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Original Article

Prevalence of *Legionella* species isolated from shower water in public bath facilities in Toyama Prefecture, Japan

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ABSTRACT

Aims: We investigated the prevalence of *Legionella* spp. isolated from shower water in public bath facilities in Toyama Prefecture, Japan. In addition, we analyzed the genetic diversity among *Legionella pneumophila* isolates from shower water as well as the genetic relationship between isolates from shower water and from stock strains previously analyzed from sputum specimens.
Methods: The isolates were characterized using serogrouping, 16S rRNA gene sequencing, and sequence-based typing.
Results: *Legionella* spp. were isolated from 31/91 (34.1%) samples derived from 17/37 (45.9%) bath facilities. Isolates from shower water and bath water in each public bath facility were serologically or genetically different, indicating that we need to isolate several *L. pneumophila* colonies from both bath and shower water to identify public bath facilities as sources of legionellosis. The 61 *L. pneumophila* isolates from shower water were classified into 39 sequence types (STs) (index of discrimination = 0.974), including 19 new STs. Among the 39 STs, 12 STs match clinical isolates in the European Working Group for *Legionella* Infections database. Notably, ST505 *L. pneumophila* SG 1, a strain frequently isolated from patients with legionellosis and from bath water in this area, was isolated from shower water.
Conclusions: Pathogenic *L. pneumophila* strains including ST505 strain were widely distributed in shower water in public bath facilities, with genetic diversity showing several different origins. This study highlights the need to isolate several *L. pneumophila* colonies from both bath water and shower water to identify public bath facilities as infection sources in legionellosis cases.

1. Introduction

Legionella pneumophila is a major causative agent of Legionnaires' disease, a severe form of legionellosis and a potentially fatal pneumonia [1]. Although 58 species and more than 70 serogroups of *Legionella* spp. have been identified [2], more than 90% of legionellosis cases are caused by *L. pneumophila* [3]. One of the 15 serogroups of *L. pneumophila*, serogroup (SG) 1, accounts for most clinical strains (84% worldwide and 80% in Japan) [3,4]. *Legionella* spp. are ubiquitously found in natural environments. They have also been found in artificial environments, such as cooling towers, baths, and decorative fountains [5–7].

According to the National Epidemiological Surveillance of Infectious Diseases, public bath facilities are a major source of infection in Japan [8]. Fatal cases of legionellosis from homes and public bath facilities have been reported [9,10]. In most cases, evidence clearly demonstrated that bath water acted as the probable infection source. However, the sources of some infections have been unclear. A legionellosis case caused by shower water in a public bath facility in Japan was reported in 2009 [11]. Although *Legionella* spp. are often found in shower water from hospitals and nursing homes worldwide [12–14], the prevalence of *Legionella* spp. isolated from shower water in public bath facilities has rarely been investigated [15]. Furthermore, genetic diversity among *L. pneumophila* isolates from shower water and

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genetic relationships between isolates from shower water and from clinical specimens have not been analyzed by molecular typing techniques.

When a case of legionellosis is reported, it is important to identify the source of infection by molecular typing methods for public health purposes. Sequence-based typing (SBT) using defined regions of seven genes (*flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, and *neuA* (or *neuAh*)) was proposed as the identification strategy for *L. pneumophila* by the European Working Group for *Legionella* Infections (EWGLI) [16–18]. Recent studies revealed significant correlations between the SBT sequence type (ST) and the whole genome-based genotype [19,20]. Thus, SBT has been commonly used as a molecular typing method to characterize *L. pneumophila* strains in recent studies [21–23].

In this study, we investigated the prevalence of *Legionella* spp. isolated from shower water in public bath facilities in Toyama Prefecture, Japan. The isolates were characterized using serogrouping, 16S rRNA gene sequencing, and SBT. The aim of this study was to characterize the genetic relationship between isolates from shower water and stock strains previously analyzed from sputum specimens and to establish that shower water in public bath facilities could serve as reservoirs for *Legionella* and potentially cause legionellosis.

2. Materials and methods

2.1. Water samples

Nine public health centers (in Niikawa, Uozu, Chubu, Toyama, Imizu, Takaoka, Himi, Tonami, and Oyabe district) participated in a survey of *Legionella* spp. in 37 public bath facilities from September 2012 to December 2014 throughout Toyama Prefecture, Japan. Shower water (500 ml each) and bath water (1 l each) were collected in sterile specimen bottles. Shower water was flushed for about 10 s before sampling. Free residual chlorine concentrations (mg/l) of shower water samples were measured on site by the *N*,*N*-diethyl-*p*-phenylenediamine method using several commercial kits (Nippon Soda, Tokyo, Japan; Suzuken, Tokyo, Japan; Sibata Scientific Technology, Tokyo, Japan; Wako Pure Chemical Industries, Osaka, Japan). Water samples were then tested in our laboratory for *Legionella* spp.

2.2. Identification of Legionella spp. from water samples

Water samples were concentrated 100-fold by filtration through a 0.22 μm-pore-size polycarbonate membrane (Millipore, Billerica, MA, USA). Concentrated samples were divided into three portions (0.1 ml each) and cultured without pretreatment, after heat treatment (50 °C for 20 min) and after acid treatment [equal volumes of 0.2 mol/l KCl–HCl buffer (pH 2.2) for 4 min at 24 °C], on glycine–vancomycin–polymyxin B-cycloheximide agar plates (Nikken Bio Medical Laboratory, Kyoto, Japan). Unconcentrated water samples were also plated. The agar plates were incubated at 35 °C for 7 days in a humidified chamber. Smooth gray colonies with the characteristic outward structures (cut-glass-like or mosaic-like appearance) viewed under a stereomicroscope with oblique illumination [24] were subcultured onto buffered charcoal-yeast extract agar plates with L-cysteine (bioMérieux, Lyon, France) and blood agar plates (Eiken Chemical, Tokyo, Japan). The colonies growing only on buffered charcoal-yeast extract agar plates were presumed to belong to the genus *Legionella*. The detection limit of the procedure was 100 cfu/l. Species and serogroups of *Legionella* isolates were determined using a latex agglutination test kit (Oxoid, Hampshire, United Kingdom) and slide agglutination with commercial antisera (Denka Seiken, Tokyo, Japan). Furthermore, 16S rRNA genes of some isolates were sequenced as described previously [25].

2.3. Bacterial strains

Thirty-eight *L. pneumophila* strains isolated in this study, single representative isolates from each serogroup in each water sample, were analyzed by SBT. Among these, the STs of 2 isolates (LG2051 and LG2055) obtained in this study were analyzed in our previous study [25]. In addition, 22 isolates from shower water in 11 public bath facilities collected from the Takaoka Public Health Center and Toyama City Health Center laboratory were also analyzed by SBT to reveal the genetic diversity in this study. The remaining isolate (LG0593 from shower water in a hospital) was obtained in our previous study [26]. As a result, 61 *L. pneumophila* isolates from shower water in 25 public bath facilities and in a hospital were analyzed in this study (Supplementary Table 1).

2.4. SBT

SBT was performed according to the protocol of the EWGLI (http://www.hpa-bioinformatics.org.uk/legionella/legionella_sbt/php/sbt_homepage.php), as described previously [16–18]. Novel alleles and STs were submitted to the EWGLI SBT database for assignment. A minimum-spanning tree with categorical coefficients of similarity and the priority rule of the highest number of single-locus variants was used to indicate differences in the number of loci among operational taxonomic units (OTUs). The tree was constructed using BioNumerics software (version 6.5; Applied Maths, Sint-Martens-Latem, Belgium).

2.5. Statistical analysis

The odds ratio (OR) calculation and the χ^2 tests were performed to compare the proportions of *Legionella*-positive and -negative samples from shower water according to the water source and free residual chlorine concentrations using Microsoft Excel (Microsoft, Tokyo, Japan). A P value of <0.05 was considered statistically significant.

2.6. Indices of discrimination (IOD)

To assess the molecular typing methods, we calculated the IODs of the isolates from shower water as described previously [27].

2.7. Nucleotide sequence accession numbers

The sequence data from this study have been submitted to the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/) under accession numbers LC120331 to LC120340.

3. Results

3.1. Isolation of Legionella spp. from shower water

Legionella spp. were isolated from 31/91 shower water samples (34.1%) derived from 17/37 (45.9%) public bath facilities. Among the 31 positive samples, 18 (19.8%) samples contained 100 to 990 cfu/l, 11 (12.1%) samples contained 1.0×10^3 to 9.9×10^3 cfu/l, and 2 (2.2%) samples contained $\geq 1.0 \times 10^4$ cfu/l. The maximum number of cfu in a sample was 1.26×10^5 cfu/l.