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ORIGINAL ARTICLE

# Molecular epidemiology of *Legionella pneumophila* serogroup 1 isolates identify a prevalent sequence type, ST505, and a distinct clonal group of clinical isolates in Toyama Prefecture, Japan

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**Abstract** We performed comparative analyses of *Legionella pneumophila* serogroup (SG) 1 isolates obtained during 2005–2012 in Toyama Prefecture, Japan, by sequence-based typing (SBT) and pulsed-field gel electrophoresis (PFGE). Seventy-three isolates of *L. pneumophila* SG 1, including 17 isolates from patients, 51 from public baths, 4 from cooling towers, and 1 from a shower, were analyzed. The isolates were classified into 43 sequence types (STs) by SBT and 52 types by PFGE. Fourteen STs were unique to Toyama Prefecture, as determined from the SBT database of European Working Group for *Legionella* Infections (EWGLI), as of October 31, 2012. ST505 strain was identified in 4 isolates from patients and 5 isolates from public baths, and these isolates belonged to 2 PFGE types. These, however, were similar because of the difference with only two restriction fragments, indicating that ST505 strain was prevalent among *L. pneumophila* SG 1 isolates in this area. ST505 strains isolated from patients and public baths were distributed along the river in a western part of Toyama Prefecture. SBT and PFGE profiles of 3 clinical isolates were identical with those of 3 environmental isolates from the suspected origins of the infection in each case, respectively. This finding suggested that SBT and PFGE were useful for epidemiological study. Furthermore, by SBT analysis, we identified a clonal group formed only by 7 clinical isolates that are not associated with bathwater, suggesting that they were derived from unrecognized sources.

**Keywords** *Legionella pneumophila* · Molecular epidemiology · Molecular typing

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## Introduction

*Legionella* are pathogenic gram-negative bacteria that cause legionellosis and are ubiquitously found in the environment. Although 55 species and more than 70 serogroups of *Legionella* spp. have been identified [1], more than 90 % of legionellosis cases are caused by *Legionella pneumophila* [2]. Among 15 serogroups of *L. pneumophila*, most clinical strains (80 %) belonged to serogroup (SG) 1 in Japan [3].

Legionellosis is usually acquired through inhalation of aerosolized water contaminated with *Legionella* spp. [4]. Legionellosis has two distinct forms: Pontiac fever, which is an influenza-like illness, and Legionnaires' disease, which is a more severe form that causes pneumonia [5, 6]. *Legionella* spp. have been found in artificial environments such as cooling towers, baths, showers, and decorative fountains [7–10]. Therefore, these facilities are potential sources of sporadic or outbreak cases of infection. In Japan, public baths are a major source of infection according to the National Epidemiological Surveillance of Infectious Diseases [11]. Fatal cases have been reported in homes and spa pools [12, 13].

When a case of legionellosis is reported, it is important to identify the source of infection by molecular typing methods for public health purposes. Pulsed-field gel electrophoresis (PFGE) is commonly used to determine the source of infection [9, 14, 15]. However, this typing

method is time consuming. Sequence-based typing (SBT) is a rapid identification method developed by the European Working Group for *Legionella* Infections (EWGLI). SBT is a sequence-based scheme comprising defined regions of seven genes (*flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, and *neuA*) for *L. pneumophila* [16–18]. Similar to PFGE, SBT has been considered to be a powerful epidemiological tool [19].

Toyama Prefecture in Japan has the largest number of patients with legionellosis per 100,000 population from 2008 to 2010 [1.98 (1.80–2.07) in Toyama Prefecture and 0.62 (0.56–0.70) in Japan] [20]. However, in many cases, the sources of infection have been unclear. Comparative analysis of *L. pneumophila* SG 1 isolates from clinical specimens and public baths in a local area has been rarely reported. In this study, we performed comparative analyses of *L. pneumophila* SG 1 isolates from clinical specimens and public baths obtained during 2005–2012 in Toyama Prefecture by SBT and PFGE, and we found that *L. pneumophila* SG 1 strain ST505 was prevalent in this area. We also found a clonal group formed only by clinical isolates distinct from bath isolates, and we discussed the origin of these clinical isolates.

## Materials and methods

### Bacterial strains

Seventy-three strains of *L. pneumophila* SG 1 were isolated and collected during 2005–2012 in Toyama Prefecture (Table 1). Fifty-one strains from 24 public baths (PB1–PB24) were isolated in our laboratory. Four strains from two cooling towers (CT1 and CT2) and 1 strain from a shower (SH1) were collected from each building. Seventeen strains from 16 patients (PA1–PA16) with legionellosis were collected from four hospitals in Toyama Prefecture. Of the 17 clinical isolates, 15 were obtained from 15 patients; the remaining 2 isolates were obtained from patient PA11 but belonged to different STs and PFGE types. The incubation period was 2–10 days, depending on the diagnosis by the physician.

### Isolation of *L. pneumophila* SG 1 from environmental sources

Water samples (500 ml) were filtered with a 0.22-μm pore size membrane (cat. no. GTTP04700; Millipore, Billerica, MA, USA) and resuspended in 5 ml distilled water. After the concentrated samples were heated at 50 °C for 20 min, they were spread onto glycine–vancomycin–polymyxin B-cycloheximide agar plates (bioMerieux, Lyon, France). These agar plates were incubated at 35 °C for 7 days in a moist chamber. Smooth gray colonies were subcultured onto buffered charcoal yeast extract (BCYE) agar plates (bioMerieux) and blood agar plates (Eiken Chemical, Tokyo, Japan). Suspected colonies that grew only on BCYE agar plates were tested by slide agglutination with commercial antisera (Denka Seiken, Tokyo, Japan) to identify *L. pneumophila* SG 1 strains among various *Legionella* spp. and serogroups.

### SBT analysis

Isolates were suspended in distilled water. The suspension was boiled at 100 °C for 10 min and then centrifuged at 20,000 *g* for 5 min at room temperature. The supernatant was used as a DNA template. Polymerase chain reaction (PCR) of the SBT scheme was carried out according to the protocol of EWGLI (http://www.hpa-bioinformatics.org.uk/legionella/legionella_sbt/php/sbt_homepage.php), as described previously [16, 17]. Novel alleles and sequence types (STs) were submitted to the EWGLI SBT database for assigning the newly identified alleles and STs. A phylogenetic tree with concatenated sequences of seven SBT alleles was constructed by the neighbor-joining method, using the MEGA4 software [21]. A bootstrapping test was performed 1,000 times. Clonal analyses were performed by using eBURST V3 (http://eburst.mlst.net). Groups were generated with single- and double-locus variants and defined as clonal groups.

### PFGE analysis

PFGE was carried out as previously described [22] with a slight modification. Genomic DNA in the plug was digested overnight with 30 U *Sfi*I (TaKaRa Bio, Shiga, Japan) at 50 °C. Electrophoresis was carried out at 6 V/cm for 19 h with the pulse time ranging from 5 to 50 s, using the CHEF DRIII system (Bio-Rad Laboratories, Hercules, CA, USA). A dendrogram showing the genetic similarity between PFGE profiles was constructed by the UPGMA method with the Fingerprinting II software (Bio-Rad Laboratories) using a Dice coefficient at 1.2 % of tolerance and 1.0 % of optimization. Reproducibility was confirmed by repeat analysis of 17 randomly selected isolates. PFGE types were defined at the 100 % similarity breakpoint given by the software. PFGE with *Sfi*I digestion had the ability to type all *L. pneumophila* isolates in this study.

### Indices of discrimination (IOD)

To assess the molecular typing methods, we calculated the IODs of isolates from patients and public baths as described previously [23].

**Table 1** Sequence-based typing (SBT) and pulsed-field gel electrophoresis (PFGE) profiles of *Legionella pneumophila* SG 1 isolates used in this study

| No. | Strain | Origin[a] | Year | Month | SBT profile | | | | | | | ST | PFGE type | Sources of infection |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | *flaA* | *pilE* | *asd* | *mip* | *mompS* | *proA* | *neuA* | | | |
| 1 | LG0002 | PA1 | 2005 | May | 6 | 10 | 19 | 3 | 19 | 4 | 6 | 502 | P39 | Unknown |
| 2 | LG0003 | PA2 | 2005 | Aug | 7 | 6 | 17 | 3 | 11 | 11 | 9 | 505[b] | P14 | Bathwater[c] |
| 3 | LG0122 | PA3 | 2006 | Sep | 8 | 10 | 6 | 15 | 51 | 1 | 6 | 353 | P6 | Unknown |
| 4 | LG0123 | PA4 | 2006 | Sep | 2 | 3 | 6 | 13 | 2 | 1 | 6 | 506[b] | P3 | Bathwater |
| 5 | LG0124 | PA5 | 2006 | Sep | 2 | 3 | 5 | 10 | 2 | 1 | 6 | 507 | P11 | Unknown |
| 6 | LG0215 | PA6 | 2006 | Oct | 7 | 6 | 17 | 3 | 11 | 11 | 9 | 505[b] | P13 | Bathwater |
| 7 | LG0232 | PA7 | 2006 | Nov | 2 | 3 | 5 | 11 | 2 | 1 | 6 | 120 | P12 | Unknown |
| 8 | LG0392 | PA8 | 2007 | Feb | 2 | 3 | 9 | 10 | 2 | 1 | 10 | 384 | P2 | Unknown |
| 9 | LG0585 | PA9 | 2008 | May | 7 | 6 | 17 | 3 | 11 | 11 | 9 | 505[b] | P13 | Unknown |
| 10 | LG0586 | PA10 | 2008 | Jun | 2 | 3 | 9 | 10 | 2 | 1 | 10 | 384 | P4 | Unknown |
| 11 | LG0604 | PA11 | 2008 | Sep | 6 | 10 | 20 | 10 | 9 | 14 | 11 | 644 | P27 | Bathwater[c] |
| 12 | LG0613 | PA11 | 2008 | Sep | 7 | 6 | 17 | 3 | 11 | 11 | 9 | 505[b] | P13 | Bathwater[c] |
| 13 | LG0716 | PA12 | 2008 | Sep | 2 | 1 | 6 | 15 | 2 | 1 | 6 | 132 | P5 | Unknown |
| 14 | LG0977 | PA13 | 2008 | Dec | 6 | 10 | 19 | 3 | 19 | 4 | 9 | 2 | P38 | Bathwater |
| 15 | LG1008 | PA14 | 2009 | Feb | 7 | 6 | 17 | 10 | 13 | 9 | 11 | 682 | P17 | Bathwater |
| 16 | LG1060 | PA15 | 2009 | Jun | 2 | 3 | 9 | 10 | 2 | 1 | 10 | 384 | P1 | Unknown |
| 17 | LG1171 | PA16 | 2009 | Dec | 4 | 7 | 11 | 3 | 11 | 12 | 9 | 42 | P24 | Bathwater |
| 18 | LG0017 | PB1 | 2005 | Aug | 6 | 10 | 19 | 28 | 19 | 4 | 11 | 763 | P37 | |
| 19 | LG0006 | PB1 | 2005 | Aug | 6 | 10 | 19 | 28 | 19 | 4 | 11 | 763 | P40 | |
| 20 | LG0007 | PB1 | 2005 | Aug | 7 | 6 | 17 | 3 | 11 | 11 | 9 | 505[b] | P14 | |
| 21 | LG0029 | PB1 | 2005 | Nov | 6 | 10 | 19 | 28 | 19 | 4 | 11 | 763 | P37 | |
| 22 | LG0030 | PB1 | 2005 | Nov | 7 | 6 | 17 | 3 | 11 | 11 | 9 | 505[b] | P13 | |
| 23 | LG1116 | PB1 | 2009 | Nov | 7 | 6 | 17 | 3 | 11 | 11 | 9 | 505[b] | P14 | |
| 24 | LG1119 | PB1 | 2009 | Nov | 2 | 10 | 14 | 10 | 19 | 4 | 3 | 285 | P32 | |
| 25 | LG0128 | PB2 | 2006 | Sep | 3 | 13 | 1 | 28 | 14 | 9 | 11 | 493 | P22 | |
| 26 | LG0129 | PB2 | 2006 | Sep | 7 | 10 | 17 | 13 | 14 | 11 | 11 | 1091[b] | P16 | |
| 27 | LG0156 | PB3 | 2006 | Oct | 6 | 10 | 15 | 28 | 4 | 14 | 11 | 278 | P43 | |
| 28 | LG0326 | PB3 | 2006 | Dec | 6 | 10 | 15 | 28 | 4 | 14 | 11 | 278 | P43 | |
| 29 | LG0347 | PB3 | 2006 | Dec | 7 | 4 | 31 | 10 | 48 | 15 | 11 | 1092[b] | P19 | |
| 30 | LG0218 | PB4 | 2006 | Oct | 3 | 13 | 1 | 3 | 14 | 9 | 9 | 664 | P22 | |
| 31 | LG0219 | PB4 | 2006 | Oct | 6 | 10 | 17 | 6 | 9 | 4 | 9 | 136 | P50 | |
| 32 | LG0254 | PB5 | 2006 | Nov | 7 | 6 | 17 | 3 | 11 | 11 | 9 | 505[b] | P13 | |
| 33 | LG0258 | PB6 | 2006 | Dec | 6 | 10 | 15 | 13 | 17 | 14 | 11 | 122 | P33 | |
| 34 | LG0478 | PB6 | 2007 | Oct | 6 | 10 | 15 | 13 | 17 | 14 | 11 | 122 | P34 | |
| 35 | LG0490 | PB6 | 2007 | Oct | 10 | 12 | 7 | 3 | 16 | 18 | 6 | 138 | P48 | |
| 36 | LG0301 | PB7 | 2006 | Dec | 10 | 12 | 7 | 21 | 16 | 18 | 9 | 769 | P49 | |
| 37 | LG0534 | PB7 | 2007 | Nov | 10 | 12 | 7 | 21 | 16 | 18 | 9 | 769 | P49 | |
| 38 | LG0449 | PB8 | 2007 | Sep | 7 | 43 | 31 | 3 | 48 | 15 | 40 | 1151 | P20 | |
| 39 | LG0453 | PB9 | 2007 | Oct | 6 | 10 | 19 | 28 | 19 | 4 | 11 | 763 | P37 | |
| 40 | LG0454 | PB9 | 2007 | Oct | 7 | 6 | 17 | 3 | 13 | 11 | 11 | 59 | P15 | |
| 41 | LG0469 | PB10 | 2007 | Oct | 6 | 10 | 15 | 14 | 21 | 7 | 6 | 1093[b] | P36 | |
| 42 | LG0516 | PB11 | 2007 | Oct | 7 | 6 | 17 | 3 | 13 | 11 | 40 | 1152[b] | P13 | |
| 43 | LG0622 | PB12 | 2008 | Sep | 6 | 10 | 20 | 10 | 9 | 14 | 11 | 644 | P29 | |
| 44 | LG0643 | PB12 | 2008 | Sep | 6 | 10 | 20 | 10 | 9 | 14 | 11 | 644 | P27 | |
| 45 | LG0646 | PB12 | 2008 | Sep | 6 | 10 | 20 | 10 | 9 | 14 | 11 | 644 | P28 | |
| 46 | LG0638 | PB12 | 2008 | Sep | 6 | 10 | 20 | 10 | 9 | 4 | 9 | 1094[b] | P30 | |

**Table 1** continued

| No. | Strain | Origin[a] | Year | Month | SBT profile | | | | | | | ST | PFGE type | Sources of infection |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | *flaA* | *pilE* | *asd* | *mip* | *mompS* | *proA* | *neuA* | | | |
| 47 | LG0641 | PB12 | 2008 | Sep | 6 | 10 | 20 | 10 | 9 | 4 | 9 | 1094[b] | P31 | |
| 48 | LG0626 | PB13 | 2008 | Sep | 7 | 6 | 17 | 3 | 11 | 11 | 9 | 505[b] | P13 | |
| 49 | LG0629 | PB13 | 2008 | Sep | 6 | 10 | 20 | 6 | 9 | 4 | 9 | 530 | P25 | |
| 50 | LG0708 | PB14 | 2008 | Sep | 6 | 10 | 15 | 28 | 21 | 14 | 11 | 1095[b] | P42 | |
| 51 | LG0709 | PB14 | 2008 | Sep | 7 | 6 | 17 | 3 | 14 | 11 | 11 | 128 | P16 | |
| 52 | LG0710 | PB14 | 2008 | Sep | 7 | 6 | 17 | 3 | 14 | 11 | 11 | 128 | P13 | |
| 53 | LG0864 | PB15 | 2008 | Nov | 7 | 6 | 17 | 3 | 13 | 11 | 11 | 59 | P13 | |
| 54 | LG0903 | PB16 | 2008 | Nov | 6 | 10 | 20 | 28 | 9 | 4 | 9 | 1097[b] | P26 | |
| 55 | LG0909 | PB17 | 2008 | Nov | 2 | 12 | 3 | 6 | 8 | 14 | 9 | 141 | P51 | |
| 56 | LG0941 | PB18 | 2008 | Nov | 23 | 10 | 3 | 3 | 8 | 4 | 9 | 1098[b] | P46 | |
| 57 | LG0954 | PB18 | 2008 | Nov | 6 | 6 | 15 | 3 | 9 | 14 | 11 | 1101[b] | P44 | |
| 58 | LG0964 | PB18 | 2008 | Nov | 7 | 6 | 17 | 6 | 13 | 11 | 9 | 1099[b] | P18 | |
| 59 | LG1132 | PB18 | 2009 | Nov | 6 | 6 | 15 | 3 | 9 | 14 | 11 | 1101[b] | P44 | |
| 60 | LG1134 | PB18 | 2009 | Nov | 10 | 22 | 7 | 3 | 16 | 9 | 6 | 162 | P47 | |
| 61 | LG1142 | PB18 | 2009 | Nov | 23 | 10 | 3 | 3 | 8 | 4 | 9 | 1098[b] | P45 | |
| 62 | LG0976 | PB19 | 2008 | Nov | 6 | 10 | 15 | 28 | 4 | 14 | 11 | 278 | P42 | |
| 63 | LG0987 | PB20 | 2008 | Dec | 6 | 10 | 19 | 28 | 19 | 4 | 11 | 763 | P41 | |
| 64 | LG1034 | PB21 | 2009 | May | 6 | 10 | 15 | 3 | 17 | 14 | 9 | 1100[b] | P52 | |
| 65 | LG1124 | PB22 | 2009 | Nov | 6 | 10 | 14 | 10 | 2 | 3 | 6 | 77 | P35 | |
| 66 | LG1156 | PB23 | 2009 | Nov | 3 | 6 | 1 | 28 | 14 | 9 | 11 | 1102[b] | P23 | |
| 67 | LG1167 | PB24 | 2009 | Nov | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | P10 | |
| 68 | LG1169 | PB24 | 2009 | Nov | 7 | 6 | 17 | 3 | 13 | 11 | 11 | 59 | P13 | |
| 69 | LG0808 | CT1 | 2008 | Oct | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | P9 | |
| 70 | LG1948 | CT2 | 2012 | Apr | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | P7 | |
| 71 | LG1949 | CT2 | 2012 | Apr | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | P8 | |
| 72 | LG1950 | CT2 | 2012 | Apr | 5 | 2 | 22 | 27 | 6 | 10 | 12 | 48 | P21 | |
| 73 | LG0593 | SH1 | 2008 | Aug | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | P7 | |

[a] *ST* sequence type, *PA* patient, *PB* public bath, *CT* cooling tower, *SH* shower

[b] Fourteen of 43 STs were unique to this area, as of 31 Oct 2012

[c] Confirmed by PFGE with environmental isolates

## Results

### SBT analysis

Seventy-three isolates were divided into 43 STs (Table 1). The IODs of 17 isolates from patients and 38 isolates from public baths were 0.934 [95 % confidence interval (CI), 0.859–1.000] and 0.986 (95 % CI, 0.971–1.000), respectively; strains obtained on the same day from the same public bath and with identical STs were represented as a single strain. Fourteen STs were unique to this area in the EWGLI SBT database, as of 31 October 2012. Among these, 9 ST505 isolates were obtained from four patients and three public baths along the Shou River (Fig. 1; LG0003, LG0215, LG0585, LG0613; LG0007, LG0030, LG1116, LG0254, and LG0626 in Table 1). The ST of 3 of 4 isolates (75 %) from cooling towers and 1 isolate from a shower was ST1. A phylogenetic tree was constructed, and seven clonal groups were generated by SBT (Fig. 2). Among the seven clonal groups (CG1–CG7), CG3 was formed by isolates from seven patients (LG0123, LG0124, LG0232, LG0392, LG0586, LG0716, and LG1060; Table 1). No environmental isolates were present in CG3. Isolates belonging to CG3 found by using eBURST V3 were also clustered using the neighbor-joining method by the MEGA4 software, as shown by the bootstrap support value of 67 %.

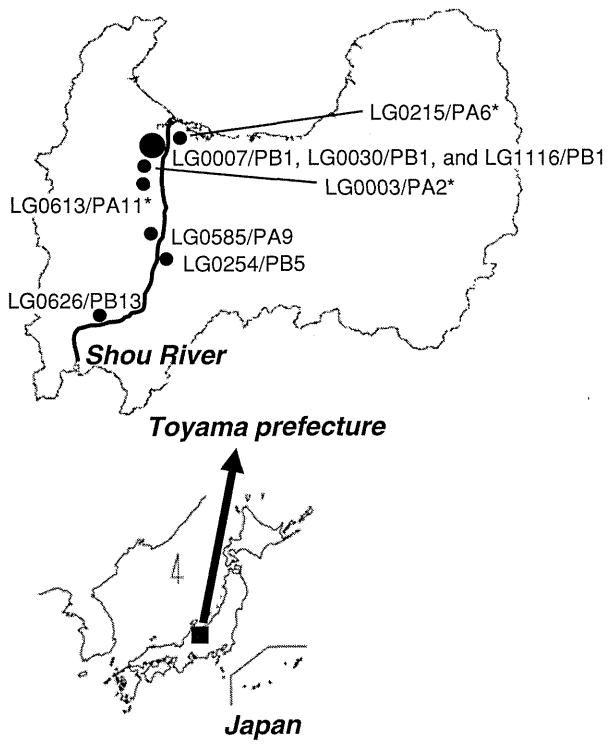


**Fig. 1** Geographic distribution of ST505 strain. *Isolate name* indicates the strain/origin as described in Table 1. The *size of the circle* indicates the number of isolates. *Asterisk* indicates the clinical isolates associated with bathwater by epidemiological investigation

PFGE analysis

A dendrogram of the PFGE pattern was constructed (Fig. 3). Figure 4 shows the original gel image of band patterns of isolates belonging to 3 STs (ST1, ST278, ST505) among 13 STs (ST1, ST59, ST122, ST128, ST278, ST384, ST505, ST644, ST763, ST769, ST1094, ST1098, ST1101) that were found in more than 1 isolate. Seventy-three isolates were divided into 52 PFGE types. The IODs of 17 isolates from patients and 46 isolates from public baths were 0.978 (95 % CI, 0.934–1.000) and 0.976 (95 % CI, 0.949–1.000), respectively; strains obtained on the same day from the same public bath and with the identical type by PFGE were represented as a single strain. Although 9 ST505 isolates belonged to 2 PFGE types (P13 and P14; Fig. 3), band patterns of these types were different by only two restriction fragments with similarity of approximately 90 % (Fig. 4). The CG3 consisting of 7 clinical isolates was split into two PFGE groups with similarity of more than 80 % each (Fig. 3). Epidemiologically unrelated ST1 isolates obtained from a cooling tower and a shower had the same PFGE type (LG0593 and LG1948; Fig. 3). However, band patterns of other isolates belonging to ST1 were different by more than three restriction fragments (Fig. 4). The other isolates from different environmental sources did not have identical PFGE types.

## Discussion

In this study, we found ST505 to be the most prevalent strain in Toyama Prefecture, Japan, and identified a clonal group (CG3, Fig. 2) formed only by seven clinical isolates that were not associated with bathwater. Travel histories of 14 of the 16 patients during the likely exposure period were available. Although patient PA5 had a history of a visit outside Toyama Prefecture, we could not identify whether this patient had been infected in Toyama Prefecture. However, the remaining 13 patients had been in Toyama Prefecture, suggesting that most patients had been infected in Toyama Prefecture. ST1 strain was isolated from public baths (1 of 51, 2.0 %), cooling towers (3 of 4, 75 %), and a shower (1 of 1, 100 %). ST1 strain was not isolated from clinical specimens in this study, although this strain has been frequently isolated worldwide from clinical specimens and environmental sources [24–26]. Cases of legionellosis from cooling towers and showers have not been reported yet in Toyama Prefecture by epidemiological investigation, but these environmental sources, as well as public baths, are still possible infection sources of legionellosis in this area.

The ST505 strain was the most frequently isolated from patients and bath facilities, and two PFGE types of the isolates were similar because of the difference with only two restriction fragments (Fig. 4), indicating that this strain was prevalent among *L. pneumophila* SG 1 isolates in this area. A recent study observed high diversity and high abundance of *Legionella* spp. in a river by 16S rRNA gene sequencing and quantitative PCR [27]. Because the ST505 isolates were obtained along the Shou River, this strain was likely to be distributed along this river and may contaminate artificial environments such as public bath facilities. Alternatively, other sources of bacterial contamination may be present upstream of the river, as reported in the previous paper in which the presence of *L. pneumophila* in the river was caused by the release of wastewater from industrial aeration ponds [28].

The isolation rates of the ST505 strain from patients and public baths were 23.5 % (4 of 17) and 9.8 % (5 of 51), respectively. Several studies of endemic clones have been reported. In Ontario, Canada, endemic ST211 (*flaA*3, *pilE*10, *asd*1, *mip*1, *mompS*14, *proA*9, and *neuA*11) and ST222 (*flaA*2, *pilE*19, *asd*5, *mip*10, *mompS*18, *proA*1, and *neuA*10) strains were detected in 7.7 % (15 of 194) and 6.7 % (13 of 194) of the total clinical isolates, respectively [29]. Thus, the higher isolation rate of clinical ST505 strain found in this study suggests that this strain may be highly

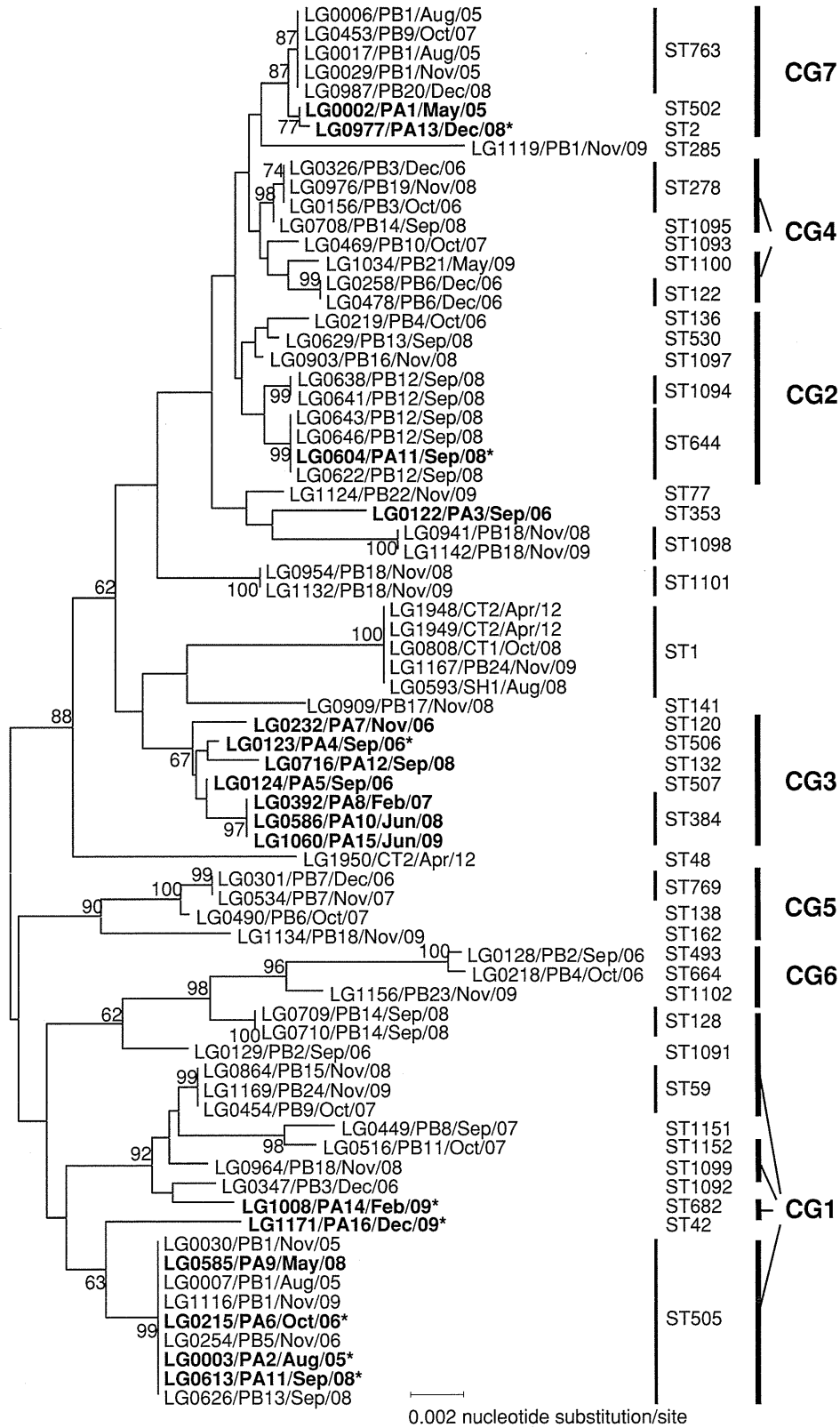


**Fig. 2** Phylogenetic analysis of the concatenated sequences (*flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, *neuA*) of *Legionella pneumophila* SG 1 isolates in this study. *Isolate name* indicates the strain/origin/month/year as described in Table 1. *Isolates in boldface* are from patients. *Asterisk* indicates the clinical isolates associated with bath water by epidemiological investigation. More than 60 % of bootstrap values are shown on the branches. Clonal groups (*CG1–CG7*) were generated with single- and double-locus variants by using eBURST V3 (http://eburst.mlst.net)

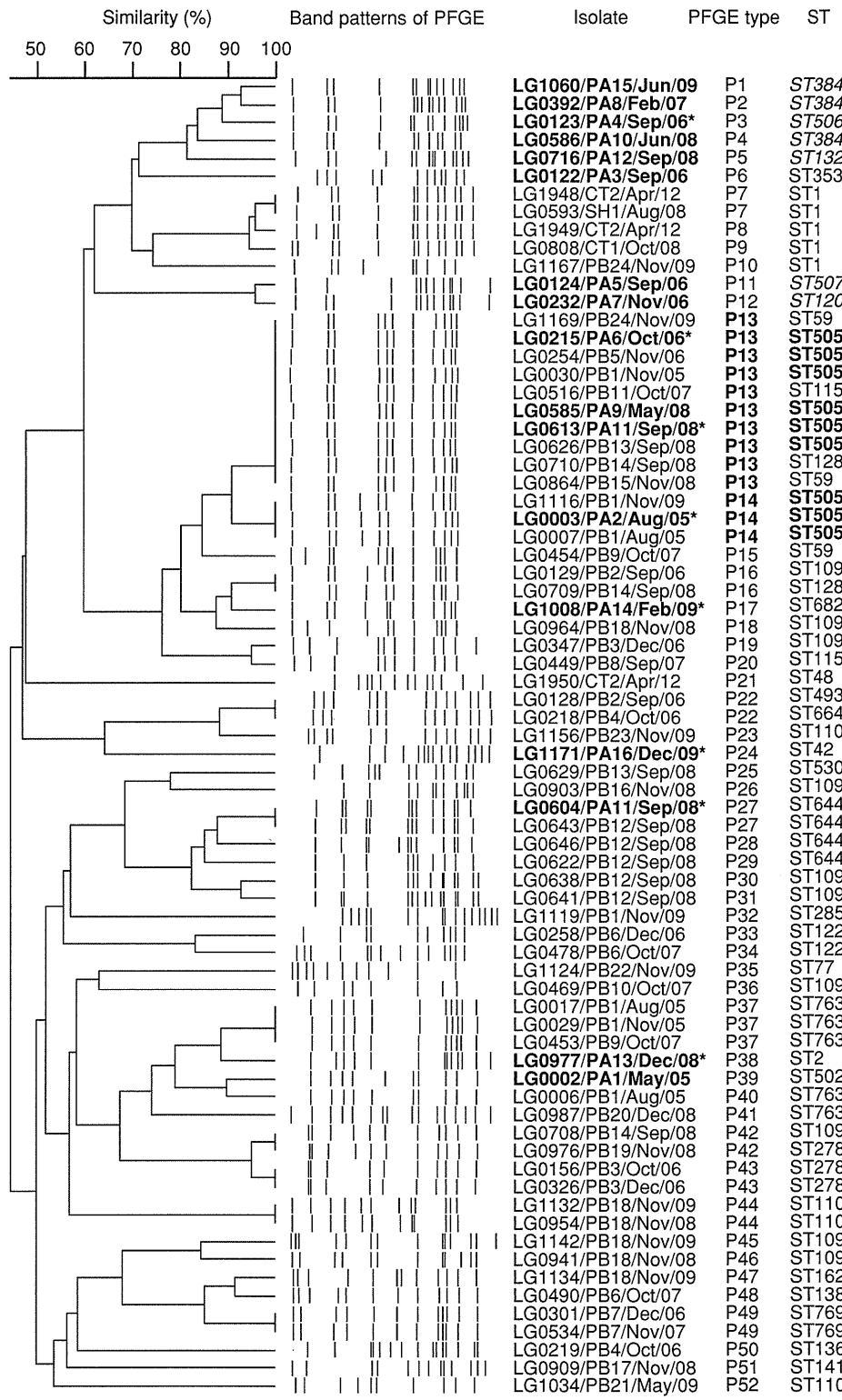


**Fig. 3** *Dendrogram* of the pulsed-field gel electrophoresis (PFGE) pattern constructed from *L. pneumophila* SG 1 isolates in this study. *Isolate name* indicates the strain/origin/month/year as described in Table 1. *Isolates in boldface* are from patients. *Asterisk* indicates the clinical isolates associated with bathwater by epidemiological investigation. Two PFGE types (P13 and P14) and ST505 are denoted by *boldface*. *Italic letters* indicate STs belonging to CG3

pathogenic. In South Korea, ST-K1 (*flaA*7, *pilE*12, *asd*17, *mip*3, *mompS*35, *proA*11, and *neuA*11) strains accounted for 36.1 % of the total isolates in hot-water samples [26]. It is notable that ST505 is a triple-locus variant of ST-K1. These endemic clones were not detected in this study. Further investigation of endemic clones is required, as our study, in addition to previous findings, suggested that it was important to determine the infection source of

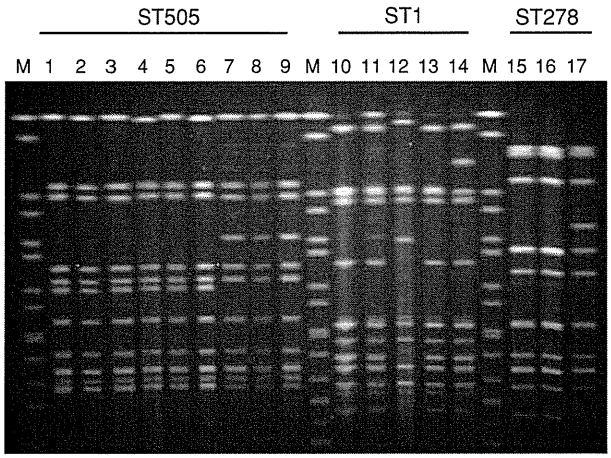


**Fig. 4** PFGE patterns with *SfiI* digestion of *L. pneumophila* SG 1 isolates. *Lanes*: *M Salmonella enterica* serovar Braenderup H9812 strain digested with *XbaI* as a size marker, *1* LG0215, *2* LG0254, *3* LG0030, *4* LG0613, *5* LG0585, *6* LG0626, *7* LG0003, *8* LG0007, *9* LG1116, *10* LG0593, *11* LG0808, 12 LG1167, *13* LG1948, *14* LG1949, *15* LG0156, *16* LG0326, *17* LG0976

legionellosis by the combination of molecular typing methods such as PFGE and SBT analyses, monoclonal antibody subgrouping [3], and epidemiological investigation in certain areas.

By SBT and PFGE analyses, LG0003 strain from PA2 and LG0007 strain from PB1 as the suspected origin of the infection in this case had the same profile (ST505 and P14; Table 1). In another case, LG0604 and LG0613 strains that were obtained on the same day from PA11 had different profiles (ST644 and P27; ST505 and P13 in Table 1). These profiles were identical with those of LG0643 strain from PB12 and LG0626 strain from PB13, respectively, that were obtained from the suspected origins of the infection. Therefore, this patient might be serially infected with two different strains by using several public baths. These findings indicated that SBT and PFGE were useful for epidemiological study and that several colonies should be isolated from a patient for epidemiological study.

By SBT analysis, the seven clinical isolates belonged to CG3 (Fig. 2), in which no environmental isolates were present. Among the seven clinical isolates, six were not associated with bathwater by epidemiological investigation. The STs of clinical strains in this clonal group were ST120, ST132, ST384, ST506, and ST507. All registered strains belonging to these STs in the EWGLI SBT database were isolated only from patients and not from the environment. Amemura-Maekawa et al. [30] suggested the possibility of habitat segregation of *L. pneumophila*. Thus, these clinical isolates belonging to the same clonal group were originally derived from unrecognized environmental sources. These STs have single-, double-, and triple-locus variants of STs belonging to group S1, which mainly consisted of isolates from soil as well as from bathwater in rare cases, but not isolates from cooling towers [30], suggesting that the clinical strains belonging to the 5 STs in this study may originate from soil. Although the LG0123 strain in CG3 (Fig. 2) was suspected to be derived from bathwater by epidemiological investigation, *L. pneumophila* SG 1 strains were not isolated from the suspected origin of the infection in this case. Our findings, in addition to those of previous reports, may reveal potential major routes of infection from soil. Alternatively, it is important to type more than one isolate from an environmental source because otherwise the causative strain might be not detected. Further investigation by SBT analysis of isolates from various environmental sources, including soil, and those from patients is required to reveal potential major routes of *Legionella* infection.

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**Conflict of interest** None.

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【短報】

# 各種水利用設備のレジオネラ属菌検出実態

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## Summary of the *Legionella* Detection in Man-made Water Systems

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We detected *Legionella* from man-made water systems by using the plate culture method, and summarized the results between January 2001 and December 2012. The detection rate of *Legionella* was 25.9 % from the cooling tower water, 14.1 % from the bath water, 4.3 % from the pool water, 5.2 % from the hot water supply, 31.9 % from the heat storage tank water and 10.6 % from the water for landscaping use. The detection rate of *Legionella* from the cooling tower water subjected to various treatment was 53.1 % with no treatment, 9.7 % with glutaraldehyde treatment, 19.2 % with isothiazolin treatment, 21.9 % with cation surfactant or the polymer treatment, and 55.0 % with chlorination. Chlorination had no effect against *Legionella*; moreover, *Legionella* contamination of more than 10000 CFU/100ml increased up to 13 % From these results, the organic biocides were estimated to show bactericidal activity against *Legionella*. Using an effective biocide against *Legionella* is very important for the control of *Legionella* contamination. (Accepted 11 July 2013)

**Key words**：Biocide（殺菌剤）/ Cooling tower（冷却塔）/ *Legionella*（レジオネラ）.

レジオネラ属菌はグラム陰性の桿菌で自然界の土壌や淡水に生息し，アメーバ類等の細菌捕食性原生動物に寄生して増殖する[1]。また，レジオネラ属菌は浴槽水や冷却水などの人工環境水で増殖し，しばしばレジオネラ症を引き起こす。国立感染症研究所感染症疫学センターの報告[2]によると，2011年には818件のレジオネラ症が報告されており，夏季に報告数が増える傾向にある（図1）。レジオネラ症の発生防止には，各種水利用設備のレジオネラ属菌抑制が重要である。浴槽水は公衆浴場法および旅館業法に定めるレジオネラ属菌の管理基準（10 CFU/100ml未満）を満たすように管理される。冷却水では第3版レジオネラ症防止指針に従い，定期的にレジオネラ属菌を検査し，その結果に基づいた対策をとる必要がある。すなわち，冷却水のレジオネラ属菌が100 CFU/100ml以上の場合は殺菌剤処理や化学的洗浄等を実施してレジオネラ属菌が不検出（10 CFU/100ml未満）になることを確認しなければならない[3]。

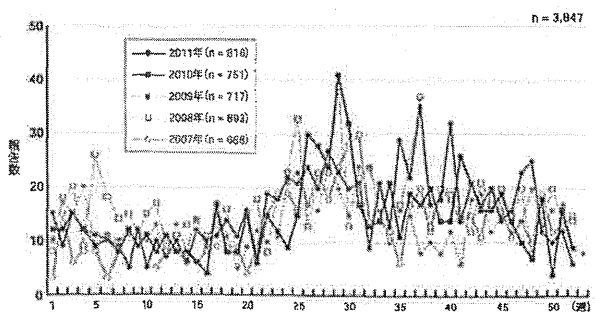


**Fig.1.** The number of cases of Legionnaire's disease reported between January 2007 and December 2011 on a weekly and yearly basis in Japan[2].

今回，著者らが実施した冷却水，浴槽水，プール水，給湯水，蓄熱槽水，修景水におけるレジオネラ属菌の検査結果を水系別に集計した。また，冷却水，浴槽水はレジオネラ属菌の検出率の年次および月次推移を，更に冷却水では殺菌剤の種類ごとにレジオネラ属菌の検出率を集計したので報告する。

2001年1月から2012年12月にかけて日本全国の建築物（ビル）や工場，医療施設，商業施設，温浴施設，温泉等における冷却水，浴槽水，プール水，給湯水，蓄熱槽水，噴水や滝水等の修景水から試料水を採水した。採水にはあらかじめ25％チオ硫酸ナトリウム水溶液を1 ml添加して高圧蒸気滅菌した500ml容のポリプロピレン製容器を用いた。採水した試料水は冷蔵状態（4～6℃）で保存し，速やかに検査した。

レジオネラ属菌の検査方法はISO 11731[4]に準じた方法で行った。すなわち，試料水400mlを冷却遠心（6400×g，30min）またはろ過濃縮法にて100倍に濃縮し，等量の0.2M HCl-KCl緩衝液（pH 2.2）を加えて10min室

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温に放置後，200 μl を GVPC 培地（MERCK）に接種した。37℃のインキュベーター内で培養し，6日後に培地を観察してレジオネラ属菌と判断される集落数を計数した。これらの集落を血液寒天培地（5％量の馬脱繊血を添加した普通寒天培地）と BCYE α 培地[5]に接種して，37℃のインキュベーター内で培養した。2日後にそれぞれの培地を観察し，血液寒天培地に発育せずBCYE α 培地に発育した集落をレジオネラ属菌とした。なお，この試験の検出下限は10 CFU/100ml である。ただし，上記手法でレジオネラ属菌の検査をした場合，GVPC 培地全体にレジオネラ属菌以外の細菌類や真菌類が発育し，レジオネラ属菌の検出ができない場合がある。その場合にレジオネラ属菌の検出を優先させるため，0.2M 酸性リン酸緩衝液（pH 2.2）による酸処理[6]，および抗生物質の添加量と添加する抗真菌剤の種類を増やした CAT α 培地[7]を使用して検査した結果も合わせて集計した。

図2は2001年1月から2012年12月の各種水系のレジオネラ属菌の検出状況を示している。冷却水の25.9％，浴槽水の14.1％，プール水の4.3％，給湯水の5.2％，蓄熱槽水の31.9％，修景水の10.6％からレジオネラ属菌が検出されており，冷却水，蓄熱槽水のレジオネラ属菌検出率が比較的高かった。

次に冷却水と浴槽水の年ごとのレジオネラ属菌検出率の推移を図3に示す。冷却水は若干の検出率低下が見られ，検出率30から25％程度を推移した。一方，浴槽水の検出率は2001年は30％だったが，2003年にかけて大きな低下が見られ，その後13％程度の検出率で推移した。図4は冷却水と浴槽水のレジオネラ属菌検出率の季節変動を見るために，2001年から2012年の検出率を月別に示している。冷却水は夏季から秋季にかけて検出率が高く，冬季から春季には低くなる傾向にあった。一方，浴槽水の検出率に季節変動は認められなかった。

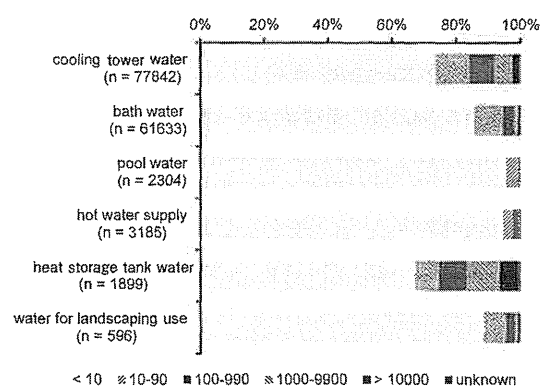


Fig.2. Distribution of detected *Legionella* counts in various man-made water systems between January 2001 and December 2012. "Unknown" in the figure indicates that the presence of *Legionella* is unknown due to the overgrowth of non-target microorganisms.

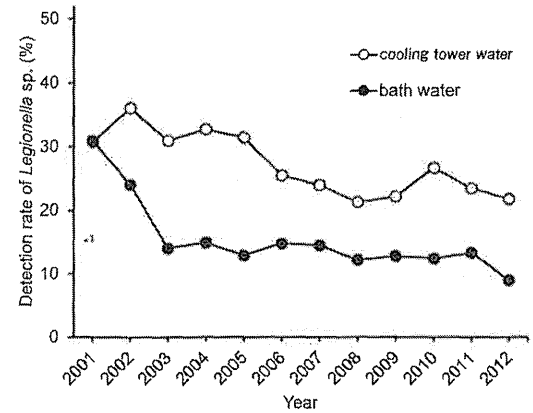


Fig.3. Trend of the detection rate of *Legionella* species in cooling tower water and bath water samples.

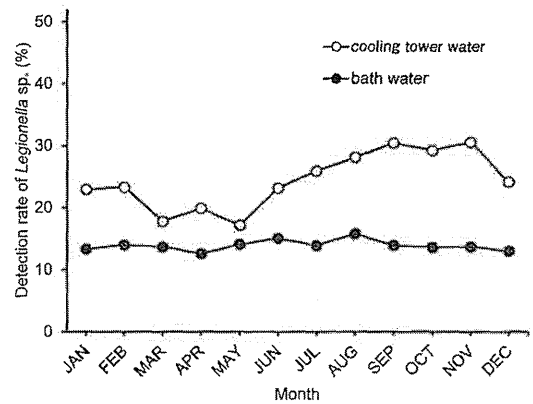


Fig.4. Detection rate of *Legionella* species in cooling tower water and bath water samples between January 2001 and December 2012 by the month.

次に，冷却水に使用する殺菌剤の種類別の効果を見るために，殺菌剤処理の履歴が明確な冷却水のみを抽出して処理剤別に集計した結果を図5に示す。冷却水の水処理に使用される有機系殺菌剤はイソチアゾリン系，カチオン系，グルタルアルデヒドに分類した。また，次亜塩素酸ナトリウムに代表さる酸化性殺菌剤を塩素系として集計した。各処理のレジオネラ属菌検出率は殺菌剤無処理が53.1％，グルタルアルデヒド処理が9.7％，イソチアゾリン系処理が19.2％，カチオン系処理が21.9％，塩素系処理が55.0％であった。

無処理の冷却水と比較すると，有機系殺菌剤処理ではレジオネラ属菌の検出率は明らかに減少しており，冷却水の有機系殺菌剤処理はレジオネラ属菌汚染防止のための一定の効果が認められると判断した。しかしながら，有機系殺菌剤で処理していてもレジオネラ属菌が検出されることがある。その要因としては殺菌剤の注入量不足や殺菌剤の消費，分解等によって，系内の殺菌剤濃度が有効濃度以上に保たれていないことが考えられた[3]。

一方，塩素系処理では無処理と同等以上（55.0％）のレジオネラ属菌が検出されている。また，無処理と比較して10000 CFU/100ml 以上のレジオネラ属菌が検出さ

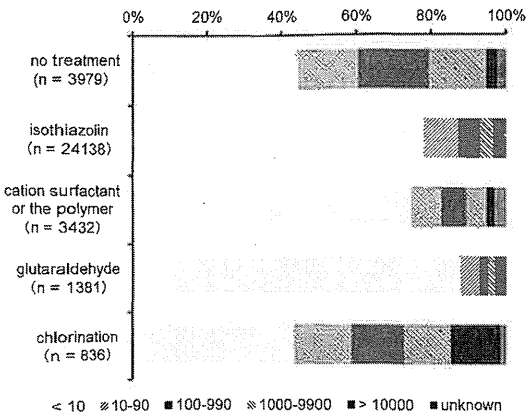


**Fig.5.** Distribution of detected *Legionella* counts in cooling tower water samples untreated or treated with various biocides between January 2001 and December 2012. "Unknown" in the figure indicates that the presence of *Legionella* is unknown due to the overgrowth of non-target microorganisms. The treatment with organic biocides showed bactericidal activity against *Legionella*, but chlorination had no effect.

れる検体の割合が2.9％から13％に上昇している。今回の解析結果では，塩素系処理は冷却水系のレジオネラ属菌の抑制効果が認められなかった。環境中でのレジオネラ属菌の増殖にはアメーバ類が大きく関わっているが，Schook らは *Acanthamoeba polyphaga* に感染して増殖した *Legionella pneumophila* は BCYE *α* 寒天培地で増殖した *L. pneumophila* と比べて塩素剤に対する抵抗性が64倍上昇したと報告している[8]。また，Chang らは *Hartmannella vermiformis* に感染して増殖した *L. pneumophila* は *A. castellanii* に感染して増殖した *L. pneumophila* よりも塩素剤に対して抵抗性が上昇したと報告している[9]。このように，環境中のレジオネラ属菌には塩素系薬剤が効きにくい可能性があり，今後更なる調査が必要である。

我が国では浴槽水のレジオネラ汚染が注目されているが，1994年に東京都で冷却塔の冷却水を感染源とするレジオネラ症の集団感染が報告されており[10]，冷却水の衛生管理も重要である。冷却水を感染源とするレジオネラ症の発生を抑えるためには，レジオネラ属菌検査による汚染実態の把握とレジオネラ属菌の殺菌対策を継続的に行い，冷却水中のレジオネラ属菌数を抑制していくことが重要である。

図1は国立感染症研究所感染症疫学センターの許可を得て転載した。

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