III	Olda	d	B5, Sa5, Sp3
27 July	Pt.	*L. pneumophila*/277532	III	Olda	d	B3, Sa2, Sp3
5 Sept	Pt.	*L. pneumophila*/295676	III	Olda	d	B3, Sa2, Sp3
22 Sept	Pt.	*L. pneumophila*/304500	III	Olda	d	B3, Sa2, Sp3
7 Oct	Pt.	*L. pneumophila*/312421	III	Olda	d	B3, Sa2, Sp3
Patient 7						
19–20 March	3B-083	*L. pneumophila*/9433	II	Olda	b	B4, Sa3, Sp1
20–22 March	CVICU Bed 9	*L. pneumophila*/9434	III	Olda	d	B3, Sa2, Sp3
23–31 March	4B-094	*L. pneumophila*/9435	II	Olda	b	B4, Sa3, Sp1
13 Apr	CCU Bed 14	*L. pneumophila*/9436	VI	Untyp		B7, Sa7, Sp4
14 Apr	Pt.	*L. pneumophila*/409916	III	Olda	d	B6, Sa6, Sp2
15 Apr	Pt.	*L. pneumophila*/411950	III	Olda	d	B6, Sa6, Sp2
17 Apr	Pt.	*L. pneumophila*/411324	III	Olda	d	B6, Sa6, Sp2
21 Apr	Pt.	*L. pneumophila*/410689	III	Olda	d	B6, Sa6, Sp2
22 Apr	Pt.	*L. pneumophila*/414568	III	Olda	d	B6, Sa6, Sp2
23 Apr	Pt.	*L. pneumophila*/408189	III	Olda	d	B6, Sa6, Sp2
28 Apr	Pt.	*L. pneumophila*/408811	III	Olda	d	B6, Sa6, Sp2

* Refers to the days during which the patient resided in the room or unit shown in the second column. It also refers to the date(s) on which *L. pneumophila* was isolated from the indicated patient.
† Water from the room which the patient occupied or an isolate from the patient.
‡ Denotes the environmental isolate that is identical to the patient isolate.
Plasmid type, as defined in methods sections.
MAB, Monoclonal antibody type.
Small fragment restriction endonuclease patterns were determined by conventional slab gel electrophoresis.
Large fragment restriction endonuclease restriction patterns were resolved by pulsed field gel electrophoresis.
ND, not done. In both instances this was because the organism could not be recovered from storage.
Untyp, untypable.

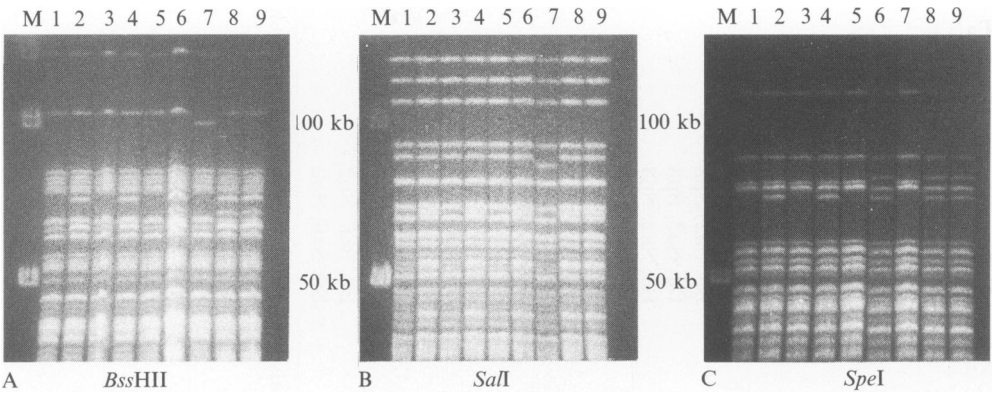

Fig. 1. Pulsed-field gel electrophoresis of *L. pneumophila* chromosomal DNA following digestion with *Bss*HII (panel A), *Sal*I (panel B), and *Spe*I (panel C). All isolates were associated with patient 1. M refers to molecular mass markers. Lanes 1–9 in each panel represent isolates 4707, 4700, 4699, 4689, 4705, 4690, 134515, 136788 (Table 1). Note that the profiles in lanes 6, 8 and 9 are identical. The isolate in lane 6 was recovered from the potable water of a room that this patient occupied 3 days before *L. pneumophila* (isolate 134515) was isolated from her respiratory sections. She had occupied 6 different rooms prior to this one. *L. pneumophila* was recovered from potable water samples from 5 of these 6 rooms. These isolates are shown in lanes 1–5. The isolate in lane 7 was recovered from the potable water source for SICU bed 7 where the patient was located when Legionnaires' disease was diagnosed.

obtained from the room(s) which the patients occupied. This table also categorizes each of the 38 isolates according to plasmid content, monoclonal antibody type and restriction endonuclease analysis of chromosomal DNA. Fig. 1 depicts the banding patterns obtained upon PFGE of the *Bss*HI, *Sal*I and *Spe*I digests of the clinical and environmental isolates associated with patient 1. Fig. 1 clearly demonstrates that only one of the potable water isolates strain 4706 (lane 6) was identical to the patient isolates (lanes 8 and 9).

By PFGE, small fragment electrophoresis patterns and plasmid complements, 4 of the 7 patients had an isolate of *L. pneumophila* that was identical to an isolate obtained from the potable water in one of the rooms in which the patient had stayed (Table 1). Restriction endonuclease analysis of chromosomal DNA by PFGE discriminated best among the isolates. Nine distinct patterns were evident with PFGE, compared with 3 types by plasmid profiling and the 2 each with MABs and small fragment DNA electrophoresis.

Table 2 gives details about the movements of patients with nosocomial Legionnaires' disease. These patients were moved frequently – from 2 to 6 times (mean 3·57) before Legionnaires' disease was diagnosed.

DISCUSSION

This study shows that all sources of potable water to which a patient is exposed should be cultured during investigations of nosocomial legionellosis. Since we did not culture the water from the various rooms until after we had isolated *Legionella pneumophila* from a patient, the question arises as to the stability of the Legionella population colonizing a particular outlet. In a previous study, we found that a site

Table 2. *Details of the movements of 7 patients with nosocomial Legionnaires' disease and correlation of plasmid analysis of isolates of* Legionella pneumophila *from patient and environment*

	Patient number						
	1	2	3	4	5	6	7
Date of admission	10 Sept 1990	19 Dec 1990	6 Dec 1990	21 Jan 1990	24 Jan 1990	24 June 1991	19 Mar 1992
Days after admission that LD developed	22	40	10	18	30	28	25
No. of moves before LD was diagnosed	5	7	3	2	2	4	3
Plasmid type of patient isolate of *Legionella pneumophila*	III	III	II	II	II	III	III
Correlation of plasmid type of patient and environmental isolates with move number	4	5	1	1 and 2	None*	2 and 4†	2
No. of days spent in room in which legionella was probably acquired	2	4	3				3
Days after admission to room noted above that LD was diagnosed	6	16	4				24
Pulsed field data in agreement with plasmid data	Yes	Yes	No‡	Yes§	NA	Yes¶	Yes

* Potable water in both rooms negative for legionella.
† Data for move no. 3 not shown. This move was for a few hours only.
NA, not applicable.
‡ Suggests no correlation between potable water and patient isolate.
§ Allowed discrimation between isolates that were the same by plasmid profile.
¶ As for pt. no. 4.

colonized by a particular strain tended to yield the same strain (based on the plasmid content) for months [8]. However, in the current study we have shown that PFGE of large DNA fragments can differentiate between isolates that carry the same plasmids, react with the same monoclonal antibodies and demonstrate the same small fragment DNA (high frequency cutters) pattern. Patient 4 in Table 1 is such an example.

Intermittent low concentrations of Legionella from a previously positive site, i.e. below the limits of detection using our methods [8], is the likely explanation for the negative water cultures in the rooms occupied by patient 5 and some of the rooms occupied by patient 2.

A limitation of our study is the possibility that the patients ingested water from sites in the hospital other than the room(s) which they occupied. Thus 2 of the 7

patients with no corresponding water isolates may have acquired the isolate at another site in the hospital or even outside the hospital.

Stout and colleagues [13] studied 20 patients with culture confirmed community-acquired Legionnaire's disease. They compared environmental and patient isolates using *Eco*R1, *Eco*RV and *Hin*dIII digests of chromosomal DNA. For 8 of the 20 patients, identical isolates of *Legionella pneumophila* were recovered from the patient and the potable water to which he or she had been exposed in the 2 weeks prior to onset of symptoms.

At our hospital there are no sources of legionella other than the potable water. There are no cooling towers within miles of our hospital and 5 of the 7 cases occurred during the winter months. In a previous case-control study we showed that microaspiration of contaminated potable water was the likely mode of acquisition of legionellosis at our hospital [2]. Provision of sterile potable water to patients who are receiving corticosteroids and to organ transplant patients has reduced the number of cases of nosocomial Legionnaires' disease at our hospital. Several of the cases in the current study occurred as a result of a lapse in the policy, i.e. patients ingested contaminated water.

Our study raises additional questions that need to be addressed in future studies of nosocomial legionellosis. For example, why, when susceptible patients are exposed to multiple sources of contaminated water, does transmission occur from one source and not from others? Careful observational studies are necessary to determine whether host or microbial characteristics are the determining factors. Thus, more water may be ingested at one site than at another site; showering may occur at one location and not at another; bathing the face with contaminated water may be done at one site and not at the other.

The microenvironment of the organisms may vary from site to site or the organisms at one site may be more virulent. Lowry and colleagues [14] observed sternal wound infections with *L. dumoffii* and *L. pneumophila* after nurses used tap water to remove povidone-iodine from patients' chests within the first 24 h after surgery. One of the most interesting comments in this report was: 'it is remarkable that a single strain of *L. dumoffii* caused serious infection at our institution for several years but was isolated from hospital tap water on only one occasion' [13]. This observation highlights the questions raised above.

The usual incubation period for Legionnaires' disease is 2–10 days [15] although Kirby and colleagues [16] noted apparent incubation periods of 26 and 28 days among 65 patients with nosocomial Legionnaires' disease. We previously reported a patient who had been colonized with *L. pneumophila* for 63 days before the onset of pneumonia [17]. Colonization of the oropharyngeal mucosa by *L. pneumophila* and later aspiration into the lungs is an attractive hypothesis; however, one study which examined the oropharynx for colonization by legionella concluded that this rarely if ever occurs [18]. The study by Blatt and colleagues suggests that aspiration of contaminated water is one mechanism whereby Legionella reaches the lung [19].

A large number of methods have been used to try and characterize legionella isolates for epidemiologic purposes. Such methods include plasmid profiling [5], definition of surface antigens by monoclonal antibodies [20], alloenzyme and restriction endonuclease analysis [21, 22], and ribotyping [23]. Recently ar-

bitrarily primed polymerase chain reactions have been used to generate an array of strain specific amplicons which can then be sorted by agarose gel electrophoresis [23].

Monoclonal antibody typing is easy to perform but variation of this phenotypic characteristic can occur in a single parent strain within a particular environmental site [20].

We found that PFGE allowed us to differentiate among environmental isolates that were the same by all other parameters tested. Isolates such as 4689 and 4706 associated with Patient 1, 4838 and 4840 associated with patient 4 and 9362 and 9366 associated with patient 8 are such examples. Schoonmaker and colleagues [12] found that PFGE resolved 14 different patterns among 32 *L. pneumophila* serogroup 1 and serogroup 6 isolates. They also found that PFGE subdivided isolates of the same ribotype. We also found that isolates that were identical by PFGE were different when all typing methods were considered – for example isolates 4689 and 4700 from patient 1 were PFGE type B2, Sa2, Sp2; however, 4689 was plasmid type III and 4700 was plasmid type VI.

Another question that has to be considered in an investigation such as ours pertains to the significance of variation in fragmentation patterns. Are the nucleotide changes great enough to make one isolate different from another? The stability of *L. pneumophila* isolates recovered from patients 6 and 7 over a 9 and a 2 week period respectively suggests that even minor differences are important. Bialkowska-Hobrzanska and colleagues [24] suggested that 2 strains were identical when numerical analysis of restriction endonuclease fragmentation patterns demonstrated percentage similarity patterns values of $> 95\%$.

We conclude that all sources of potable water to which a patient was known to be exposed should be cultured in investigations of nosocomial Legionnaires' disease. PFGE of large DNA fragments provide a more extensive differentiation among isolates of *L. pneumophila* than do plasmid content, MABs, or restriction endonuclease small fragmentation patterns obtained with enzymes that are frequent DNA cutters.

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