IV. 研究成果の刊行物・別刷

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Note

Detection of *Legionella* Species in Environmental Water by the Quantitative PCR Method in Combination with Ethidium Monoazide Treatment

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We detected *Legionella* species in 111 bath water samples and 95 cooling tower water samples by using a combination of conventional plate culture, quantitative polymerase chain reaction (qPCR) and qPCR combined with ethidium monoazide treatment (EMA-qPCR) methods. In the case of bath water samples, *Legionella* spp. were detected in 30 samples by plate culture, in 85 samples by qPCR, and in 49 samples by EMA-qPCR. Of 81 samples determined to be *Legionella*-negative by plate culture, 56 and 23 samples were positive by qPCR and EMA-qPCR, respectively. Therefore, EMA treatment decreased the number of *Legionella*-positive bath water samples detected by qPCR. In contrast, EMA treatment had no effect on cooling tower water samples. We therefore expect that EMA-qPCR is a useful method for the rapid detection of viable *Legionella* spp. from bath water samples.

Key words : Bath / Cooling tower / Ethidium monoazide (EMA) / *Legionella* / Quantitative polymerase chain reaction (qPCR).

Legionella species are Gram-negative bacteria that inhabit man-made water environments such as bath and cooling tower water. Inhalation of aerosolized water from *Legionella* contaminated sources can result in a severe form of pneumonia called Legionnaires' disease (Vogel and Isberg, 1999). Therefore, the control of *Legionella* contamination in water systems is very important, and the effectiveness of the treatment to control it is evaluated by monitoring for the presence of *Legionella*.

In general, the conventional plate culture method has been used for the detection of *Legionella* spp. from environmental water samples. However, more than 1 week is needed to obtain results because growth of *Legionella* spp. on the selective agar is very slow. In contrast, gene amplification methods such as polymerase chain reaction (PCR) or loop-mediated isothermal amplification (LAMP) detect *Legionella* spp. within a few hours (Furuhata et al., 2005; Inoue et al., 2004a). However, the number of *Legionella*-positive samples detected by the PCR and LAMP methods is higher than that by the plate culture method, because gene amplification methods detect not only viable, but also dead *Legionella* (Ng et al., 1997).

Ethidium monoazide (EMA) treatment is known to

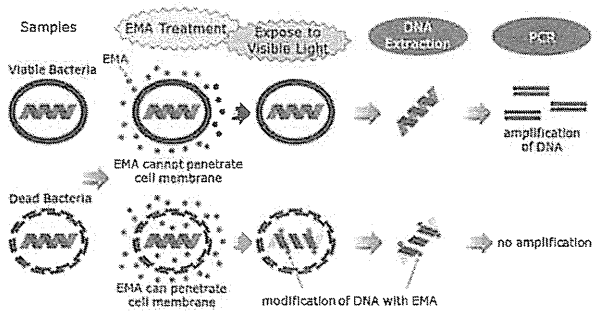

FIG. 1. The principle of ethidium monoazide (EMA) treatment on bacterial cells. This figure was modified and reprinted by courtesy of Takara Bio Inc. from the catalogue of the company.

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eliminate the PCR amplification of DNA in dead cells (Nogva et al., 2003). The principle of EMA treatment is shown in FIG. 1. EMA is a DNA-intercalating dye that can penetrate membrane-damaged cells such as dead cells, form covalent links with DNA, and cleave to genomic DNA into pieces following exposure to visible light. EMA-treated DNA is not a target for amplification by PCR, and therefore viable *Legionella* are selectively detected by PCR when combined with EMA treatment. The effect of EMA treatment on *Legionella* detection by PCR has been published (Chang et al., 2009; Chang et al., 2010; Chen et al., 2010; Delgado-Viscogliosi et al., 2009; Qin et al., 2012).

In this paper, we report a comparison of the detection of *Legionella* spp. by the plate culture method, quantitative PCR (qPCR) method and qPCR method in combination with EMA treatment (EMA-qPCR).

All water samples from different baths (111 samples) and cooling towers (95 samples) were collected between September and December 2012. These samples were collected in sterile 500-ml polypropylene bottles with sodium thiosulfate, and were examined as soon as possible.

Legionella spp. were detected according to the standard method (ISO11731, 1998). That is, collected water samples were concentrated 100-fold by centrifugation (6400×g, 30 min) or filtration (pore size: 0.45 μm, cellulose acetate, Advantec, Japan). A portion of the concentrated samples (500μl) was pretreated with acid-phosphate buffer (Inoue et al, 2004b), and inoculated onto GVPC selective agar plates (Merck, Japan). The plates were incubated at 37℃ for 6 to 8 days, and the colonies of *Legionella* spp. that grew on GVPC selective agar plates were enumerated. The detection limit of this method is 10 CFU/100 ml. The species of the isolated *Legionella* strains (up to 10 strains per sample) were identified by using the immune serum aggregation assay (Denka Seiken, Japan) and the DNA-DNA hybridization assay (Kyokuto Pharmaceutical Industrial, Japan). *Legionella* strains that could not be identified by the DNA-DNA hybridization assay were identified by evaluating the 16S rRNA gene partial sequences.

Viable *Legionella* Selection Kit for PCR Ver. 2.0 (Takara Bio, Japan) was used as an EMA reagent. EMA treatment was carried out according to the manufacturer's instructions. The 100-fold concentrated 1 ml samples were further concentrated to a final volume of 40μl. To these samples were added 10μl of reaction buffer, 2.5μl of dilution buffer and 2.5μl of EMA reagent. The samples were then mixed gently using a vortex type mixer, and incubated in the dark for 15 min at room temperature. Subsequently, the samples were exposed to visible light for 15 min using a LED Crosslinker 12 (Takara Bio, Japan). After EMA treatment, *Legionella* DNA was extracted and purified using NucleoSpin Tissue XS (Takara Bio, Japan) according to the manufacturer's instructions. For each sample, 20 μl of purified DNA solution was obtained. In addition, purified DNA solutions that had not been treated with EMA were prepared as controls.

Cycleave PCR *Legionella* (16S rRNA) Detection Kit (Takara Bio, Japan) was used as a qPCR reagent. Reaction mixtures for qPCR were prepared in 0.2 ml PCR tubes according to the manufacturer's instructions. Purified DNA template solutions (5μl) were added to the prepared reaction mixtures (20μl), and were subject to PCR amplification in the Thermal Cycler Dice Real Time System *II* (Takara bio, Japan). The PCR program parameters were: initial denaturation step of 10 s at 95℃ followed by 45 cycles of denaturation for 5 s at 95℃, annealing for 10 s at 55℃, and extension for 20 s at 72℃. DNA amplification was detected by monitoring the fluorescence at 2 wavelengths (FAM and ROX). The amplified 16S rRNA gene of *Legionella* and the internal control gene were detected by FAM and ROX, respectively. The samples containing no amplified DNA or late amplified internal control DNA due to the

TABLE 1. Distribution of *Legionella* counts by the plate culture method, and the detection results by qPCR and EMA-qPCR methods from bath water samples.

Legionella counts [a] (CFU/100ml)	No. of samples	qPCR		EMA-qPCR	
		Positive	Negative	Positive	Negative
Less than 10	81(73.0%)	56(65.9%)	25(96.2%)	23(46.9%)	58(93.5%)
10-40	14(12.6%)	13(15.3%)	1(3.8%)	10(20.4%)	4(6.5%)
50-90	2(1.8%)	2(2.4%)	0(0%)	2(4.1%)	0(0%)
100-990	11(9.9%)	11(12.9%)	0(0%)	11(22.4%)	0(0%)
1000-9900	3(2.7%)	3(3.5%)	0(0%)	3(6.1%)	0(0%)
Subtotal	30(27.0%)	29(34.1%)	1(3.8%)	26(53.1%)	4(6.5%)
Total	111(100%)	85(100%)	26(100%)	49(100%)	62(100%)

[a] Measured by the plate culture method

TABLE 2. Distribution of *Legionella* counts by the plate culture method, and the detection results by qPCR and EMA-qPCR methods from cooling tower water samples.

Legionella counts [a] (CFU/100ml)	No. of samples	qPCR		EMA-qPCR	
		Positive	Negative	Positive	Negative
Less than 10	55(67.1%)	53(66.2%)	2(100%)	53(66.2%)	2(100%)
10-100	13(15.9%)	13(16.2%)	0(0%)	13(16.2%)	0(0%)
100-990	7(8.5%)	7(8.8%)	0(0%)	7(8.8%)	0(0%)
1000-9900	4(4.9%)	4(5.0%)	0(0%)	4(5.0%)	0(0%)
10000-99000	3(3.7%)	3(3.8%)	0(0%)	3(3.8%)	0(0%)
Subtotal	27(32.9%)	27(33.8%)	0(0%)	27(33.8%)	0(0%)
Total	82(100%)	80(100%)	2(100%)	80(100%)	2(100%)

[a] Measured by the plate culture method

presence PCR inhibitors were eliminated from the data analysis.

TABLE 1 shows the results of *Legionella* spp. detection from bath water samples by the plate culture, qPCR and EMA-qPCR methods. *Legionella* spp. were detected in 30 (27%) of 111 samples by the plate culture method, and all strains tested were identified as *Legionella pneumophila* (30/30 samples, 100%). In the case of bath water samples, the internal control gene was amplified from all samples tested, indicating the absence of PCR inhibitors. Of 81 samples that were *Legionella*-negative by the plate culture method, 56 and 23 samples were positive by the qPCR and EMA-qPCR method, respectively. That is, EMA treatment prior to qPCR reduced the number of *Legionella*-positive bath water samples by half. Out of 30 *Legionella*-positive samples identified by the plate culture method, one sample (10 CFU/100 ml) and four samples (10, 20, 20 and 30 CFU/100 ml) were judged *Legionella*-negative by the qPCR and EMA-qPCR method, respectively. In addition, one *Legionella*-negative sample by the qPCR method changed to *Legionella*-positive by the EMA-qPCR method. It was thought that these inconsistencies were errors involving *Legionella* counts near the detection limit of the qPCR and EMA-qPCR methods.

TABLE 2 shows the results of *Legionella* detection from cooling tower water samples by the plate culture, qPCR and EMA-qPCR methods. We tested 95 samples, but were unable to detect *Legionella* in one sample due to the overgrowth of non-target microorganisms on GVPC agar plates, and were unable to amplify internal control DNA from 12 samples due to the presence of residual PCR inhibitors. Therefore, these 13 samples were eliminated from the data analysis. *Legionella* spp. were detected in 27 (33%) of 82 samples by the plate culture method, and these strains tested were identified as *L. pneumophila* (20/27 samples, 74%), *L. pneumophila* and *L. quinlivanii* (1/27, 4%), *L. pneumophila* and *L. gratiana* (1/27, 4%), *L. feeleii* (2/27, 7%), *L. busanensis* (1/27, 4%), and *Legionella* sp. (2/27, 7%). Of 55 samples that were found to be *Legionella*-negative by the plate culture method, 53 samples were positive in both the qPCR and EMA-qPCR methods. That is, no effect of EMA treatment was observed.

Legionella counts by the plate culture method and *Legionella* 16S rRNA gene copies by the qPCR method were plotted as scatter diagrams (FIG. 2 and FIG. 3). No correlation for both the bath water and cooling tower water samples was seen. Therefore, it seems that

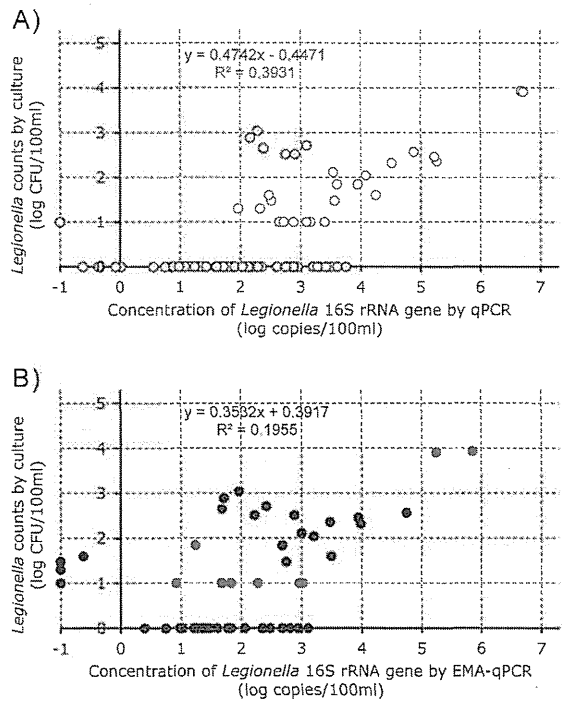

FIG. 2. Comparison of the results of the detection of *Legionella* by the plate culture and qPCR (A) or EMA-qPCR (B) methods from bath water samples.

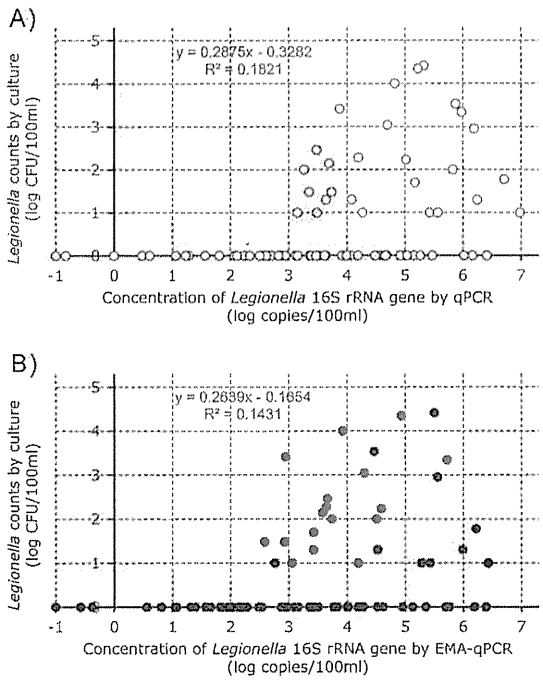

FIG. 3. Comparison of the results of the detection of *Legionella* by the plate culture and qPCR (A) or EMA-qPCR (B) methods from cooling tower water samples.

Legionella counts by the plate culture method cannot be expected based on the results of EMA-qPCR method.

In this study, our experimental data indicate that the EMA-qPCR method is useful for the rapid detection of viable *Legionella* spp. from bath water samples. In contrast, the effect of EMA treatment was not recognized in cooling tower water samples. The cause of the lack of effect of EMA treatment on cooling tower water samples is unknown, but we propose that the inhibition of EMA treatment could have been due to the water quality and/or the sludge in samples, and/or the presence of viable but nonculturable (VBNC) *Legionella*. Further research will be necessary to characterize the inhibition of EMA treatment in cooling tower water samples by using Control Test Kit (Viable Bacteria Selection, Takara Bio, Japan), because the sludge in the samples will especially inhibit transmission of the visible light. We expect that the presence of VBNC *Legionella* in environmental water is an important factor in the difference in the results between EMA-qPCR and the plate culture method, because VBNC *Legionella* are detected by the EMA-qPCR method but not by the plate culture method. The analysis of VBNC *Legionella* in environmental water is now in progress.

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MICROBES AND ENVIRONMENTS

Short Communication

Molecular Characterization of Viable *Legionella* spp. in Cooling Tower Water Samples by Combined Use of Ethidium Monoazide and PCR

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Viable *Legionella* spp. in environmental water samples were characterized phylogenetically by a clone library analysis combining the use of ethidium monoazide and quantitative PCR. To examine the diversity of *Legionella* spp., six cooling tower water samples and three bath water samples were collected and analyzed. A total of 617 clones were analyzed for their 16S rRNA gene sequences and classified into 99 operational taxonomic units (OTUs). The majority of OTUs were not clustered with currently described *Legionella* spp., suggesting the wide diversity of not-yet-cultured *Legionella* groups harbored in cooling tower water environments.

Key words: clone library, Ethidium monoazide (EMA), *Legionella*, quantitative polymerase chain reaction (qPCR), Viable but nonculturable (VBNC)

Legionella species are Gram-negative bacteria that are ubiquitously found in natural and man-made water systems. In aquatic environments, *Legionella* spp. can proliferate as the intracellular parasites of free-living protozoa (8, 19). The human inhalation of aerosols from *Legionella*-contaminated waters, mainly from cooling tower waters and bath waters, often results in a severe form of pneumonia called Legionnaires' disease (legionellosis) (22). Therefore, the control of *Legionella* populations in water systems and monitoring for *Legionella* contamination are very important areas in public health microbiology.

The populations of *Legionella* spp. in environmental water samples have so far been estimated by culture-based plate counting and culture-independent molecular methods using the quantitative polymerase chain reaction (qPCR). Many attempts to grow environmental strains of *Legionella pneumophila*, the main causative agent of legionellosis, on plate media have been successful, and have provided insights into the ecology of *L. pneumophila* in natural environments (9). Regarding molecular methods, *Legionella* genus-specific (14) and *L. pneumophila* species-specific (13) PCR assays have been developed and proven to be valuable tools for investigating *Legionella* contamination in water systems. The molecular detection of *Legionella* spp. by conventional PCR methods could not previously distinguish viable bacterial cells from viable but nonculturable (VBNC) and dead cells (15, 24). However, the use of DNA-intercalating dyes such as ethidium monoazide (EMA) and propidium monoazide (PMA) before PCR was recently found to be effective for the specific amplification of DNA from *Legionella* cells maintaining membrane integrity (17). EMA and PMA can penetrate membrane-damaged cells and form covalent links with DNA, and such labeled genomic DNA within damaged cells is degraded upon exposure to visible light. The use of EMA (2–4, 6, 11, 18) and PMA (3, 20, 25) for the PCR quantification of *Legionella* has been described previously. However, to the best of our knowledge, the *Legionella* groups detected by the EMA- or PMA-treated PCR method have not yet been fully characterized.

Therefore, the aim of the present study was to determine whether the *Legionella* groups detectable by EMA-treated PCR belonged to known *Legionella* spp.. We compared the diversities of viable *Legionella* groups in six cooling tower water samples and three bath water samples by constructing clone libraries. As a result, 617 clones from *Legionella* spp. were recovered and their sequences determined from the water samples.

Water samples were collected from six different cooling towers (sample ID; CTW-A, -B, -C, -G, -H, and -I) and three different baths (BW-D, -E, and -F) between November 2012 and January 2014. Water quality control management prior to our water sampling is described in Supplementary Table S1. Samples were taken in sterile 500-mL polypropylene bottles with 0.05% (w/v) sodium thiosulfate, kept in the dark at 4°C until microbiological plating and DNA extraction, and used for these analyses within 3 d.

Legionella populations in water samples were enumerated according to the standard culture method (12). Briefly, water samples were subjected to centrifugation at 6,400×*g* for 30 min and the precipitate was suspended in one-100th the volume of the initial water sample. A portion of the suspension was mixed with the same volume of acid-phosphate buffer (10), and after 10 min, inoculated onto GVPC selective agar plates (Merck, Tokyo, Japan). These plates were incubated at 37°C for 8 d. Isolates of *Legionella* from the GVPC selective agar plates (50 to 100 strains per sample, except for the very low-CFU sample [four strains, CTW-G] and the sample with CFUs below the detection limit [CTW-H]) were tested by the immune serum aggregation assay (Denka Seiken, Niigata, Japan) and DNA-DNA hybridization assay (Kyokuto Pharmaceutical Industrial, Tokyo, Japan). 16S rRNA gene sequences were determined for strains that were negative for both assays, as described below.

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L. pneumophila Phladelphia-1 (ATCC 33152) was used as the standard in qPCR assays, and cultured on a buffered charcoal yeast extract medium supplemented with 2-ketoglutarate (BCYEα) (7) at 30°C. The genomic DNA of *L. pneumophila* cells was extracted by the alkaline-boil method of Beige *et al.* (1) and purified using a NucleoSpin gDNA Clean-up kit (TaKaRa Bio, Otsu, Japan) according to the manufacturer's instructions. The copy number of *Legionella* 16S rRNA genes was calibrated using a Cycleave PCR *Legionella* (16S rRNA) Detection Kit (TaKaRa Bio).

A Viable *Legionella* Selection Kit for PCR ver.2.0 (TaKaRa Bio) including the EMA treatment was used for the clone library construction, as described by the manufacturer. Briefly, 1 mL of the 100-fold concentrated water sample was further concentrated to a final volume of 40 μL and mixed with 10 μL of the kit reaction buffer, 2.5 μL of the kit dilution buffer, and 2.5 μL of EMA reagent. After gently mixing using a vortex mixer and incubating in the dark for 15 min at room temperature, the samples were exposed to visible light for 15 min in a LED Crosslinker 12 (TaKaRa Bio). Thereafter, DNA was extracted and purified from each sample as described above.

Populations of *Legionella* spp. and *L. pneumophila* were quantified by qPCR using the primer pairs LEG-225F (5'-AAG ATT AGC CTG CGT CCG AT-3') and LEG-858R (5'-GTC AAC TTA TCG CGT TTG CT-3') (14), and *Lmip*L920 (5'-GCT ACA GAC AAG GAT AAG TTG-3') and *Lmip*R1548 (5'-GTT TTG TAT GAC TTT AAT TCA-3') (13), respectively. PCR reaction mixtures (30 μL) contained 5 μL of template DNA, 1 μL of 10 μM forward primer, 1 μL of 10 μM reverse primer, 0.15 μL of Ex *Taq* polymerase, 2.4 μL of dNTPs, 3 μL of 10×Ex buffer (TaKaRa Bio), and 1 μL of 1,000 dilutions of SYBR Green I dye (Lonza, ME, USA) with dimethyl sulfoxide in a Thermal Cycler Dice Real Time System *II* (TaKaRa Bio). The PCR program parameters were: an initial denaturation step of 2 min at 95°C followed by 45 cycles of denaturation for 15 s at 95°C, annealing for 30 s at 65°C (LEG primer pair) or 50°C (*Lmip* primer pair), and extension for 60 s at 72°C. A melting curve analysis was performed to detect the presence of primer dimers after the final extension by increasing the temperature from 50 to 95°C in 0.5°C increments every 10 s. The calibration qPCR was performed using *L. pneumophila* DNA, and the copy numbers of *Legionella* 16S rRNA genes were quantified as described previously: PCR performance was confirmed to be reproducible at the threshold cycles (Ct) <37 (11). Furthermore, the ratios of *L. pneumophila* were calculated from the amounts of *Legionella* 16S rRNA genes and *L. pneumophila mip* genes. To construct clone libraries, PCR using primers LEG-225F and LEG-858R was carried out according to the protocol of Nishizawa *et al.* (16) to minimize PCR bias: an initial denaturation step of 2 min at 95°C followed by each threshold cycle as determined by qPCR, denaturation for 15 s at 95°C, annealing for 30 s at 65°C, and extension for 60 s at 72°C. The reaction mixture (30 μL) was composed of 5 μL of template DNA, 1 μL of 10 μM LEG-225F primer, 1 μL of 10 μM LEG-858R primer, 0.15 μL of Ex *Taq* polymerase, 2.4 μL of dNTPs, and 3 μL of 10×Ex buffer (TaKaRa Bio) in a Thermal Cycler SP (TaKaRa Bio). The PCR products were purified by using a QIAquick PCR purification kit (Qiagen, CA, USA), ligated with the vector pMD20-T using a Mighty TA-cloning kit (TaKaRa Bio), and the ligation products were used to transform *E. coli* DH5α Competent Cells (TaKaRa Bio) according to the manufacturer's instructions. The nucleotide sequences of clones were determined with a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, CA, USA) using M13 primer RV (5'-CAG GAA ACA GCT ATG ACC-3') or M13 primer M4 (5'-GTT TTC CCA GTC ACG AC-3') according to the manufacturer's instructions and were read on an Applied Biosystems 3130xl genetic analyzer. Operational taxonomic units (OTUs) were defined as sequences with at least 99% similarity of all clones based on an analysis using Mothur platform software (http://www.mothur.org). The phylogenetic tree was constructed by the neighbor-joining method using MEGA5 software. Diversity indices (Chao 1, Simpson, Shannon-Wiener, and Good's coverage) were calculated on Mothur platform software at a cut-off level of 0.01 (99% sequence identity with gaps) in the average neighbor method.

The 16S rRNA gene partial sequences were deposited in DDBJ with accession numbers AB857847 to AB858225 and AB933772 to AB934017.

Fig. 1 shows a graphical representation of the relationships between viable population densities of *Legionella* spp. in the tested water samples determined by the standard culture method (horizontal axis) and those by the EMA-qPCR method targeting the *Legionella* 16S rRNA genes (vertical

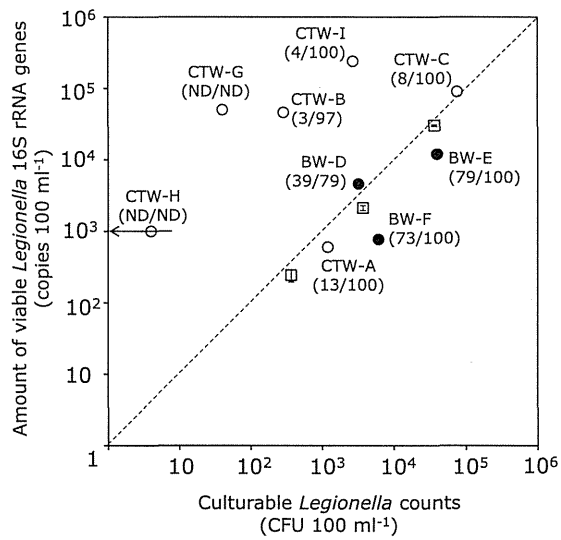

Fig. 1. Viable population densities of *Legionella* spp. in tested cooling tower (open circles), bath (solid circles) water samples, and cell suspensions of *L. pneumophila* ATCC33152 (open squares) determined by the standard culture method (horizontal axis) and EMA-qPCR targeting 16S rRNA genes (vertical axis). CTW-A to -C and -G to -I, and BW-D to -F represent the sample ID. The number pair in parentheses under the sample ID shows the percentage of the *Legionella pneumophila* population density relative to the total *Legionella* spp. population density, which was estimated by qPCR specific for *L. pneumophila* (left figure) and the identification of isolates (right figure); ND, not detected. Arrow on the sample CTW-H symbol shows that the sample harbored <10 CFU 100 mL^{-1} of *Legionella* spp. The relationship between CFU 100 mL^{-1} (x) and the 16S rRNA gene copy number 100 mL^{-1} (y) in *L. pneumophila* ATCC33152 suspensions was approximated as a dotted straight line, $y = 0.45x^{1.05}$ ($r^2 = 0.996$) (duplicate determinations).

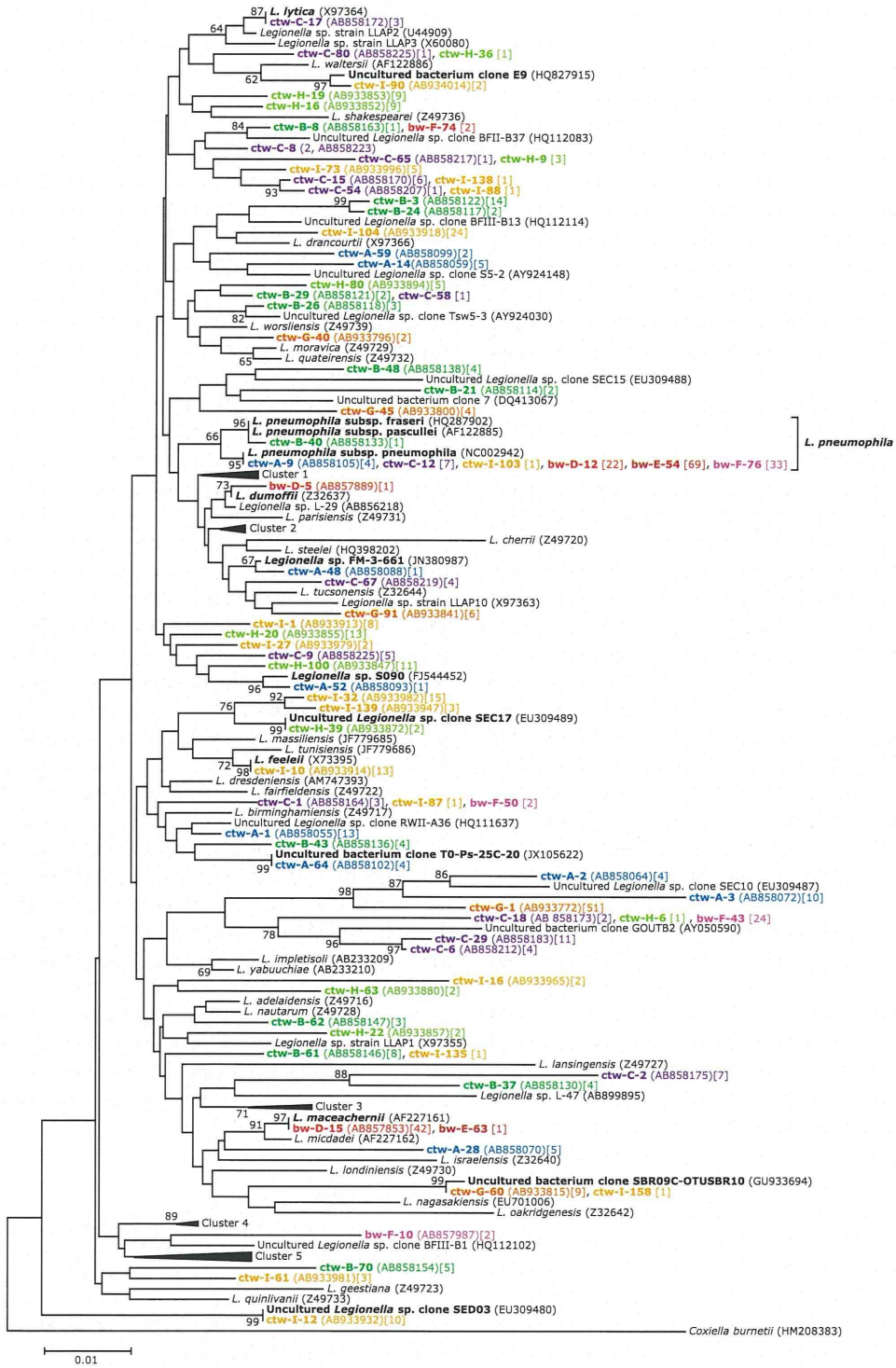

Fig. 2. Neighbor-joining tree based on the alignment of 616-bp 16S rRNA gene sequences of 79 major representative *Legionella* clones, the *Legionella* spp. that have been described to date, and the related known uncultured *Legionella* clones. Numbers at nodes are bootstrap percentages (based on 1,000 resamplings); only values above 60% are shown. The sources of the clones were expressed in different color letters: CTW-A, blue; CTW-B, green; CTW-C, purple; CTW-G, orange; CTW-H, lime green; CTW-I, yellow; BW-D, red; BW-E, brown; BW-F, pink. After the representative clone, the accession number of the representative clone and the number of similar sequences (based on a 1% cut-off) are given in parentheses and square brackets, respectively. Cluster 1 includes *L. anisa* (Z32635), *L. bozemanii* (Z49719), *L. wadsworthii* (Z49738), *L. gormanii* (Z32639), and *L. steigerwaltii* (Z49737). Cluster 2 includes *L. sainthelensi* (Z49734), *L. santicrucis* (HF558374), *L. longbeachae* (AY444740), *L. cicinatiensis* (Z49721), and *L. gratiana* (Z49725). Cluster 3 includes *L. beliardensis* (AF122884), *L. busanensis* (AF424887), and *L. gresilensis* (AF122883). Cluster 4 includes *L. rubrilucens* (Z32643), *L. taurinensis* (DQ667196), and *L. erythra* (Z32638). Cluster 5 includes *L. brunensis* (Z32636), *L. cardiac* (JF831047), *L. hackliae* (M36028), *L. jamestownensis* (Z49726), *L. jordanis* (Z32667), and *L. spintensis* (M36030).

axis). When *L. pneumophila* ATCC33152 was used as a positive control, the relationship between CFU 100 mL^{-1} (x) and the 16S rRNA gene copy numbers 100 mL^{-1} (y) was approximated as an equation, $y = 0.45x^{1.05}$ ($r^2 = 0.996$). Culturable *Legionella* counts ranged from <10 to 7.6×10^4 CFU 100 mL^{-1} and the copy numbers of *Legionella* 16S rRNA genes from viable cells were between 6.0×10^2 and 2.4×10^5 100 mL^{-1}. Four (CTW-B, -C, -G, and -I) of the six cooling tower water samples contained approximately 10^5 100 mL^{-1} *Legionella* 16S rRNA gene copies, which was approximately 100-fold higher than that in the other samples (CTW-A and -H) and 10- to 100-fold higher than that in the bath water samples. *Legionella* viable counts for all bath water samples and the three cooling tower water samples (CTW-A, -C, and -I) were $>10^3$ CFU 100 mL^{-1}, which was higher than that in the other cooling tower water samples. Four sample plots (CTW-A, CTW-C, BW-D, and BW-E) were close to the positive control line, while the plots of the other cooling tower water samples (CTW-B, -G, -H, and -I) deviated markedly upward from the line, suggesting that these samples contained larger *Legionella* populations that were unable to grow under the tested culturing conditions than the culturable ones, which was also found in our recent study (11). The identification of isolates by the immunoassay and the DNA-DNA hybridization assay revealed the dominance of *L. pneumophila*, accounting for >79% of the total *Legionella* populations, except for the very low-CFU sample (only four isolates for CTW-G) and one sample that was below the detection limit (CTW-H). The sequences of the 16S rRNA genes from all four isolates from CTW-G and one from CTW-B were 100% identical to those from *Legionella* sp. LC2720 and *Legionella* sp. L-29, respectively. Approximately 20% of the isolates from BW-D were identified as *Legionella dumoffii*.

Fig. 2 shows the neighbor-joining tree based on the *Legionella* 16S rRNA gene partial sequence (616 bp) from the cooling tower and bath water samples. A total of 617 clones (cooling tower waters: 417 clones, bath waters: 200 clones) were recovered from the water samples and classified into 99 OTUs at a cut-off level of 0.01 (99% sequence identity). Good's coverages of these libraries were 82.9% to 96.0% (cooling tower waters) and 96.9% to 98.6% (bath waters). The most abundant OTU, represented by clone ctw-A-9 (137 clones, 22% of all clones), clustered with the *L. pneumophila* group (Fig. 2). The dominance of *L. pneumophila* in BW-E and BW-F was confirmed by the clone library analysis, accounting for 99% and 51% of clones, respectively. In the other bath water sample (BW-D), *L. pneumophila* was also the main member (34%) of the clone library. In contrast, the percentage of *L. pneumophila* clones was very low in the cooling water samples: less than the detection limit for CTW-G and CTW-H and 1 to 11% for the other cooling tower water samples. The second most abundant OTU, represented by clone bw-D-15 (43 clones, 7% of all clones), was affiliated with the *L. maceachernii* cluster and accounted for 65% of the clones from BW-D and 1% of those from BW-E. The other clones that clustered with known *Legionella* spp. were *L. feeleii* (13 clones, 2% of all clones), *L. lytica* (three clones, 0.5% of all clones), and *L. dumoffii* (one clone, 0.2% of all clones).

Although the clone sequences that clustered with the *L. maceachernii* sequence were abundant in BW-D, this organism was not detected by the plate culture method. These results may be explained by either its VBNC state or a failure to outcompete *L. pneumophila* in the culture. On the other hand, *Legionella* sp. L-29 and *Legionella* sp. LC2720 were not detected by the clone libraries from CTW-B and -G, respectively. It is likely that although the plate culture method detected their very low population densities, the coverage of our clone library was too low to detect them.

Diversity indices were calculated and are summarized in Supplementary Table S2. The Chao1 values of these libraries were 10 to 67 (cooling tower water) and 2 to 7 (bath water). The Simpson ($1/\lambda$) values of these libraries were 2.08 to 13.32 (cooling tower water) and 1.03 to 2.58 (bath water). The Shannon-Wiener (H') values of these libraries were 1.14 to 2.67 (cooling tower water) and 0.07 to 1.06 (bath water). All these indices suggested that the diversity of *Legionella* communities present in cooling tower water was higher than that in bath water, and may be explained by differences in water treatments. Bath water was cleaned with a higher concentration of chlorine for a shorter period of time than cooling tower water, which may have resulted in the selective survival of chlorine-resistant strains. Further studies will be needed to clarify the relationship between the diversity of *Legionella* floras and the treatment of water systems.

A number of clones (390 clones, 63% of all clones) showed less than 99% similarity to the sequences of the known culturable *Legionella* spp. strains or uncultured *Legionella* clones. Thirty clones (7 OTUs) were closely related to the uncultured *Legionella* sp. clone SEC03 (10 clones) from the cooling tower water (23), the uncultured bacterium clone SBR09C-OTUSBR10 (10 clones), the uncultured bacterium clone T0-Ps-25C-20 (21) (four clones), the uncultured *Legionella* sp. clone SEC17 (23) (two clones), the uncultured bacterium clone E9 (two clones), *Legionella* sp. FM-3-661 (one clone), and *Legionella* sp. S090 (5) (one clone).

In conclusion, our results showed that the EMA-PCR method was capable of revealing more diverse *Legionella* groups than the standard culture method and is, thus, a better tool for monitoring *Legionella* contamination in various environments.

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We express our sincere thanks to Dr. Fumiaki Kura and Dr. Junko Amemura-Maekawa of the National Institute of Infectious Diseases for reviewing our experimental data. We thank Ms. Tomoko Takama and Ms. Asako Morishita in our laboratory for the plate culture data collection and excellent laboratory assistance in the molecular biological experiments, respectively. This work was partially supported by the Health and Labor Science Research Grants (H25-kenki-009).

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Supplementary tables

Table S1. Water quality control management before water sampling

Sample ID	Sampling site	Treatment condition	Concentration of biocide	Sampling date
Cooling tower waters				
CTW-A	a building in Niigata	no treatment	none	November 6, 2012
CTW-B	a plant in Tokyo	continuous supply of CMI [a]	<0.1 mg L^{-1} (CMI)	February 7, 2013
CTW-C	a factory in Yamanashi	intermittent supply [b] of CMI	<0.1 mg L^{-1} (CMI)	February 14, 2013
CTW-G	a factory in Saitama	continuous supply of CMI	<0.1 mg L^{-1} (CMI)	December 3, 2013
CTW-H	a building in Kanagawa	continuous supply of CMI	<0.1 mg L^{-1} (CMI)	January 17, 2014
CTW-I	a factory in Fukuoka	continuous supply of stabilized chlorine	2.0 mg L^{-1} (total chlorine)	November 29, 2013
Bath waters				
BW-D	a sports club in Kanagawa	continuous supply of free chlorine	1.0 mg L^{-1} (free chlorine)	March 21, 2013
BW-E	a hotel in Miyagi	continuous supply of free chlorine	no data (free chlorine)	April 23, 2013
BW-F	a sports club in Osaka	continuous supply of free chlorine	0.5 mg L^{-1} (free chlorine)	May 15, 2013

[a] 5-Chloro-2-methyl-4-isothiazolin-3-one

[b] The water treatment chemicals including CMI were batch fed as necessary.

Table S2. Diversity indices of *Legionella* communities

Sample	No. of clones analyzed	No. of OTUs obtained	Chao1	Simpson (1/λ)	Shannon-Wiener (*H'*)	Good's coverage (%)
CTW-A	51	11	15	7.87	2.16	92.2
CTW-B	58	17	23	10.60	2.53	87.9
CTW-C	62	16	28	13.32	2.63	87.1
CTW-G	75	8	10	2.08	1.14	96.0
CTW-H	66	19	30	9.71	2.45	84.8
CTW-I	105	29	67	9.91	2.67	82.9
BW-D	65	3	3	1.90	0.71	98.5
BW-E	70	2	2	1.03	0.07	98.6
BW-F	65	7	7	2.58	1.16	96.9

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Case report

A case of pneumonia caused by *Legionella pneumophila* serogroup 12 and treated successfully with imipenem

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ABSTRACT

The patient was an 83-year-old man hospitalized for *Haemophilus influenzae* pneumonia, who developed recurrent pneumonia after improvement of the initial episode. *Legionella pneumophila* serogroup 12 was isolated from the sputum, accompanied by increased serum antibody titers to *L. pneumophila* serogroup 12. Therefore, the patient was diagnosed as having *Legionella* pneumonia caused by *L. pneumophila* serogroup 12.

Case reports of pneumonia caused by *L. pneumophila* serogroup 12 are rare, and the case described herein is the first report of clinical isolation of this organism in Japan. When the genotype was determined by the protocol of The European Working Group for *Legionella* Infections (Sequence-Based Typing [SBT] for epidemiological typing of *L. pneumophila*, Version 3.1), the sequence type was ST68. Imipenem/cilastatin therapy was found to be effective for the treatment of *Legionella* pneumonia in this patient.

1. Introduction

Legionella infection is caused by organisms of the genus *Legionella*, which are Gram-negative bacilli. *Legionella pneumophila* strains are the predominantly isolated in clinical practice. *L. pneumophila* has been classified into 15 serogroups, of which serogroup 1 is most frequent cause of *Legionella* pneumonia, whereas *L. pneumophila* serogroup 12 is rarely responsible. Only four cases of pneumonia caused by *L. pneumophila* serogroup 12 have been reported to date [1–4]. Herein, we report a case of pneumonia caused by *L. pneumophila* serogroup 12, which is the first report of clinical isolation of this organism in Japan.

2. Case report

The patient was an 83-year-old man with a 3-day history of cough, sputum expectoration and anorexia, who was brought to our hospital by ambulance because of weakness of both legs in the beginning of January, 2012; he was then admitted to the hospital with the diagnosis of pneumonia. The patient had underlying diabetes mellitus, and had been on treatment with oral prednisolone 10 mg/day for the interstitial pneumonia. He gave no history of visits to hot spring facilities or circulation-type baths. Gram staining of the sputum on admission revealed phagocytosed Gram-negative bacilli, and sputum culture grew *Haemophilus influenzae*. The patient improved after 10 days of treatment with piperacillin/tazobactam (TAZ/PIPC) (12.5 g/day). Because the patient also had concomitant asthmatic symptoms, the steroid dose was increased to 80 mg of methylprednisolone/day (Fig. 1). Chest radiography carried out on the 13th hospital day revealed infiltrative opacities in the right upper lung field, with deterioration of the infiltrative opacities in the lower lung fields bilaterally that had improved once with TAZ/PIPC (Fig. 2A). Chest computed tomography (Fig. 2B and C) also confirmed the presence of infiltrative opacities in the right upper and both lower lobes. Anorexia was the only subjective symptom, and there was no diarrhea. The patient was recorded to have fever (body temperature, 38 °C) only on the 18th hospital day. The patient received oxygen supplementation at 2 L/min by nasal

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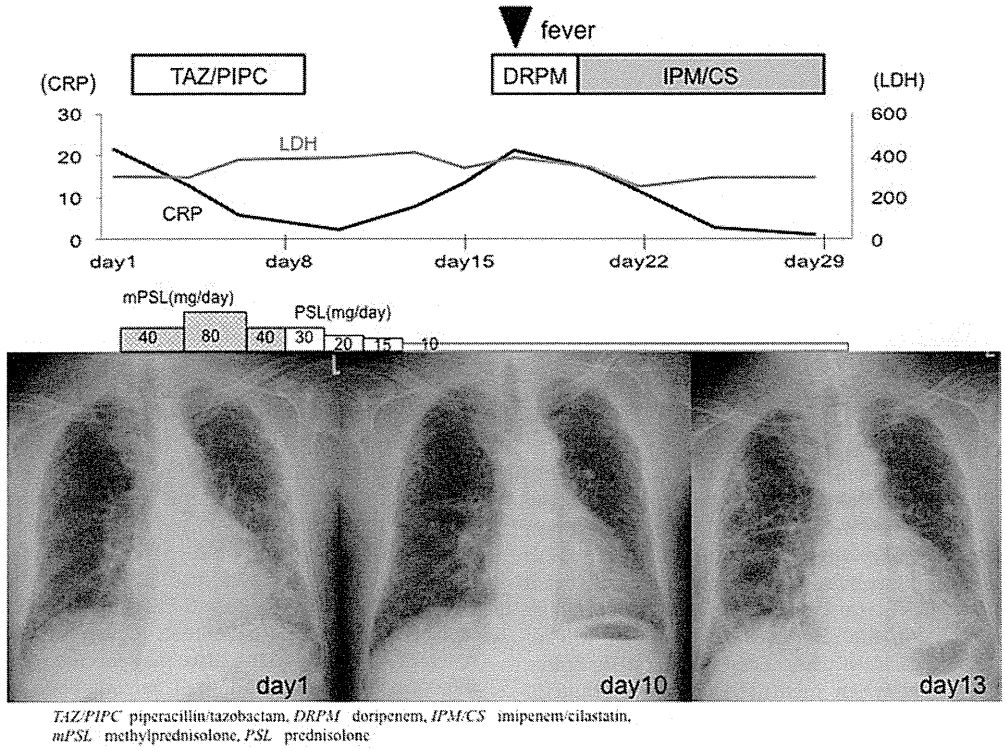

TAZ/PIPC piperacillin/tazobactam, *DRPM* doripenem, *IPM/CS* imipenem/cilastatin, *mPSL* methylprednisolone, *PSL* prednisolone

Fig. 1. Clinical course.

cannula; however, the oxygen flow needs to be increased temporarily to 3 L/min from 17th hospital day to 26th hospital day. Laboratory data obtained on the 13th hospital day were as follows: WBC count 17,400 cells/μL (stab cells 2.0%, segmented cells 89.0%), and serum CRP 7.63 mg/dL, revealing a tendency towards increase in the values of these parameters. Serum LDH was increased to 416 IU/L. The other parameters were BUN 24.0 mg/dL, serum creatinine 1.08 mg/dL, Na 136 mEq/L, K 3.8 mEq/L, Cl 98 mEq/L, fasting blood glucose 113 mg/dL, and HbA1c 6.2%. Gram staining of the sputum on the 13th hospital day showed phagocytosed Gram-negative bacilli. Doripenem (750 mg/day) therapy was initiated on the 17th hospital day. The

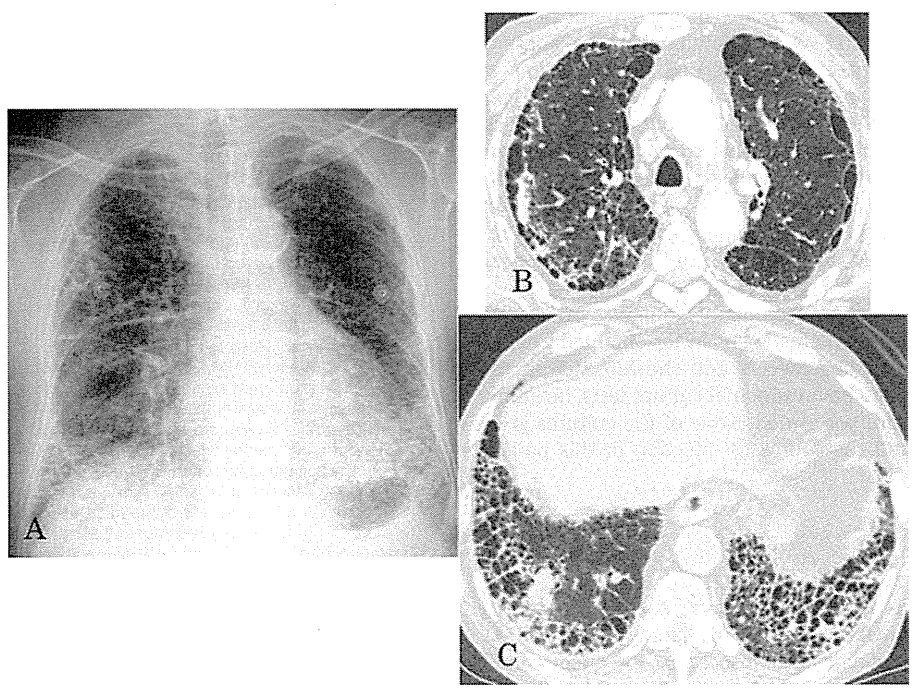

Fig. 2. Chest radiography and chest computed tomography on the 13th hospital day demonstrates the infiltrative opacities in the right upper and both lower lobes.

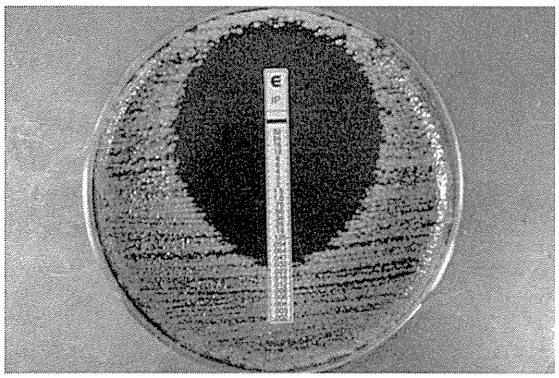

Fig. 3. Result of imipenem Etest for *Legionella pneumophila*.

doripenem therapy was switched to imipenem/cilastatin (1.5 g/day) therapy on the 20th hospital day because of elevation of the hepatic enzyme levels, and the patient's clinical condition improved thereafter. Because sputum obtained on the 13th hospital day cultured by the conventional method did not reveal any growth of bacteria, BCYE agar culture was carried out, which grew *Legionella* colonies on the 32nd hospital day. The MIC of imipenem for the isolated strain using Etest (AB Biodisk, Solna, Sweden) was 0.047 μg/ml when measured by the method of Murakami et al. [5] (Fig. 3). The slide agglutination test carried out using monovalent immune sera (Denka Seiken, Tokyo, Japan) identified the *Legionella* bacterium isolated from the sputum as *L. pneumophila* serogroup 12. The serum antibody titer on the 32nd hospital day was determined using microplate agglutination test kit (Denka Seiken, Tokyo, Japan) and in-house heat-killed *L. pneumophila* antigens (serogroups 7–15) [6]. The serum antibody titer determined was 1:512 for *L. pneumophila* serogroup 6 and 1:8192 for *L. pneumophila* serogroup 12, showing a distinct single high-titer. The serum antibody titers against *L. pneumophila* serogroups 1 to 5 and 7 to 11 and 13 to 15 were <1:16. A definitive diagnosis of infection caused by *L. pneumophila* serogroup 12 was made in our patient because *L. pneumophila* serogroup 12 was isolated from the sputum, accompanied by an increase of the serum antibody titer to *L. pneumophila* serogroup 12. Urinary *Legionella* antigen (BinaxNOW®) was negative throughout the course of the illness. The genotype was determined according to the protocol of the European Working Group for *Legionella* Infections (EWGLI; http://www.ewgli.org/) (Sequence-Based Typing (SBT) for epidemiological typing of *L. pneumophila*, Version 3.1). The results showed (3, 13, 1, 28, 14, 9, 3) for (*flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, *neuA*), and the sequence type was ST68.

Because the possibility of nosocomial infection was considered to be highly likely in the patient, testing of swabs obtained from 13 sites in the hospital, including faucets and showerheads in the bathrooms of the ward, and environmental culture tests, including of the shower water, were performed. None of the cultures grew *Legionella* organisms, and the source of infection in this patient remained unclear.

3. Discussion

Fifty-seven species of *Legionella* are currently known (http://www.bacterio.net/legionella.html, as of January 22, 2014), among which *L. pneumophila* is the most frequently encountered causative organism of *Legionella* pneumonia. Among the 15 serogroups of *L. pneumophila*, serogroup 12 was first identified in 1987 [1]. Among the 5370 clinical strains whose genotypes are registered in the EWGLI database, there are 11 strains of serogroup 12 including our case as of January 21, 2014. Our present case is the first report of clinical isolation of this serogroup in Japan. Recently, 2 new cases of infection with *L. pneumophila* serogroup 12 including our case were recorded in the surveillance data of legionellosis in Japan [7]. A review of the literature collected within the scope of our search yielded 4 cases of pneumonia caused by serogroup 12 [1–4]. Clinical data were available for 2 of these patients, both of whom were cases of nosocomial infection occurring in immunosuppressed patients, just as in our patient [1,2].

The SBT proposed by the EWGLI is a technique to determine the base sequence by PCR amplification of some parts of 7 genes, i.e., *flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, and *neuA*. The genotype of *L. pneumophila* in this case was ST68, which was found in 18 clinical strains in the EGWLI database as of January 21, 2014. Of these 18 strains, serogroup 6 accounted for the majority (11 strains), followed by serogroup 12 (4 strains). ST68 was previously isolated in 1 case of serogroup 6 in Japan, and therefore our case represents the second case of isolation of the ST68 genotype.

Our patient showed an increase of the serum antibody titer to *L. pneumophila* serogroup 6. Because cross-reactions between serogroups 6 and 12 have been reported previously [1], a cross-reaction in our patient also may occur. However, the case of infection with more than one *L. pneumophila* serogroup cannot be ruled out.

In a study by Murakami et al., the MIC of imipenem was 0.023–0.064 μg/ml for all 23 clinical isolates of *Legionella*, indicating good sensitivity of this organism to imipenem. The MIC for the isolate in our patient reported here was also within the above range. In general, it is considered that imipenem exerts no effect on *Legionella* bacteria *in vivo* because of its poor transfer into macrophages [8,9]. On the other hand, the efficacy of this drug for this infection has also been occasionally reported [10–15].

Ramirez JA et al. reported imipenem showed superior bactericidal activity against intracellular *L. pneumophila* compared to erythromycin in an *in vitro* model [13].

Our patient with *Legionella* pneumonia reported here improved with imipenem/cilastatin therapy, imipenem/cilastatin may be effective for the treatment of *Legionella* pneumonia in some cases.

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話題の環境衛生

モノクロラミン消毒による浴槽水の衛生対策

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1．はじめに

循環式浴槽では肺炎の起因菌であるレジオネラ属菌の汚染が問題となっており，死亡例を含む集団感染が繰り返されたことから[1, 2]，厚生労働省の指導のもとで緊急避難的に遊離塩素消毒による管理が行われた。しかし，今日に至るまで，遊離塩素消毒を行っているにも関わらず浴槽からレジオネラ属菌が検出されることがあり[3]，遊離塩素消毒が全ての浴槽の安全を担保するとは言い難い状況にある。その原因として，井水や温泉水など多様な水質の存在，高 pH の条件下では遊離塩素消毒の効果が期待できないこと等が考えられる。また塩素臭などが敬遠されて遊離塩素の使用が必ずしも徹底されない恐れもあり，多方面から代替消毒方法が求められている。そこで，厚生労働科学研究班では，米国の水道で実用化されているモノクロラミン（結合塩素の一種）消毒[4, 5]に着目し，モノクロラミン消毒の入浴施設への応用について検討を行なった。本稿では，浴槽水のモノクロラミン消毒について解説するとともに，実際の入浴施設への導入事例等を紹介したい。

2．遊離塩素消毒の問題点

都道府県や政令市の浴槽水等の衛生管理を定めた条例では，循環式浴槽の消毒に使われる遊離塩素の濃度を 0.2 または 0.4mg/L 以上に保ち，かつ最大 1.0mg/L を超えない範囲で適切に管理することが求められている。しかし，実際の入浴施設では，アルカリ泉質の影響や温泉の泉質による遊離塩素の消失によって十分な消毒効果が得られない場合もあり，レジオネラ属菌が検出されることがある。

遊離塩素の殺菌力は，次亜塩素酸（HClO）と，それより殺菌力が落ち同じ殺菌速度を得るために約 80 倍の濃度が必要と言われる次亜塩素酸イオン（ClO^-）の存在比により決まる[6]。アルカリ泉質の温泉に次亜塩素酸ナトリウムを注入した場合は，殺菌力の劣る次亜塩素酸イオンの存在割合が高くなるため，遊離塩素の殺菌力は大きく低下する。

また，アンモニアを含む温泉水に遊離塩素を添加した場合，遊離塩素濃度は速やかに低下する。**図 1** に，アンモニア態窒素（アンモニウムイオン中の窒素の量）1mg/L を含む工業用水（pH7.5）に遊離塩素を 1.95 ～ 11.7mg/L（0.5 ～ 3.0 モル当量）となるように添加した後，40 ℃，1 時間静置後の各塩素濃度とアンモニア態窒素濃度を示した。アンモニア態窒素 1mg/L を含む水において，遊離塩素を検出するのに必要な遊離塩素の投入量は 8mg/L 以上（不連続点塩素処理に相当）という配管洗浄濃度に匹敵する高濃度なものであることがわかる。また，やっと検出された遊離塩素もアンモニアを含む新たな水が追加されると，遊離塩素と速やかに反応するため，遊離塩素は再度検出されなくなることが容易に推測できる。このようにアンモニアを含む水で遊離塩素濃度を一定に保つことは非常に困難

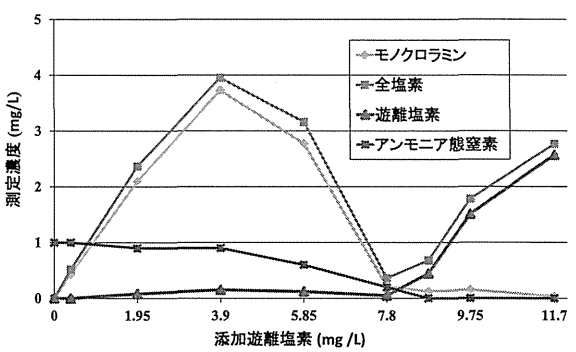


図 1　アンモニア態窒素を含む工業用水に遊離塩素を添加した時の各塩素濃度

といえる。

アンモニアは温泉成分の分析項目ではないので，施設ではその含有の有無を把握していないことが多いが，アンモニアを含む温泉水はかなりの頻度でみられると報告[7]されている。

アンモニア成分以外に，遊離塩素消毒が有効でない温泉の泉質として，鉄・マンガン，硫化水素，フミン酸質などの有機物や，酸性では塩素ガスが発生する恐れがあるpH4以下の酸性泉などがあげられる[8]。

前述の浴槽水の遊離塩素消毒による塩素臭に加えて，有害なトリハロメタン等の消毒副生成物の生成も問題となりえる。

3. 代替消毒法としてのモノクロラミン消毒

米国の水道で採用されていたモノクロラミン消毒は，国内では水道法施行規則第17条（水道の結合残留塩素による消毒は0.4mg/L以上，著しく汚染される恐れがある場合1.5mg/L以上と規定）に記載されている結合塩素に相当し，国内でも飲用に適用可能と考えられる。

3.1 モノクロラミンの殺菌効果と安全性

図2に示すように，3mg/Lのモノクロラミンは，高pH域（pH8.8），40℃の環境下で10^6CFU/mLのレジオネラ属菌を10分間で殺菌できた[9]。アルカリ泉での殺菌効果が期待できる成績である。また，同じ3mg/Lの濃度のモノクロラミンによってレジオネラ属菌の増殖宿主であるアメーバの栄養体やシストの不活化が確認できた[10]。なお，モノクロラミンを浴用に使用した場合に皮膚への刺激性が心配されるが，実験動物を用いた皮膚刺激試験では56mg/Lの濃度のモノクロラミンも無刺激物と判定され，入浴に差し支えないと推測された[11]。

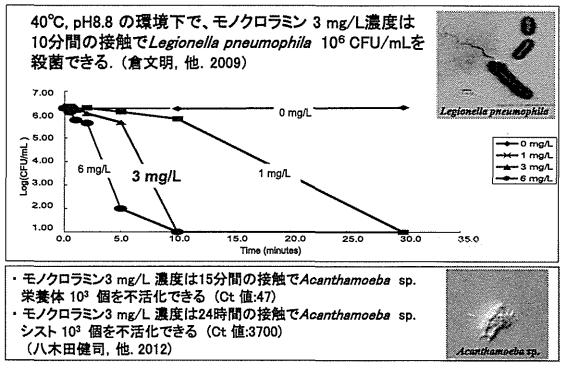


図2 モノクロラミンのレジオネラ，アメーバに対する殺菌・不活化効果

3.2 モノクロラミン消毒の事前適合性試験

温泉水におけるモノクロラミンの安定性を知るために，少量の温泉源泉水にモノクロラミンを3mg/Lの濃度になるよう添加し，ウォーターバスで40℃に保温し，一定時間ごとに，モノクロラミン濃度を測定した。

図3に，アンモニア態窒素を0.4mg/L含む温泉施設の源泉水を使ってモノクロラミン等の安定性試験を行なった結果を示した。本温泉水中ではモノクロラミン濃度は6時間以上安定して維持された。一方，遊離塩素濃度は速やかに低下し30分後には30％に減少した。このように温泉源泉水におけるモノクロラミンの濃度安定性を事前に知ることで，その温泉水におけるモノクロラミン消毒の有効性を事前に予測できた。モノクロラミン消毒の現場への適用判断が容易になると思われる。

なお，遊離塩素消毒が効きにくい温泉成分と言われ

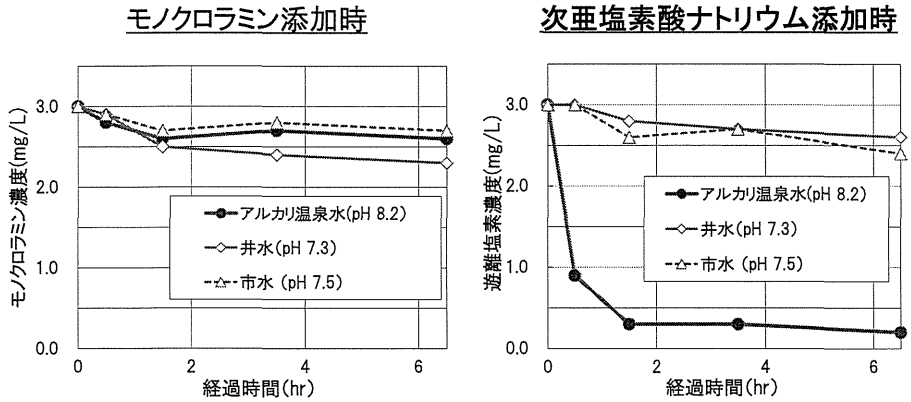


図3 アンモニア態窒素を含む温泉源泉水におけるモノクロラミン等の安定性試験

るアンモニア態窒素，フミン酸，鉄イオン，ヨウ化物イオン，臭化物イオン，硫黄のモノクロラミンに与える影響を調べたところ，モノクロラミンへの影響がほとんどなかったのは，アンモニア態窒素とフミン酸であった。鉄イオンは，モノクロラミンへの影響が見られたものの，濃度低下は遊離塩素よりも小さかった。ヨウ化物イオンは，濃度依存的にモノクロラミン濃度を速やかに低下させた。臭化物イオンは反応速度が遅かったが，ヨウ化物イオンと同様な傾向にあった。硫黄は，モノクロラミンと遊離塩素のいずれでも，添加直後に急速な濃度低下が確認され，系内の硫黄の消費後に塩素濃度が安定した[12]。以上の結果から，モノクロラミンに対しても影響を与える成分が存在することが確認できたが，遊離塩素よりは濃度維持が可能な結果が得られており，遊離塩素処理に替えて温泉施設への適用が期待できる。

4．入浴施設へのモノクロラミン消毒の導入事例

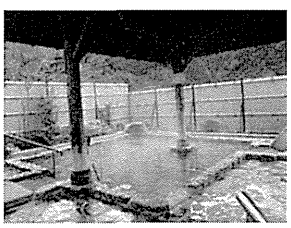
4.1　循環式浴槽施設への導入

泉質の異なった営業中の温泉施設3ヶ所の循環式浴槽（図4）で，モノクロラミン消毒の検証試験を行った。対象施設は，1日平均利用者数300～600人規模の露天風呂浴槽（総容量6.5～28㎥）で，泉質は塩化物泉，炭酸水素塩泉で，pHは7.8，8.2，9.0の弱アルカリからアルカリ性であり，アンモニア態窒素を0.4，1.9，4.3mg/L含んでいた。

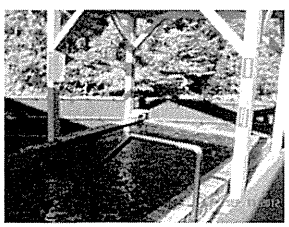
事前に源泉水を使ってモノクロラミン消毒の適合性試験を行ない，各泉質におけるモノクロラミン濃度の安定性を確認した上で，営業施設での実証試験を行った。

4.2　モノクロラミンの生成・注入・測定方法

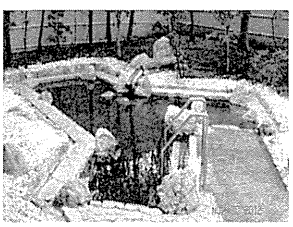
モノクロラミンは，水道水または井戸水に，12％次亜塩素酸ナトリウム溶液と20％塩化アンモニウム溶液を順次添加して生成した。この時の両剤のモル比



・泉質：ナトリウム－炭酸水素塩泉、pH9.0、アンモニア態窒素 1.9 mg/L含む
・公衆浴場の露天風呂
・浴槽容量：20m³
・利用者数：平均319人/日



・泉質：ナトリウム－塩化物泉、pH7.8、アンモニア態窒素 4.3 mg/L含む
・公衆浴場の露天風呂
・浴槽容量：6.5m³
・利用者数：平均587人/日



・泉質：ナトリウム－カルシウム塩化物泉、pH8.2、アンモニア態窒素 0.4 mg/L含む
・ホテルの露天風呂
・浴槽容量：28m³
・利用者数：平均480人/日

図4　モノクロラミン消毒を実施した営業中の温泉施設の概要

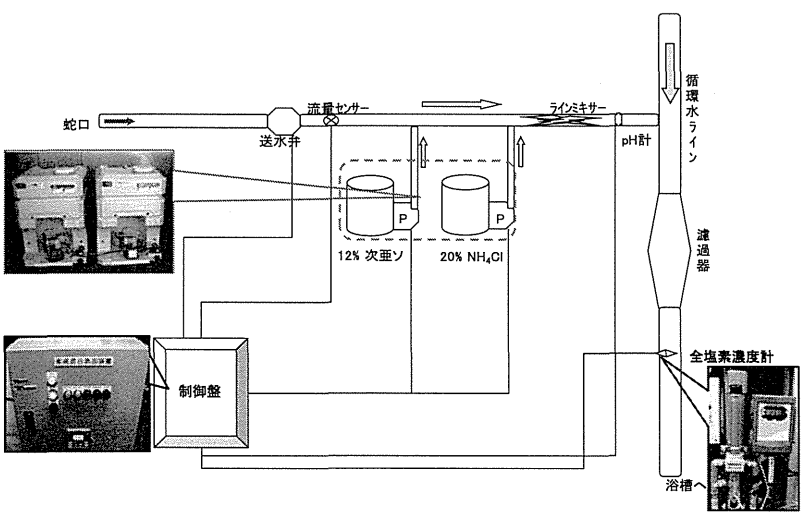


図5　モノクロラミンの自動生成・注入・測定方法

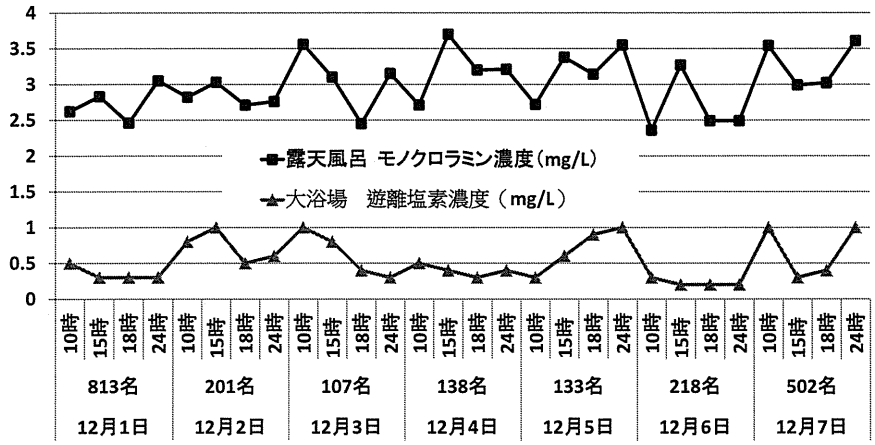


図６　モノクロラミン消毒浴槽と遊離塩素消毒浴槽の各塩素濃度と入浴者数

は遊離塩素に対してアンモニアを過剰（1 ： 2.5）とし，ジクロラミンおよびトリクロラミン生成の抑制に努めた。

モノクロラミンの注入方法は，ヒトの手による投入も可能[13]であるが，機械による注入が便利で確実である。機械による注入はモノクロラミン生成装置と全塩素濃度測定計器を組み合わせて濃度を自動制御する方法（図5）[14]と，新湯の追加量に応じ一定量のモノクロラミンを注入し，濃度を一定に保つタイマー注入方式[15]のいずれかを採用した。モノクロラミンは循環系統のろ過器前に注入した。

図６に，一施設のモノクロラミン消毒浴槽と対照浴槽である遊離塩素消毒浴槽で測定した各塩素濃度と入浴者数を示した。モノクロラミン消毒の露天風呂浴槽水の濃度は入浴者数にかかわらず一定の濃度範囲（2.5 ～ 3.5mg/L）を安定して維持することができた。モノクロラミン濃度が，入浴者数にかかわらず安定して保たれることは，新湯の補給に合わせてタイマー注入する方式の施設でも同様であった。

4.3　モノクロラミンの消毒効果

モノクロラミン濃度を自動制御する方法と，タイマー注入方式のいずれを用いた施設でも，レジオネラ属菌は一切検出されず，レジオネラ属菌の増殖の場となる自由生活性アメーバも不検出であった。代表して一施設の微生物検査と各種塩素濃度の結果を**表１**に示した。他の一施設では事前に実施した遊離塩素管理時の検査で，温泉水に含まれるアンモニアの影響で浴槽水からは遊離塩素は検出されず（不連続点塩素処理が出来ていないため），レジオネラ属菌が検出された。一方，モノクロラミン消毒時にはレジオネラ属菌，アメーバともに不検出であり，モノクロラミン消毒の優位性が確認された。

本稿では詳しく述べないが，鉄イオンを含む鉄泉の温泉施設や井戸水の沸かし湯[15]でもモノクロラミン消毒の有効性が確認できた。

また，浴槽水の消毒だけでなく，モノクロラミン消毒によるバイオフィルム除去や形成抑制効果も明らかになった。各施設のヘアキャッチャー接続配管内面を手で拭き取ったところ，モノクロラミン消毒実施時に

表１　アンモニア態窒素 0.4mg/L を含む温泉営業施設のモノクロラミン消毒の各種塩素濃度と微生物検査結果

		対照浴槽	１週目	２週目	３週目	４週目	５週目	６週目
微生物検査	レジオネラ属菌数(CFU/100mL)	＜10	＜10	＜10	＜10	＜10	＜10	＜10
	アメーバ数(個/50mL)	＜1	＜1	＜1	＜1	＜1	＜1	＜1
	従属栄養細菌数(CFU/mL)	＜1	1.1×10^2	5.2×10^2	1.0×10^3	1.7×10^3	1.6×10^4	7.2×10^2
塩素濃度	モノクロラミン(mg/L)	0.1	2.6	3	3.4			2.2
	ジクロラミン(mg/L)	0.1	＜0.1	＜0.1	＜0.1			0.1
	トリクロラミン(mg/L)	＜0.015	＜0.015	＜0.015	＜0.015			＜0.015
	遊離塩素(mg/L)	1.2	＜0.1	＜0.1	＜0.1			＜0.1
現場簡易検査	モノクロラミン(mg/L)	0.35	3.6	3.2	3.5	4.0	3.3	3.7
	全塩素(mg/L)	1.5	3.0	4.0	4.0	4.0	4.0	4.0
	遊離塩素(mg/L)	1	0.1	0.1	＜0.1	＜0.1	＜0.1	＜0.1
	遊離アンモニア(mg/L)	ND	0.32	0.55	0.4	0.25	0.35	0.3