Use of sequence-based typing for investigation of a case of nosocomial legionellosis

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A fatal case of nosocomial legionellosis in a low prevalence region (Calgary, Alberta, Canada) prompted investigation into the source of infection. Hospital water systems contaminated with *Legionella pneumophila* have been shown to pose a risk to compromised patients. Typing of an *L. pneumophila* serogroup 1 strain isolated from the patient using sequence-based typing (SBT) and amplified fragment length polymorphism (AFLP) analysis linked it to a persistent and widespread strain isolated from the hospital water system establishing a nosocomial mode of acquisition. Different SBT and AFLP patterns were determined for non-epidemiologically linked cases and isolates from different hospitals.

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INTRODUCTION

Legionella pneumophila infection has been associated with fountains and respiratory therapy equipment suggesting that transmission occurs via inhalation (Woo *et al.*, 1992). Microaspiration of contaminated potable water is another mode of transmission (Blatt *et al.*, 1993; Stout & Yu, 1997; Yu, 1993). The incidence of legionellosis in Alberta (Canada) is low compared to the eastern provinces of Ontario and Quebec, with 0.047 human cases per 100 000 per year (Public Health Branch, Alberta Health and Wellness), and thus a case of nosocomial pneumonia due to *L. pneumophila* serogroup 1 that contributed to the death of a patient in an acute-care hospital in Calgary, Alberta, raised concern and prompted the analysis of water supplies.

Due to the ubiquitous presence of legionellae in nature, discriminatory molecular methods are required for investigation of clinical and environmental strains to determine the source of infection. Recently sequence-based typing (SBT) has been described as a simple, rapid, discriminatory and portable method for typing *L. pneumophila* strains (Gaia *et al.*, 2003, 2005). Based on the sequence of six target genes, strains are assigned an SBT pattern number by the

European Working Group for Legionella Infections (EWGLI: www.ewgli.org). In this report, we used SBT to study the link between a rare nosocomial case of *L. pneumophila* infection and environmental isolates. We also performed amplified fragment length polymorphism (AFLP) analysis (Valsangiacomo *et al.*, 1995) for confirmation of the SBT types.

METHODS

Isolation of *L. pneumophila*. *L. pneumophila* was isolated from bronchoalveolar lavage specimens on buffered charcoal yeast extract medium and from potable water by standard methods (Greenberg *et al.*, 1998). Isolates from two other non-epidemiologically related community-acquired cases (C03B09917 and C04B12826) and *L. pneumophila* strain ATCC 33153 were included as controls. Colonies with *Legionella* morphology were identified using routine clinical laboratory methods and tested by the MONOFLUO *Legionella pneumophila* IFA test kit (Bio-Rad). Identification was confirmed by cellular fatty acid analysis, using the MIDI system with Sherlock software version 4.5, and by 16S ribosomal sequencing at the National Microbiology Laboratory, Winnipeg, Canada. Isolates were identified as serogroup 1 using monoclonal antibodies (ProLab).

SBT of *L. pneumophila*. Genomic DNA was extracted from study isolates using the QIAamp DNA Mini kit (Qiagen). Six genes, *flaA*, *pilE*, *asd*, *mip*, *mompS* and *proA*, were amplified using the primers and amplification protocol provided by the EWGLI. Amplified products were sequenced in both directions using the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing kit in the ABI PRISM 3100-Avant Genetic Analyser (Applied Biosystems). Data collection used the Applied Biosystems Software v2.0. The sequences were

Abbreviations: AFLP, amplified fragment length polymorphism; ER, emergency room; EWGLI, European Working Group for Legionella Infections; ICU, intensive care unit; SBT, sequence-based typing.

The GenBank accession numbers for the novel alleles of *mompS* and *proA* are DQ309456 and DQ309457, respectively.

edited and analysed using BioEdit v7.1.1 (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). SBT allele numbers were assigned to the strains based on the EWGLI database.

AFLP. For the AFLP analysis, DNA was extracted using the TaKaRa DEXPAT method (TAKARA Bio Inc), and PCR, restriction digests and agarose gel electrophoresis were carried out according to methods described previously (Fry *et al.*, 1999; Valsangiacomo *et al.*, 1995).

RESULTS AND DISCUSSION

Patient and environmental isolates

A 74-year-old woman was admitted to hospital 1 due to a myocardial infarction. She was intubated in the emergency room (ER) and transferred to the intensive care unit (ICU), where she was stabilized. On day 10 she developed pneumonia, and *L. pneumophila* serogroup 1 was isolated from a bronchoalveolar lavage specimen. The infection was defined as nosocomial, based on the onset and the incubation period. She died on day 24 of cardiac complications. *L. pneumophila* serogroup 1 was isolated from potable water in the ER and ICU in the hospital, as well as from water samples from other hospitals in the city, but not from the residence of the index patient.

Typing of *Legionella* environmental and clinical isolates

The SBT allele numbers based on the six target genes are summarized in Table 1. The new alleles from this study have been submitted to the EWGLI SBT database and the National Center for Biotechnology Information database (GenBank accession numbers are: C04B12826 *mompS*, DQ309456; and C04B12826 *proA*, DQ309457). AFLP patterns of the control and samples are shown in Fig. 1.

Fig. 1. AFLP patterns for *L. pneumophila* serogroup 1 patient and environmental isolates. Lanes: 1, 100 bp ladder; 2, patient 1, hospital 1; 3, ICU sink 1, hospital 1; 4, ICU sink 2, hospital 1; 5, ER sink 1, hospital 1; 6, ATCC 33153; 7, patient 2, hospital 3; 8, patient 3, hospital 3; 9, ICU sink 1, hospital 3; 10, ICU sink 1, hospital 2; 11, ICU sink 1, hospital 4; 12, Negative PCR control.

The alphabetical designations based on the electrophoresis pattern are included in Table 1.

SBT and AFLP of the samples (Table 1) revealed that the isolate from the index (deceased) patient (C03B16848, hospital 1) had the same sequence type profile across the six target genes (1,4,3,1,1,1) as isolates recovered from water in

Table 1. Summary of SBT and AFLP patterns of *L. pneumophila* serogroup 1 patient and environmental isolates

Sample identification	Type of specimen*	Isolation origin, hospital, date of collection	SBT allele numbers (<i>flaA</i> , <i>pilE</i> , <i>asd</i> , <i>mip</i> , <i>mompS</i> , <i>proA</i>)	AFLP pattern type
C03B16848	BAL	Patient 1, hospital 1, 27 November 2003	(1,4,3,1,1,1)	A
C03B17576	Water	ICU sink 1, hospital 1, 11 December 2003	(1,4,3,1,1,1)	A
C03B17881	Water	ICU sink 2, hospital 1, 17 December 2003	(1,4,3,1,1,1)	A
C04B04689	Water	ICU sink 1, hospital 1, 23 March 2004	(1,4,3,1,1,1)	ND
C04B05011	Water	ER sink 1, hospital 1, 30 March 2004	(1,4,3,1,1,1)	A
C05B03153	Water	ER triage sink, hospital 1, 21 March 2005	(1,4,3,1,1,1)	ND
C04B07897	Water	ICU sink 1, hospital 2, 1 June 2004	(1,4,3,1,1,1)	A
C03B09917	BAL	Patient 2, hospital 3, 15 July 2003	(3,4,1,1,14,9)	B
C04B12826	BAL	Patient 3, hospital 3, 9 September 2004	(12,9,2,5,27,20)	E
C04B11181	Water	ICU sink 1, hospital 3, 13 August 2004	(6,10,15,28,21,3)	C
C04B11757	Water	ICU sink 1, hospital 4, 26 August 2004	(7,6,17,3,11,11)	D
ATCC 33153		ATCC control strain	(7,10,17,10,5,4)	F

ND, Not determined.

*BAL, Bronchoalveolar lavage.

nearby ICU sinks (C03B17576 and C03B17881, hospital 1). Further sampling of hospital sinks outside of the ICU in hospital 1 ($n=35$ samples) showed that 14 % of water samples harboured *L. pneumophila* including that from the ER sink where the patient was admitted (C04B05011, hospital 1), and these isolates had the same SBT type as the patient. Water from the index patient's residence was negative for *Legionella* consistent with the nosocomial acquisition of infection. Potable water supplies in hospital wards housing highly compromised patients in three additional hospitals were tested for *L. pneumophila*. Water obtained from a nearby hospital (C03B07897, hospital 2) that is supplied from the same water reservoir as hospital 1 contained *L. pneumophila* with the same sequence profile, 1,4,3,1,1. *L. pneumophila* isolated from water from two more distant hospitals in the same city (C04B11181, hospital 3; and C04B11757, hospital 4) had different *L. pneumophila* SBT types. These hospitals derive their water from a different water reservoir, and these isolates were included as non-epidemiologically related controls.

The two non-epidemiologically linked community-acquired cases (C03B09917 and C04B12826 from hospital 3) had different SBT types and AFLP patterns compared to the isolates from the index patient and from water of the various hospitals, consistent with their community-acquired source of infection.

The water system of the affected hospital was superheated (greater than 71 °C for more than 30 min) and flushed as this approach could be instituted quickly to suppress *L. pneumophila* from the water supply to the affected wards. Despite this treatment, it was still possible to isolate *L. pneumophila* with the same sequence profile after 3 months (C04B04689 and C04B05011, hospital 1). A copper-silver ionizer was installed to control *Legionella* in the water system, but *L. pneumophila* of the same SBT type persisted in the water (C05B03153, hospital 1).

Although this is a small study, the finding of identical SBT types in the patient and hospital water sources in units occupied by the patient strongly implicates the hospital potable water system as the source of the nosocomial infection. *L. pneumophila* SBT type 1,4,3,1,1 has been shown to be a widespread environmental strain, isolated in Japan (Amemura-Maekawa *et al.*, 2005) and Europe (Scaturro *et al.*, 2005), thus calling into question the discriminatory power of SBT for this strain. In this study, however, we were able to establish an epidemiological link between the patient and water isolates and show that this strain was not seen in other patients or in non-epidemiologically linked water samples in this region. The results were also confirmed by AFLP, a highly discriminatory method for *Legionella* typing (Fry *et al.*, 2000).

This study extends recent reports (Amemura-Maekawa *et al.*, 2005; Scaturro *et al.*, 2005; Perola *et al.*, 2005) by demonstrating that SBT using the EWGLI database for *L. pneumophila* allele designations is useful for investigation of

nosocomial legionellosis cases and hospital environmental strains. As the clinical and epidemiological interpretation of environmental isolates is controversial, a typing method with this level of discriminatory power has great potential for assisting in epidemiological studies. Until the epidemiology of environmental strains is better characterized, however, the discriminatory power of SBT can be increased by using monoclonal antibody serogrouping (Gaia *et al.*, 2005) and confirmatory methods such as AFLP or PFGE (Amemura-Maekawa *et al.*, 2005).

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