表 1. モノクロラミン 1 時間作用試験結果

シスト	実 Ct 値	シスト数(プレート当たり接種量)			
消毒条件		10	100	1000	
0mg/L 15 分	0	5 / 5	5 / 5	5 / 5	対照
0mg/L 15 分	0	4 / 5	5 / 5	5 / 5	
3mg/L 15 分	63	5 / 5	5 / 5	5 / 5	
3mg/L 15 分	47	4 / 5	5 / 5	5 / 5	
30mg/L 15 分	443	5 / 5	5 / 5	5 / 5	
30mg/L 15 分	445	0 / 5	0 / 5	0 / 5	
30mg/L 60 分	1806	0 / 5	0 / 5	0 / 5	Ct 値 1700 で 3-log 以上の不活化
30mg/L 60 分	1650	0 / 5	0 / 5	0 / 5	
栄養体	実 Ct 値	栄養体数(プレート当たり接種量)			
消毒条件		10	100	1000	
0mg/L 15 分	0	5 / 5	5 / 5	5 / 5	対照
3mg/L 15 分	47	0 / 5	0 / 5	0 / 5	Ct 値 45 で 3-log 以上の不活化

表 2. モノクロラミン 24 時間作用試験結果

	実 Ct 値	シスト数(プレート当たり接種量)			
消毒条件		10	100	1000	
0mg/L 0 分	0	3 / 3	3 / 3	3 / 3	対照
0mg/L 24 時間	0	3 / 3	3 / 3	3 / 3	
3mg/L 5 時間	810	3 / 3	3 / 3	3 / 3	
3mg/L 24 時間	3700	0 / 3	0 / 3	0 / 3	Ct 値 3700 で 3-log 以上の不活化

III. 研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Amemura-Maekawa J, Kikukawa K, Helbig J, Kaneko S, Suzuki-Hashimoto A, Furuhata K, Chang B, Murai M, Ichinose M, Ohnishi M, Kura F and the Working Group for *Legionella* in Japan	Distribution of monoclonal antibody subgroups and sequence-based types among *Legionella pneumophila* serogroup 1 isolates derived from cooling tower water, bath water and soil in Japan.	Applied and Environmental Microbiology		in press	
Taguri T, Oda Y., Sugiyama K., Nishikawa T, Endo T, Izumiyama S, Yamasaki M., and Kura F	A rapid detection method using flow cytometry to monitor the risk of legionellosis in bath water.	Journal of Microbiological Methods	86(1)	25-32	2011
西山明宏，石田直，興梠陽平，小西聡史，坪内和哉，伊賀知也，國政啓，岩破将博，福山一，仲川宏昭，伊藤明広，生方智，吉岡弘鎮，橘洋正，有田真知子，橋本徹，前川純子	*Legionella pneumophila* serogroup3による呼吸器感染症の4症例	感染症学雑誌	85(4)	373-379	2011

IV. 研究成果の刊行物・別刷

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A rapid detection method using flow cytometry to monitor the risk of *Legionella* in bath water

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ABSTRACT

Legionella species are the causative agents of human legionellosis, and bathing facilities have been identified as the sources of infection in several outbreaks in Japan. Researchers in Japan have recently reported evidence of significant associations between bacterial counts and the occurrence of *Legionella* in bathing facilities and in a hot tub model. A convenient and quantitative bacterial enumeration method is therefore required as an indicator of *Legionella* contamination or disinfection to replace existing methods such as time-consuming *Legionella* culture and expensive *Legionella*-DNA amplification. In this study, we developed a rapid detection method (RDM) to monitor the risk of *Legionella* using an automated microbial analyzing device based on flow cytometry techniques to measure the total number of bacteria in water samples within two minutes, by detecting typical patterns of scattered light and fluorescence. We first compared the results of our RDM with plate counting results for five filtered hot spring water samples spiked with three species of bacteria, including *Legionella*. Inactivation of these samples by chlorine was also assessed by the RDM, a live/dead bacterial fluorescence assay and plate counting. Using the RDM, the lower limit of quantitative bacterial counts in the spiked samples was determined as 3.0×10^3 (3.48 log) counts mL^{-1}. We then used a laboratory model of a hot tub and found that the RDM could monitor the growth curve of naturally occurring heterotrophic bacteria with 1 and 2 days' delayed growth of amoeba and *Legionella*, respectively, and could also determine the killing curve of these bacteria by chlorination. Finally, samples with ≥3.48 or <3.48 log total bacterial counts mL^{-1} were tested using the RDM from 149 different hot tubs, and were found to be significantly associated with the positive or negative detection of *Legionella* with 95% sensitivity and 84% specificity. These findings indicated that the RDM can be used for *Legionella* control at bathing facilities, especially those where the effectiveness of chlorine is reduced by the presence of Fe^{2+}, Mn^{2+}, NH_4^+, skin debris, and/or biofilms in the water.

1. Introduction

Legionellae are common bacteria in aquatic environments and inhabit moist soil along with protozoa and other bacterial communities (Fields et al., 2002; Rogers and Keevil, 1992; Rowbotham, 1980). *Legionella* species are the causative agents of human legionellosis and transmission occurs via aerosols containing *Legionella* cells. Several man-made devices are common sources of *Legionella* dissemination, including domestic hot water systems, cooling towers and fountains. Hot tubs have been identified as one of the most probable sources of *Legionella* infection in Japanese outbreaks (Kuroki et al., 2009; Nakamura et al., 2003; Okada et al., 2005), and for this reason the chlorination of bath water has been regulated. The local and national government guidelines indicate that the free residual chlorine concentration (FRC) of bathtub water should be maintained at 0.2 to 0.4 mg L^{-1} and at less than 1.0 mg L^{-1} (Ministry of Health, Labour and Welfare, 2003). Treatment with this level of chlorine has been shown to be critical for disinfection during *in vitro* experiments using tap water (Kuchta et al., 1983) and potable water (Phe et al., 2005). However, it is not always easy to maintain the effective chlorine concentration in bathing facilities

Abbreviations: cfu, colony forming units; FCM, flow cytometry; FL, fluorescence intensity; FRC, free residual chlorine concentration; FSC, forward scattered light; MTAB, myristyl trimethyl ammonium bromide; pfu, plaque forming units; RDM, rapid detection method.

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because some hot spring waters include components or pollutants that react with chlorine, including Fe^{2+}, Mn^{2+}, NH_4^+, skin debris and biofilms.

Flow cytometry (FCM) is a single cell analytical technology for measuring the quantity and fluorescence of microbial populations from fluid sources (Álvarez-Barrientos et al., 2000; Davey and Kell, 1996). These functions, along with high speed, high reproducibility, and the ability to carry out multi-parametric analyses, make FCM a very useful analytical tool in a range of scientific fields including research on cell viability, DNA quantification, cell cycle studies, and antibiotic susceptibility (Álvarez-Barrientos et al., 2000; Davey and Kell, 1996). Microbiologically, this method has been applied to the quality control of many industrial processes to detect microbial species, for example, the processing of drinking water, sewage, sea water, beverages, and foodstuffs, using the features of FCM for the continuous and quantitative analysis of microbial kinetics (Falcioni et al., 2006; Grégori et al., 2001; Hammes et al., 2008; Laplace-Builhé et al., 1993; Lebaron et al., 2001; Phe et al., 2004, 2005).

In samples from bath water, several Japanese researchers have indicated that there was a significant association between *Legionella* contamination and bacterial counts (heterotrophic or standard plate counts). For example, Inoue et al. (2007) reported that among using 433 bath water samples, *Legionella*-positive samples showed significantly increased heterotrophic plate counts (as analyzed using the Mann–Whitney *U* test). Meanwhile, Kura et al. (2008) demonstrated that heterotrophic plate counts of over 200 cfu mL^{-1} (colony forming units per milliliter) and standard plate counts of over 30 cfu mL^{-1} were significantly associated with the presence of *Legionella* by testing 112 bath water samples (and multiple logistic regression analysis). In addition, Ohata et al. (2006) demonstrated using the model hot tub system that the growth of naturally occurring *Legionella* was associated with the growth of heterotrophic organisms, as determined by plate counts, in unchlorinated water. Recently, three studies using hot water and/or water from a mud spring recreational area also revealed that heterotrophic plate counts were significantly associated with *Legionella* contamination (Edagawa et al., 2008; Hsu et al., 2009; Bargellini et al., 2011). By contrast, early studies demonstrated no correlation between *Legionella* contamination and plate counts in samples from cooling tower water (Yamamoto et al., 1992) and in eyewash stations (Paszko-Kolva et al., 1991). This discrepancy may depend on environmental temperatures.

We have previously proposed a rapid detection method (RDM) using fully automated microbial analyzing apparatus to monitor chlorine oxidation of bath water (Taguri et al., 2007). This system, which was based on FCM, could measure changes in total bacterial counts in a specific area of the scattergram produced by the instrument and estimate the extent of disinfection after chlorination. In this study, we applied the RDM to analyze bath water sanitation by monitoring bacterial counts.

The aims of our study were to use the RDM: 1) to determine the appropriate gating for total bacterial counts in bath water, with or without chlorine disinfection, sanitation of bathing facilities, 2) to confirm the association between the total bacterial count determined by RDM and the occurrence of *Legionella* in a model hot tub, and 3) to examine the practical application of this detection system. Achievement of these aims would allow for real-time monitoring of the risk of *Legionella* infection in bathing facilities, even in hot springs with chlorine inhibitors. Our RDM system could create a new criterion for the sanitation control of bath water and implementation of such methodology would reduce the risk of *Legionella* infection in hot tub facilities.

2. Materials and methods

2.1. Bath water samples for spike testing

Bath water from facilities using salt/bicarbonate spring, salt spring, simple acid spring, simple alkaline spring, and well water were sampled in polypropylene tubes from bathing facilities in Nagasaki, Japan, in May, 2009, and the samples were characterized within 24 h (Table 1). The remainder of each sample was preserved at 4 °C and filtered through a 0.2-μm pore size cellulose acetate membrane to eliminate some microorganisms within 1 month. Before spike testing, we confirmed the chemical value of each parameter. The bath waters used for sampling had usually been treated with chlorine, so the water samples for culturing were neutralized by the addition of sodium thiosulfate at the sampling time. Heterotrophic bacterial counts were measured by culturing on R2A agar medium (Becton Dickinson, Sparks, MD, USA) following the method of Inoue et al. (2007). Briefly, after diluting to an appropriate concentration, 0.1 mL samples were inoculated onto R2A agar medium and incubated at 42 °C for 7 days. Results were expressed as colony forming units (cfu) per milliliter. The isolation of *Legionella* was performed according to the "Manual of the countermeasure against legionellosis" (Anonymous, 1999). Briefly, samples were concentrated by filtration through a 0.45-μm pore size polycarbonate membrane filter (HTTP04700; Millipore, Ireland). The membrane was placed into distilled water in a screw-cap sterile container and shaken vigorously, and each suspension was heated at 50 °C for 20 min, inoculated onto glycine vancomycin polymyxin cycloheximide agar (bioMérieux, France) and incubated at 37 °C for 7 to 10 days. *Legionella*-like colonies were tested for their requirement for L-cysteine, and identification was confirmed using 10 kinds of species-specific or serogroup-specific monovalent antisera (against *Legionella pneumophila* serogroups 1 to 6, *L. bozemanii* serogroup 1, *L. dumoffii*, *L. gormanii* serogroup 1, and *L. micdadei*; Denka Seiken, Tokyo, Japan). Some serologically unidentified isolates were confirmed by polymerase chain reaction with specific

Table 1
Physicochemical and microbiological characteristics of hot spring source water.

	Salt/bicarbonate spring	Salt spring	Simple acid spring[a]	Simple alkaline spring[a]	Well water
pH[b]	7.4	7.0	2.6	8.0	7.0
Cation contents (mg L^{-1})[c]					
NH_4^+ (as NH_4–N)	1	10	20	0.5	0.2
Mn^{2+}	<0.5	1	<0.5	<0.5	<0.5
Fe^{2+}	<0.2	0.2	10	<0.2	<0.2
Free residual chlorine (mg Cl_2 L^{-1})[d]	<0.05	<0.05	<0.05	<0.05	<0.05
Heterotrophic plate counts (log cfu mL^{-1})[e]	<2.30	<2.30	<2.30	4.13	5.18
Legionella counts (cfu 100 mL^{-1})[f]	<10	<10	<10	<10	<10
Disinfection of bath water	Yes[g]	Yes	Yes	Yes	Yes

[a] Simple spring was defined as the spring containing under 1000 mg kg^{-1} dissolved substances.
[b] This was tested by colorimetry.
[c] These were tested by using a colorimetric reagent kit (Pack Test, KYORITSU CHEMICAL CHECK Lab., Corp., Japan).
[d] This was tested by using *N,N*-dimethyl-*p*-phenylenediamine method (DPD; Hack, Loveland, CO, USA).
[e] This was counted by plating on the R2A plates. cfu, colony forming unit.
[f] This was determined by using 100 times filter concentrating method on the GVPC plates.
[g] Disinfection of bath water was practiced by chlorine in these facilities.

primers for the *Legionella* 5S rRNA gene (Cycleave PCR Legionella Detection Kit, Takara Bio, Shiga, Japan).

2.2. Bacterial strains, media, and growth conditions

Bacterial strains used in this study are shown in Table 2. *L. pneumophila* (American Type Culture Collection, ATCC, 33152) was purchased as Culti-Loops from AMCO (Tokyo, Japan). The strain preserved at −80 °C was restored on buffered charcoal yeast extract (bioMérieux) agar incubated at 37 °C for 3 days. It was then passaged onto new agar and incubated at 37 °C overnight prior to testing. *Escherichia coli* strain IFO3972 (ATCC 8739) and *Staphylococcus aureus* strain IFO12732 (ATCC 6538P) were purchased from the Institute for Fermentation Osaka (Osaka, Japan), and cultivated using nutrient agar (Eiken Chemical, Tokyo, Japan) at 37 °C overnight. *Rhizobium radiobacter* strain JCM 20371 (ATCC 19358) was purchased from a commercial bank (RIKEN BioResource Center, Saitama, Japan) and incubated on trypticase soy agar (Becton Dickinson) at 25 °C overnight. All strains were adjusted to approximately 10^8 cells mL^{-1} in autoclaved 0.85% saline, and appropriately diluted samples were analyzed by the RDM.

2.3. Rapid detection method based on flow cytometry techniques

The RDM was performed using a microbial analyzing device, BACTANA, which was manufactured based on FCM techniques to enumerate bacteria and was provided by Sysmex Corporation (http://www.sysmex.co.jp/). The flow cell was constructed out of fused silica, and the chassis and the flow channel were cubed and spherical in appearance, respectively. The sheath and sample flow was of the syringe-type. The light source was equipped with a semiconductor laser, and the excitation and fluorescence wavelengths were 633 nm and 660 nm, respectively. The optical detector consisted of a photodiode and a photomultiplier tube, detecting forward scattered light (FSC) and fluorescence intensity (FL), respectively. A highpass filter with a threshold of 650 nm was used in the FL detector (Table 3).

Compensation of the RDM was carried out using proofreading solution (Bactcheck, Sysmex) containing latex particles. The FSC variation was constantly adjusted in gain inputs of the photodiode, and the FL variation was uniformly regulated in voltage inputs of the photomultiplier. A new material, methine dye (BactQuick dye, BactQuick™ kit composed of a dye and a diluent), was used as a fluorescence reagent for the RDM for analyzing nucleic acids, including DNA and RNA (Akai et al., 1995).

All of the reagents and instruments for the RDM, including the dye, diluent (in the BactQuick™ kit), sheath fluid (BQS-900A), and test tubes (SU-40), were supplied by Sysmex Corporation. The diluent contained 0.1% myristyl trimethyl ammonium bromide (MTAB) for effective staining of microbes and was optimized at pH 2.5 for measuring bacteria in clinical samples such as urine. However, in this study, it was used at pH 7.0 for calculating bacteria in environmental samples such as alkaline springs. The protocol for the RDM was performed as previously described (Oyane et al., 2009). Where necessary, samples were filtered through a filter bag (Stomafilter; GSI Creos, Tokyo, Japan) to eliminate the spring scales. About 200 μL of each of the water samples was applied to the test tubes and these were assigned into the appropriate sample table (using five samples at once) in the apparatus. After the measurements had commenced, 340 μL of the diluent and 50 μL of each water sample was first absorbed into another test tube. Then, after the addition of 10 μL of the fluorescence reagent, the suspension was stirred and stained at 40 °C for 20 s. Finally, after the suspension was applied into a charging line, 48 μL of the suspension was fixed and injected into the flow cell with sheath fluid using a syringe and was measured by the detector. All of the processes were performed automatically, taking approximately 2 min.

Table 2
Summary of bacterial strains used in the study[a].

Species	Strain	Phenotype	Alternate strain name or source
Legionella pneumophila	Philadelphia-1	Serogroup 1	ATCC 33152
Escherichia coli	IFO 3972		ATCC 8739
Staphylococcus aureus	IFO 12732		ATCC 6538P
Rhizobium radiobacter	JCM 20371		ATCC 19358

[a] ATCC, American Type Culture Collection.; IFO, Institute of Fermentation Osaka; and JCM, Japan Collection of Microorganism.

Table 3
Optical summary in flow cytometry.

Parameter	Component	Optical path	Detector	Signal
FSC_H	N/A	N/A	Diode	Height
FL	HP 650	passed	PMT	Height

Abbreviations: FSC_H: forward scattered light_high voltage, N/A: not applicable, FL: fluorescence intensity, HP 650: highpass filter, and PMT: photomultiplier.

These data were downloaded and analyzed by BACTAnalyzer (Sysmex) as soon as each measurement had been taken. According to the manufacturer's instructions (Sysmex Co., 2006), values of the two parameters, FSC and FL, were obtained from flowing bacterial cells and were presented as a scatter plot.

2.4. Gating of the FCM dot plot for evaluation of the effect of chlorination on bath water

The counting gate for FCM was designed according to the manufacturer's instructions (Sysmex Co., 2006). Conventional testing could not correctly detect bacterial cells by FL and FSC because of low sensitivity along with electrical noise and/or waste particles in the samples, such as spa water scales. The manufacturer adopted an original detection method developed by Inoue et al. (2000) for the counting of bacterial cells. They used a surfactant, 0.1% MTAB, to clearly observe events close to the origin, including cryptic bacteria, in the scattergrams (Fig. 1a and b). As the calculation technique was optimized for enumerating well-grown bacterial species under eutrophic conditions over a short time period, such as the bacteria found in urine, environmental oligotrophic bacteria were not fully recovered by applying this method.

In this study, the change in FL and/or FSC of bacterial cells after incubation with or without disinfection by chlorine was investigated by RDM. Three log cfu mL^{-1} of *E. coli*, *S. aureus*, and *R. radiobacter* suspensions were prepared in autoclaved R2A broth medium (0.5 g yeast extract (Difco, Detroit, MI, USA), 0.5 g proteose peptone No. 3 (Difco), 0.5 g casamino acids (Difco), 0.5 g glucose, 0.5 g soluble starch, 0.3 g sodium pyruvate, 0.3 g K_2HPO_4, and 0.05 g $MgSO_4 \cdot 7H_2O$, made up to 1.0 L in distilled water, and adjusted to pH 7.2), and incubated at 37 °C (30 °C for *R. radiobacter*) for 24 h. The suspensions were partially sampled at 0, 2, 4, 6, 22, and 24 h, and detected by RDM. In the RDM, along with 0.1% MTAB, the bacterial cells of each sample were observed predominantly in the gated area in the scattergrams (Fig. 1b).

For chlorination, the sampled bacterial suspensions were washed twice with phosphate-buffered saline (PBS, pH 7.2; Sigma, St. Louis, MO, USA) and starved at 37 °C for 24 h (Saby et al., 1997, with slight modifications). The cells were disinfected with commercial sodium hypochlorite (Wako Pure Chemical Industries, Saitama, Japan) at 20 mg L^{-1} for 120 min. The cell scattering and fluorescence events observed before chlorination (Fig. 1b), clearly disappeared, particularly in the gated area, after full chlorination (Fig. 1c), irrespective of the noise in the system (Fig. 1d) and the source of the spa water. The gate was set between 26 and 224 channels to avoid overlapping with several sources of noise, i.e., machinery noise and/or noise from spring scales in the FL 0–25 channels, and the noise from larger particles and

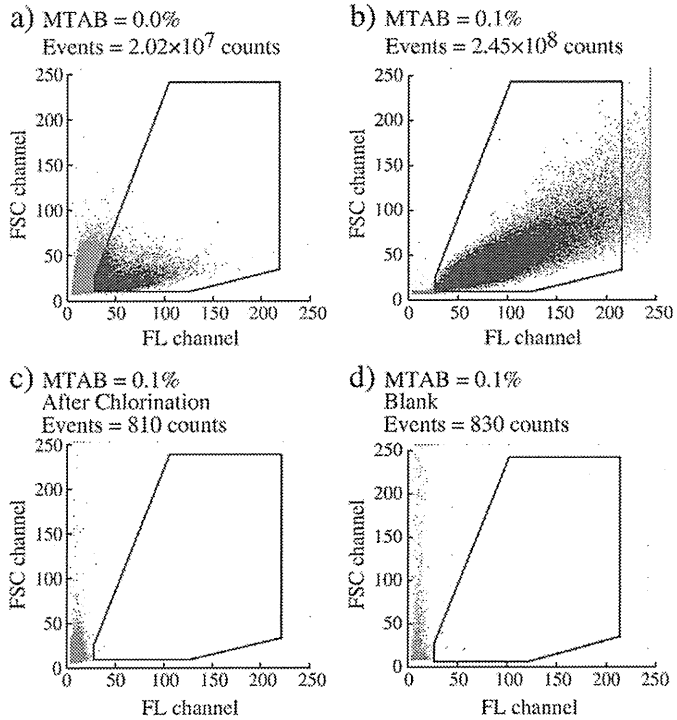

Fig. 1. Fluorescence light (FL) vs. forward scattered light (FSC) in the rapid detection method system, showing specific gating clusters for treatment with (a) 0.0% and (b) 0.1% myristyl trimethyl ammonium bromide (MTAB) at pH 7.0, for disinfection with (c) 20 mg L^{-1} of the free residual chlorine (FRC) for 120 min followed by 0.1% MTAB treatment, and for (d) PBS with 0.1% MTAB as a blank. A representative event count in each specific gating area is presented.

agglutinated cells in the FL 225–250 channels. In this scattergram, the counting area was defined as a specific area (solid line), and cell counts in this area were expressed as total bacterial counts per milliliter.

2.5. Enumeration of bacterial cells using the RDM

To evaluate the effectiveness of our RDM, first we selected simple alkaline spring and well water samples, in which autochthonous heterotrophic bacteria were abundant. Each water filtrate was used to dilute autochthonous bacteria as a positive control. Following the method of Hammes et al. (2008), the bacterial counts in samples diluted with each water filtrate (0.2-μm pore size mixed cellulose ester membrane; Millipore) were determined by plate counting and the RDM. Each dilution was carried out in triplicate and a total of nine dilutions ranging from 0.1% to 100% were made (N = 27 in total). Next, to estimate the influence of different bacterial species and the source of spa water on this method, five independent sources of water were tested and PBS was included as a control (see Table 1). These water samples were filtered through a 0.22-μm pore size polyethersulfone membrane filter (Millipore) three times. Each standard strain (described above) was added to each filtrate, and the resulting suspensions of approximately five log counts mL^{-1} were measured by the RDM. Spiked samples diluted to 10, 5, 3, 2, 1, and 0.5% and each original suspension were also measured by the RDM and plate counting, and triplicate counts of each suspension were compared.

2.6. Chlorine efficacy on spring source water spiked with bacteria

The influence of bacterial species and the source of spa water were investigated in the water samples spiked with five log counts of bacteria mL^{-1}, prepared as described above, by the RDM, fluorescence microscopy, and plate counting. Sodium hypochlorite was added to adjust each sample to a 20 mg L^{-1} final concentration. If necessary, the reagent was added to maintain this concentration for 120 min. The bacteria in each suspension were counted by these three methods pre- and post-chlorination. The RDM was performed as described above. For fluorescence microscopy, one milliliter of suspension was filtered through a 0.2-μm pore size polycarbonate membrane filter (K020N025A; Advantec Toyo, Tokyo, Japan). The membrane was then stained using the BacLight LIVE/DEAD bacterial viability kit (Molecular Probes, Leiden, The Netherlands), according to the manufacturer's instructions, and 3 μL of a mixture of dyes was added to each membrane. Each sample was incubated at room temperature for 15 min, and direct bacterial counts on each membrane were detected using epifluorescence microscopy, ECLYPSE E800 (Nikon, Tokyo, Japan). For plate counting, each sample was neutralized in 0.05% sodium thiosulfate (Wako Pure Chemical Industries), and pre- and post-chlorinated samples were counted after 10-fold dilution and 100× concentration by filtration, respectively.

2.7. Collection of water samples from the model hot tub system

To confirm the relationship between bacterial counts and the occurrence of *Legionella*, duplicate experiments using the model hot tub system (bathtub size = 2 m^3) were carried out, following the method of Ohata et al. (2006). Immediately before each experiment, the following three steps were carried out: 1) hyper-chlorination of bath water to a final concentration of 50 mg L^{-1} sodium hypochlorite for 3 h; 2) high-pressure washing of bathtubs; and 3) rinsing of bathtubs in three changes of bath water.

After the first experiment had commenced, organic matter was accumulated by bathing 16 volunteers and the chlorine concentration was automatically adjusted to 0.2 to 0.4 mg L^{-1} up to day 4. Chlorination was stopped from day 4 to day 10. After restarting chlorination, the chlorine concentration of the bathtub water was maintained at 0.2 to 0.4 mg L^{-1} until the end of the experiment. In the second experiment, 10 volunteers were used and disinfection was halted between day 6 and day 12. Twenty-one and 24 samples in total in the first and second experiments, respectively, were taken from the bathtub and filter tank. After measuring the FRC, all of these samples were neutralized and measured by RDM and heterotrophic plate counting, then 14 samples from the first experiment and 13 samples from the second experiment were subject to *Legionella* plate counting and amoeba plate counting. The RDM, plate counting, and FRC measurements were performed as described above.

Amoeba plate counting was performed in accordance with the method of De Jonckheere et al. (1991). Briefly, one milliliter of the original sample and 50× concentrated samples, obtained by centrifugation at 1000 *g* for 5 min, were inoculated onto amoeba culture medium and the medium was incubated at 30 °C for two weeks. After fledged amoebae were identified morphologically, amoeba plate counts were determined by enumerating plaque forming units (pfu) per 50 mL. The amoeba medium was made from 1.5% non-nutrient agar (Bacto-Agar, Becton Dickinson) and heat-treated *E. coli* strain DH1. After autoclaving, approximately 20 mL of each of the media were poured into sterile Petri dishes (90 mm × 15 mm; Eiken Chemical). The thickness of the agar plates was roughly 3.0–5.0 mm. About 9–10 log bacterial counts mL^{-1} of the strain were suspended in distilled water and heated at 60 °C for 1 h, and then about 0.5 mL of the suspension was spread onto the agar plates. After drying, the plates were used for amoeba plate counting.

2.8. Collection of water samples from public spas

One hundred forty-nine bathtub water samples (from 24 salt springs, 26 bicarbonate springs, 10 simple springs, and six acid springs; 22 well waters and 61 tap waters) from different hot tub systems in 66 public bath facilities, sampled between April 2007 and March 2009 in Nagasaki, Japan, were used in this study. After measuring the FRC, each

sample was collected in a polypropylene sample tube with 0.05% sodium thiosulfate to neutralize the chlorine. Total bacterial counts, heterotrophic plate counts, and the *Legionella* plate counts were measured as described above. We defined total bacterial counts and heterotrophic plate counts as the bacterial counts from FCM and the bacterial colony counts on R2A agar incubated at 42 °C for 7 days, respectively. The former indicated live or dead (morphologically preserved) cells, while the latter included only culturable cells under bath water conditions.

2.9. Statistical analyses

All experiments were carried out at least in duplicate. The significance of the results was analyzed using the student's t test and the χ square test. The Statistical Package for the Social Sciences (SPSS Japan Inc., version 13.0 J, Tokyo, Japan) was used for the analyses.

3. Results

3.1. Inhibitors of chlorine in hot springs

Salt spring and simple acid spring water contained 10 mg L^{-1} of NH_4^+ and 1 mg L^{-1} of Mn^{2+}, and 20 mg L^{-1} of NH_4^+ and 10 mg L^{-1} of Fe^{2+}, respectively. The inhibition of chlorine activity was observed in these water samples, in which concentrations of chlorine were detected to be null even "under chlorination" (Table 1).

3.2. Enumeration of bacterial cells by the RDM

Both simple alkaline spring and well water samples showed a large number of autochthonous heterotrophic bacteria (Table 1) and a high linearity between the RDM results and the plate counting results ($R^2 = 0.967$ and 0.993, respectively). Coefficients of variation (CV) over 4.0 log counts mL^{-1} were consistently below 5% of the mean values. However, lower bacterial counts per mL^{-1} were accompanied by higher CV: approximately 10% at 3.00 to 3.70 log counts mL^{-1} and over 50% at 2.70 log counts mL^{-1}. Therefore, in this study, the ratio of 10% was employed as the standard of variation. The lowest mean values, each CV of which was ≤10% of the mean, were observed in simple alkaline spring and well water samples, where values of 3.38 log counts mL^{-1} and 3.46 log counts mL^{-1}, respectively, were recorded.

In all spiked samples, except for simple acid spring water, standard curves also showed a high linearity (Fig. 2, $R^2 = 0.989$ to 0.999) and no difference from autochthonous bacterial samples. In the spiked samples of the simple acid spring, bacteria were detected by the RDM, but were not detected by plate counting (data not shown). In the spiked samples with *L. pneumophila*, the lowest mean values, each CV of which was ≤10% of the mean, were observed in the salt/bicarbonate spring, salt spring, simple alkaline spring and well water samples, and PBS, where values of 3.32, 3.37, 3.32, 3.40, and 3.35 log counts mL^{-1}, respectively, were recorded (Fig. 2). In the spiked samples with *E. coli*, the lowest mean values for the above samples were 3.44, 3.48, 3.37, 3.43, and 3.36 log counts mL^{-1}, respectively, and in the spiked samples with *R. radiobacter*, the values were 3.35, 3.40, 3.32, 3.35, and 3.32 log counts mL^{-1}, respectively (Fig. 2).

3.3. Bacterial changes pre- and post-chlorination in hot spring waters

After sufficient chlorine treatment, the bacteria observed in a specific area of the scattergram pre-chlorination (Fig. 1b) disappeared, as shown in Fig. 1c. To overcome any inhibitory effects present in the hot spring water sample, excess chlorination of 20 mg L^{-1} for 120 min was performed. After this procedure, post-chlorinated spiked samples in salt/bicarbonate spring, salt spring, simple acid spring, simple alkaline spring and well waters, and PBS, were measured by the RDM. In samples containing *L. pneumophila*, total bacterial counts were 3.18, 3.26, 2.40, 2.77, 2.40, and 2.51 log counts mL^{-1}, respectively. In samples containing *E. coli*, total bacterial counts were 3.36, 2.82, 3.11, 2.89, 3.14, and 2.59 log counts mL^{-1}, respectively. In samples containing *R. radiobacter*, total bacterial counts were 3.43, 2.89, 3.22, 3.18, 2.98, and 3.11 log counts mL^{-1}, respectively. Taking into account these results and the effective measurement limits mentioned above, the threshold of the RDM as an indicator of chlorine activity was set at 3.48 log (3.0×10^3) counts mL^{-1}. In fluorescence microscopy, almost no live cells exhibiting green fluorescence were observed, but dead cells exhibiting red fluorescence were observed with fragmentation and cell degeneration. Some cells displaying discoloration were also observed. In the *Legionella* plate counting experiments, even using 100× concentrated samples, no bacteria were cultured (<10^2 cfu L^{-1}).

3.4. Transition of microorganisms in the water samples from the model hot tub system

We carried out two independent experiments using the model hot tub system. Heterotrophic bacteria were grown naturally for 17 h after chlorination was terminated, and amoeba and *Legionella* were also grown naturally for 1 and 2 days, respectively, after the heterotrophic bacterial growth. In this second experiment, 3.18 log cfu mL^{-1} heterotrophic bacteria were detected 17 h after chlorination was terminated, and the enumerated counts at 21, 24, and 25 h were 4.24, 5.46, and 6.18 log cfu mL^{-1}, respectively. Amoeba were first identified at 43 h following the termination of chlorination (0.75 log pfu 50 mL^{-1}), with amoeba counts of 3.14, 1.74, and 1.30 log pfu 50 mL^{-1}, at 67, 93, and 133 h, respectively. *Legionella* was first detected at 1.52 log cfu 100 mL^{-1} 67 h after chlorination ceased, with *Legionella* colony counts of 3.90 and 4.80 log cfu 100 mL^{-1}, at 93 and 133 h, respectively. The time courses of growth of these microbes were reproduced in both the bathtub and

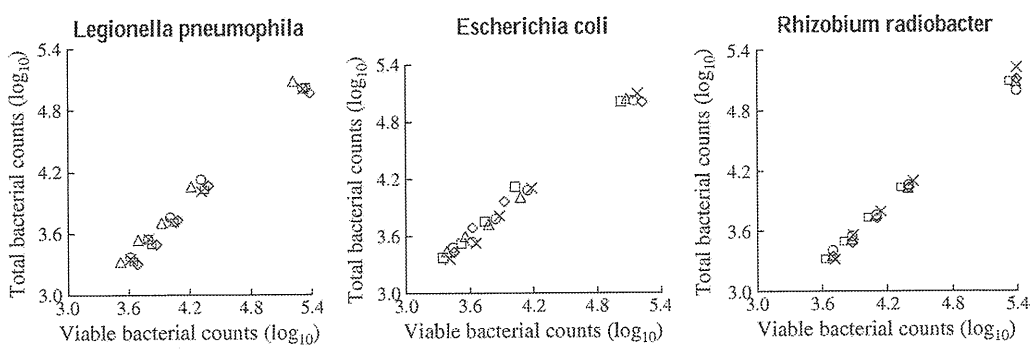

Fig. 2. The limit of determination of the total bacterial counts in the spiked samples prepared from different spring water sources. Each sample was adjusted to approximately 5.00, 4.00, 3.70, 3.48, and 3.30 log bacterial counts per milliliter by the rapid detection method, and compared with each of the culturable cell counts.

filter samples in our two experiments. The bacterial counts measured by the RDM were similar to the heterotrophic bacterial cfu values measured from the growth and killing curves, despite a few differences over the time course of growth, e.g., 3 h after chlorine treatment was restarted the RDM counts were still more than 3.48 log counts mL^{-1}, whereas heterotrophic bacteria were unculturable. These results were highly reproducible in our two experiments.

However, among the 54 samples employed in the *Legionella* test, bacterial counts by the RDM in 18 of the samples with culturable *Legionella* were over 3.48 log counts mL^{-1} and in 31 of the samples lacking culturable *Legionella* they were below that concentration. Of the remaining samples, one was taken 4 h after stopping chlorination and four were taken 3 h after restarting chlorination. The transition of FRC differed between the two experiments. The FRC was properly stabilized after disinfection in the first experiment, but fluctuated and disappeared in the second experiment due to chloramine generation (data not shown).

3.5. Comparison of RDM counts with FRC as an indicator of Legionella contamination in bathing facilities

Using 149 different water samples from hot tubs, a histogram of RDM counts with or without culturable *Legionella* was generated and is shown in Fig. 3a. Among 93 samples showing RDM counts below 3.48 log counts mL^{-1}, most did not contain culturable *Legionella* (negative predictive value: 98%; Fig. 3a). By contrast, among the remaining 56 samples showing RDM counts over 3.48 log counts mL^{-1}, 39 contained culturable *Legionella* (positive predictive value: 70%; Fig. 3a). When these results were summarized according to binary classification, the threshold value of bacterial counts by RDM (3.48 log counts mL^{-1} in this study, indicated by an arrow on Fig. 3a) clearly discriminated the "positive" samples from the "negative" samples for culturable *Legionella*. Furthermore, the sensitivity and specificity of RDM for the detection of culturable *Legionella* were 95% and 84%, respectively (inset of Fig. 3a), which were comparatively high values.

In a histogram of FRC formed in the same manner as that described above, even if the threshold was of a lower or higher standard chlorine concentration (0.2 or 0.4 mg L^{-1}, respectively), some samples with more than 0.2 or 0.4 mg L^{-1} chlorine included culturable *Legionella* (Fig. 3b). When the classification of 0.2 mg L^{-1} of FRC, the lowest concentration recommended by the government guidelines, was compared with the positivity of culturable *Legionella*, the negative predictive value and positive predictive value of FRC equated to 80% and 57%, respectively, and the sensitivity and specificity were 42% and 88%, respectively (inset of Fig. 3b).

4. Discussion

FCM is an established method in the field of microbiology conferring the advantages of high speed, high reproducibility and multi-parametric analytical potency. FCM has been successfully applied to process administration as a method for microbial enumeration in a range of industries including the processing of drinking water, sea water, waste water, beverages, and foodstuffs (Falcioni et al., 2006; Grégori et al., 2001; Hammes et al., 2008; Laplace-Builhé et al., 1993; Lebaron et al., 2001; Phe et al., 2004, 2005). Although FCM has many potential utilities, its application fields remain to be restricted because of the high cost of instrumentation and the need for well-trained operators (Álvarez-Barrientos et al., 2000; Davey and Kell, 1996). The use of the fully automated microbial analyzer in this study allows for accurate detection at low cost, by employing a simple analyzing method consisting of a single laser, a quantitative pump assembly, and original fluorochrome and surfactant, in addition to an automatic measuring process (Inoue et al., 2000; Oyane et al., 2009). Therefore, it was important that the analytical potential of the instrument used in this study was thoroughly verified.

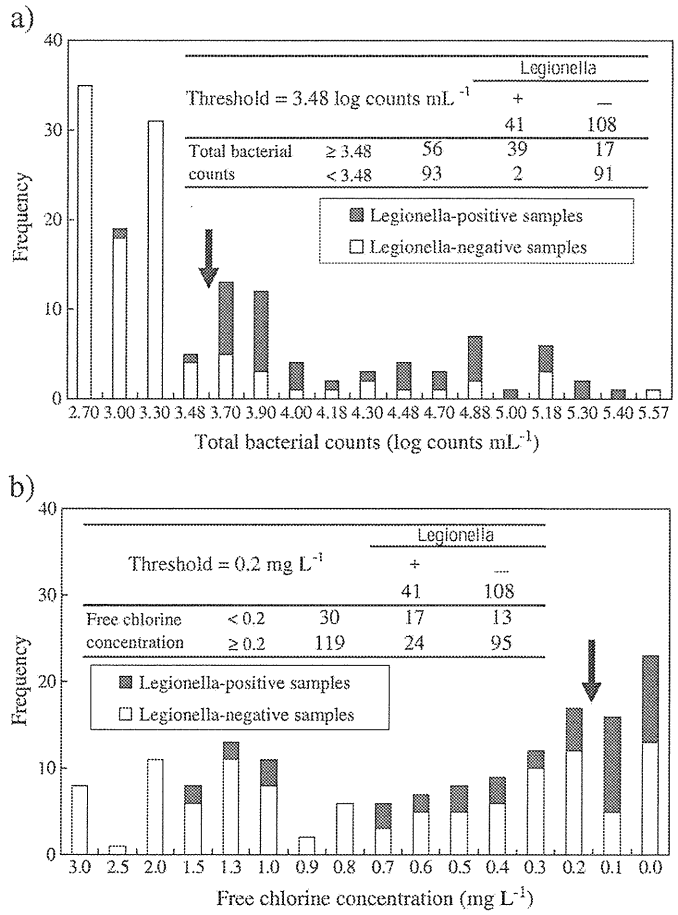

Fig. 3. Comparison of the frequency of distribution of water samples positive or negative for culturable *Legionella*. (a) Bacterial counts were measured by the rapid detection method, and (b) the free residual chlorine concentration was measured by the *N,N*-diethyl-*p*-phenylenediamine method. The arrow indicates the threshold value of each test method.

The CV of bacterial counts in this study were found to be in the range of around 10% of the mean using autochthonous spring source waters and various spiked samples, as the CV of samples at high concentration (over 4.00 log counts mL^{-1}) and at low concentration (3.00 to 3.70 log counts mL^{-1}) were consistently below 5% and around 10%, respectively, of the mean. A shorter acquisition time of 2 min might account for the variation over 50% of the mean at a lower concentration like 2.70 log counts mL^{-1}. However, the discrimination in the efficacy of chlorine disinfection was within a practical level (Fig. 3). In a previous study, a flow cytometer with equivalent potential (standard error, 10% of the mean; maximum acquisition time, 5 min) revealed a good correlation between bacterial counts from culturing and FCM in the range of 10^2 to 10^7 cfu mL^{-1} (Pinder et al., 1990). When optimized, our RDM system appeared to be comparable, with regard to bacterial enumeration, with previously reported FCM systems.

In spiked samples (Fig. 2), including five spring source water samples and the PBS control each containing one of three kinds of bacteria, there were no significant differences in the RDM values, with CV values of less than 10% of the mean observed irrespective of the bacterial species or spring water source ($p<0.05$). On the contrary, when we measured the spiked samples under post-chlorination conditions, the maximum value of chlorinated samples was 3.43 log counts mL^{-1} for *R. radiobacter* at 10^5 cfu mL^{-1} inoculated into salt/bicarbonate spring water. Although there were a few variations, all values from the post-chlorination samples were below 3.48 log counts, irrespective of the bacterial species or the spring water source. In addition, the histogram of RDM was apparently divided into two parts

based on the existence of culturable *Legionella* at a threshold line of 3.48 log counts mL^{-1} in the field study (Fig. 3). The high sensitivity and specificity of RDM for the detection of culturable *Legionella* from 149 different samples strongly suggested that a threshold existed for chlorine oxidation efficacy in RDM, irrespective of the bacterial species or the source of spring water, and the threshold corresponded to 3.48 log counts mL^{-1} (inset of Fig. 3).

Legionella was known to be sensitive to 0.2 to 0.4 mg L^{-1} of chlorine for 2 h according to the regal criterion (Ministry of Health Labour and Welfare, 2003). However, it appeared that *Legionella* could be recovered from water samples with regulative FRC. The reliability of the *N,N*-diethyl-*p*-phenylenediamine method is therefore probably overestimated in such situations. If the threshold of chlorination was higher, it could be improved in an administration setting, but it appears that not only the true positive but also the false positive rates might be increased. On the other hand, because an appropriate criterion has been set for RDM during chlorine activity, as described above, decisions based on these criteria would control not only heterotrophic bacteria but also *Legionella*.

Ohata et al. (2006) reported that *Legionella* could replicate in naturally-contaminated experimental hot tub water after the growth of heterotrophic bacteria and amoeba. In this hot tub model, these microbes were reproducibly grown in the same order, and by using the RDM we could perform real-time monitoring of heterotrophic bacteria. Therefore, a new evaluation method, bacterial counting by the RDM, could detect the risk of *Legionella* contamination quickly. Hot spring water sometimes contains natural inhibitors for chlorine disinfection and in these cases, free chlorine would probably be exhausted. In experiment two in this study, under the reduction of free chlorine, chloramine was expected to be generated immediately after re-chlorination even when tap water was used for bathing (data not shown). Throughout the experiment, a low concentration of chlorine (0.2 to 0.4 mg L^{-1}) could control not only heterotrophic bacteria but also amoeba and *Legionella*, if the appropriate prompt action was taken. This might also indicate that these protective procedures against *Legionella* could also control biofilm formation.

Our preliminary evaluation of the RDM on the disinfection of bath water suggested that the pattern of disinfection or cleanliness (Fig. 1c) determined by the RDM was closely related with breaking the chlorine demand, which is defined as the quantity of chlorine reduced or converted to inert or less active forms of chlorine by substances in the water, which, in the case of spa water, is unrelated to the bacterial species or the spring source. Further studies on our RDM system for oxidizing efficacy against bacteria are currently in progress.

Our system represents a novel screening technique to monitor the risk of *Legionella*, with potentially important practical applications. The price of a measuring instrument is 4,200,000 Japanese yen (JPY) and the estimated cost of our RDM is approximately 110 JPY per test. The maximum additional cost for maintenance and quality control for the machine is estimated to be approximately 171,000 JPY per year. It should also be noted that this method is for screening only, *Legionella* detecting methods are still necessary to identify the bacteria.

5. Conclusions

- We obtained data for the application of flow cytometric techniques to control the sanitation of public bathing facilities by estimating biocide efficacy, especially for chlorination.
- We determined a constant specific gate for total bacterial counting without any substantial noise. The results suggested that total bacterial counts in the RDM were a good indicator not only of the bacterial community present but also of the risk of *Legionella* contamination, which reflects the outcome of complicated ecology.
- We also demonstrated that RDM could enumerate bacterial species rapidly, easily, and reproducibly, irrespective of the source of spring water or the bacterial species.
- Our RDM system was able to monitor changes during bacterial disinfection because of a short acquisition time of 2 min, indicating its potential application to sanitation control in public bathing facilities.
- The effect of chlorine was clearly monitored by our RDM system. Further laboratory and field research with this system using various other disinfectants would increase confidence in and acceptance of this system in the sanitation of bathing facilities, and this will form part of our future work.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.mimet.2011.03.012.

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症　例

Legionella pneumophila serogroup 3による呼吸器感染症の4症例

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西山　明宏[1]　石田　直[1]　興梠　陽平[1]　小西　聡史[1]
坪内　和哉[1]　伊賀　知也[1]　國政　啓[1]　岩破　将博[1]
福山　一[1]　仲川　宏昭[1]　伊藤　明広[1]　生方　智[1]
吉岡　弘鎮[1]　橘　洋正[1]　有田真知子[1]　橋本　徹[1]
前川　純子[2]

（平成22年7月5日受付）
（平成23年5月6日受理）

Key words: *Legionella pneumophila*, serogroup 3, respiratory infection

序　文

抗菌薬治療の発達にも関わらず，市中肺炎はいまなお高い罹患率と致命率を呈する重要な疾患である．なかでもレジオネラ属菌による肺炎は，重症化しやすく致命率の高い肺炎として知られている．欧米に比して，本邦でのレジオネラ症の頻度は低いとされてきたが，近年その報告数が増加してきている．その背景として2003年にレジオネラ尿中抗原迅速診断キットが保険適応となったこと，および感染症法の改訂によりレジオネラ症が感染症法4類の全数把握疾患に指定されたことが考えられる．

報告数が増加するにつれて，軽症と考えられるレジオネラ症例もしばしば認められるようになってきたが，その実態についてはいまだよく知られていない．また，レジオネラ症の多くは *Legionella pneumophila* serogroup 1によるものであり，その他の血清群および菌種による報告は比較的少ない．また，肺炎以外のレジオネラ属菌による呼吸器感染症についての報告も稀である．

今回，我々は気道内定着の可能性も否定できないが，肺炎をはじめとする *Legionella pneumophila* serogroup 3（以下　SG3）が検出された呼吸器感染症3例に加え，院内発症の1症例を経験したので，文献的考察を加え報告する．

症　例

症例1

患者：66歳・男性．

主訴：湿性咳嗽．

現病歴：2008年6月中旬から，2週間続く湿性咳嗽と鼻汁を認め，近医を受診し，感冒薬を処方されるも軽快なく当院を受診した．発熱はなく，咳嗽と粘度の低い喀痰を認めるのみであった．24時間風呂や銭湯の使用，温泉への旅行歴はなく，周囲に同症状の患者も見られなかった．

既往歴：肺結核（22歳時），高血圧，糖尿病（経口血糖降下薬内服），アルコール性肝障害．

嗜好歴：喫煙歴なし，20歳から60歳まで缶ビール2缶（1缶350mL）/日．

アレルギー歴：薬剤アレルギーなし，食物アレルギーなし．

初診時身体所見：血圧124/60mmHg，体温36.7℃，脈拍68/分，呼吸回数18回/分，意識清明，胸部聴診上異常を認めず，その他に有意な所見なし

初診時検査所見：白血球6,900/μL，CRP 22.0mg/dL，LDH 204IU/L，Na 136mEq/L，胸部単純レントゲン（Fig. 1）では右中肺野及び左下肺野にすりガラス様陰影を認めた．胸部CT（Fig. 2）では両肺野に非区域性に網状影をともなうすりガラス影が多発しており，所謂crazy-paving-appearanceであった．．

提出された喀痰は粘膿性で，Gram染色ではGram陰性小桿菌と口腔内常在菌を認めた．

治療経過：日本呼吸器学会市中肺炎ガイドライン[1]による肺炎の重症度は軽症であった．非定型肺炎として妥当な画像であり，アジスロマイシン（以下AZM）500mg/日を3日間内服した．治療開始4日目で咳嗽，倦怠感，食欲は改善し，治療開始15日目に胸部レントゲン上での陰影と症状の消失を認めた．初診時の喀痰培養にて *Haemophilus　influenzae* が 10^8cfu/mL培養

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Fig. 1 Chest X-ray in case 1 showing bilateral ground-glass opacities.

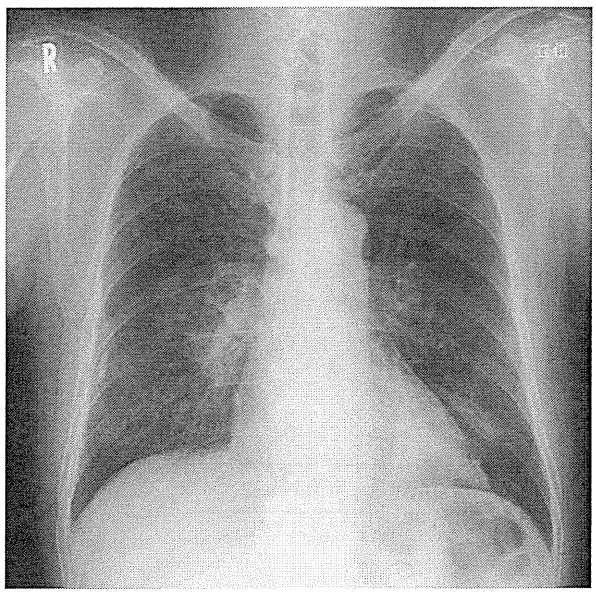
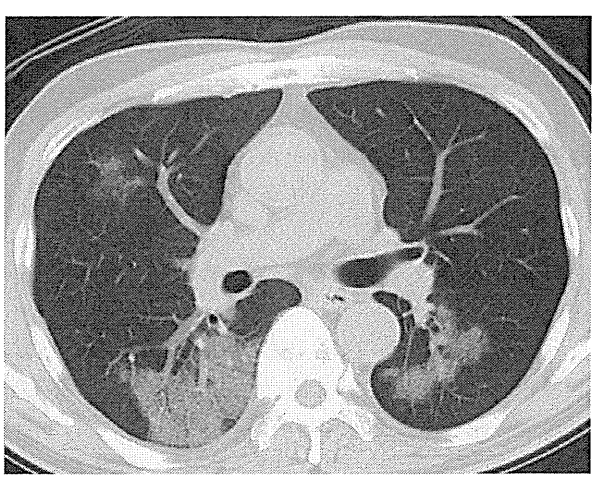


Fig. 2 Chest CT scan showing nonsegmental ground-glass opacities with reticular shadow in bilateral lung fields.



されAZMに感受性があった．その5日後にSG3が少量培養されたが，外来で経過観察したが，症状の悪化は認めなかった．治療開始11日目にレジオネラ尿中抗原を検索したが陰性であった．

症例2

患者：58歳・女性．

主訴：湿性咳嗽．

現病歴：以前から感冒後で湿性咳嗽が遷延することを認めていたが，放置していた．2008年7月に検診で胸部異常陰影を指摘され，当院紹介となった．

既往歴：特記すべき事項なし．

嗜好歴：喫煙歴なし，飲酒歴なし．

Fig. 3 Chest X-ray showing miliary nodular shadows in the left lung field.

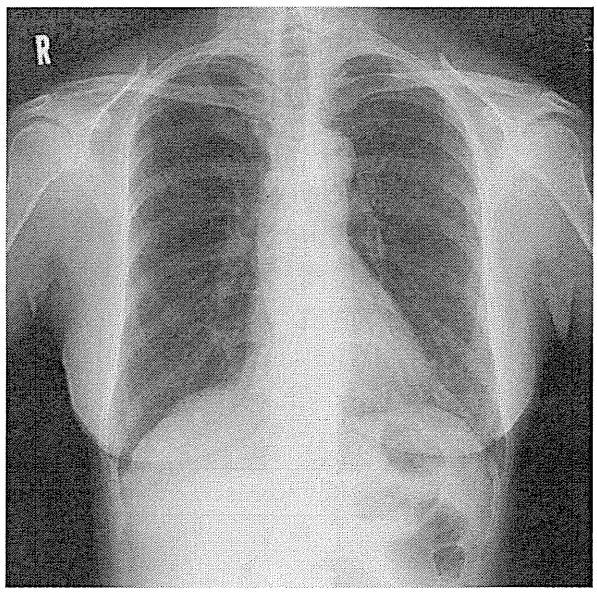
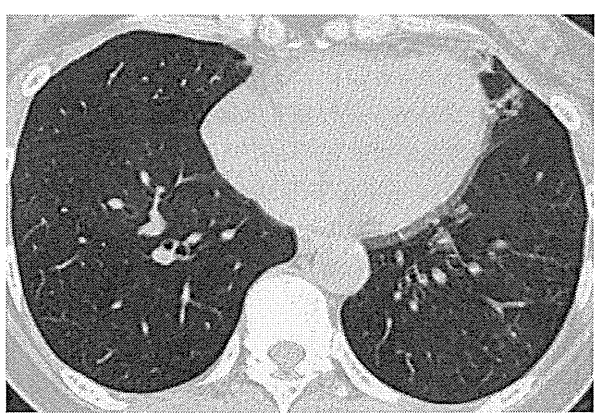


Fig. 4 Chest CT scan showing miliary nodular shadows and bronchiectasis in the left lung.



アレルギー歴：薬剤アレルギーなし，食物アレルギーなし．

初診時身体所見：体温37.0℃，脈拍96/分，SpO_2 98%（室内気），聴診上左背側にcoarse crackles とrhonchiを聴取，その他に有意な所見なし．

初診時検査所見：白血球6,600/μL，CRP 0.11mg/dL，LDH 195IU/L，Na 139mEq/L，胸部単純レントゲン写真（Fig. 3）では左中下肺野に粟粒大の粒状影を認めた．胸部HRCT（Fig. 4）では左舌区ならびに左下葉に散在性の小粒状影と気管支拡張像を認めた．

提出された喀痰の性状は粘膿性でGram染色では口腔内常在菌と思われるGram陽性球菌を認めた．

治療経過：当初，非結核性抗酸菌症などの慢性気道感染を疑ったが喀痰の抗酸菌塗抹検査は陰性であった．下気道感染症として，AZM 500mg/日を投与し

Fig. 5　Chest X-ray showing small nodular shadows and consolidation in the right middle field.

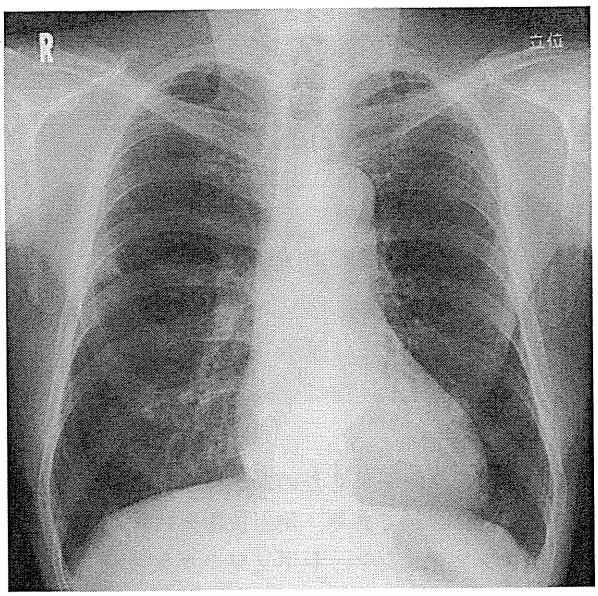
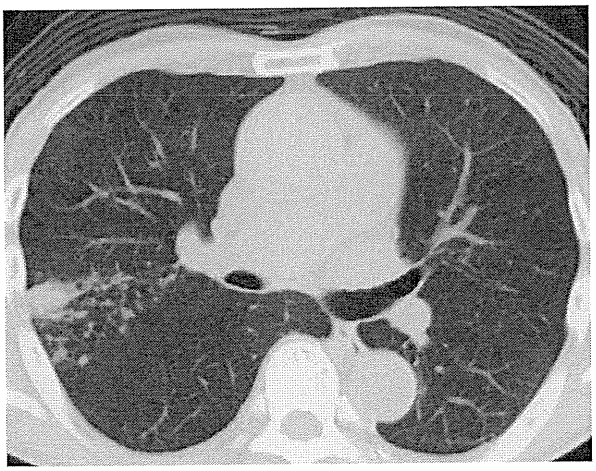


Fig. 6　Chest CT scan showing small nodular shadows along respiratory tract and consolidation in the right S2 segment.



湿性咳嗽はほぼ消失し，聴診所見も改善した．初診日から 10 日目に喀痰培養から SG3 が検出されたが，レジオネラ尿中抗原は陰性であった．抗酸菌培養は陰性であった．4 カ月後，CT を再検したところ，小粒状影は消失していた．

症例 3

患者：79 歳・男性．

主訴：胸部異常陰影．

現病歴：2004 年から右中葉に陰影を指摘されていたが，近医で経過観察されていた．2008 年 8 月に陰影の増強を認め，当院へ紹介受診となった．極く僅かに粘性の喀痰が喀出されたが，咳嗽，発熱などの症状は認めなかった．直近の旅行歴や入浴歴はないが，自宅浴槽の水は 2，3 日入れ替えなく使用していた．

既往歴：認知症．

嗜好歴：喫煙歴なし．

アレルギー歴：薬剤アレルギーなし，食物アレルギーなし．

初診時身体所見：体温 36.1℃，脈拍 56/分，SpO_2 98%（室内気），呼吸音正常，その他有意な所見なし．

初診時検査所見：白血球 5,400/μL，CRP　0.03mg/dL，LDH 224IU/L，Na 145mEq/L，胸部単純レントゲン写真（Fig. 5）では右中肺野外側に粒状影を認めた．胸部 CT（Fig. 6）では右 S2b 領域に気道散布性の粒状影と浸潤影を認めた．

提出された喀痰は粘性で Gram 染色では口腔内常在菌と思われる Gram 陽性球菌を認めた．抗酸菌塗抹は陰性であった．

治療経過：画像上は非結核性抗酸菌症を疑わせる所見であり，症状がないため経過を追った．しかし，初診時に採取した誘発喀痰から SG3 が培養されたので，AZM 500mg/日を 3 日間投与した．初診から 1 カ月後の喀痰の性状に変化はなく，再検すると SG3 は消失していた．2 カ月後の胸部単純レントゲン写真では右中肺野の粒状影は軽快していた．抗酸菌に関しては，初診時の喀痰から培養されなかったが，1 カ月後に再検した喀痰から *Mycobacterium avium* complex（MAC）が培養された．またレジオネラ属菌検出後，尿中抗原を検索したが陰性であった．

症例 4

患者：60 歳・女性．

主訴：呼吸困難，発熱．

現病歴：2009 年 6 月より当院血液内科にて多発性骨髄腫の治療を受けていた．2010 年 2 月に骨髄移植目的に入院．移植後にサイトメガロウィルス感染症，生着症候群，血球貪食症候群，出血性膀胱炎・ヒトポリオーマ BK ウィルス腎症，腎後性腎不全からの心不全などを発症し，加療されていた．4 月下旬に呼吸状態の悪化と発熱を認め，酸素投与を開始したが，翌日に呼吸状態はさらに増悪し，集中治療室にて気管挿管・人工呼吸器管理となり呼吸器内科に紹介された．気管挿管後に検体採取のために気管支鏡にて気管支洗浄を施行し，同検体から SG3 のみが培養された．

既往歴：特記すべき事項なし．

嗜好歴：喫煙・飲酒なし．

アレルギー歴：薬剤アレルギーなし，食物アレルギーなし．

気管挿管時身体所見：体温 38.8℃，脈拍 94/分，SpO_2 94%（ベンチュリーマスク 12L　50%），呼吸回数 32 回/分，両肺野で coarse crackles を聴取，下腿浮腫を

Fig. 7 Chest X-ray showing extensive bilateral groung-glass opacities and cardiomegaly.

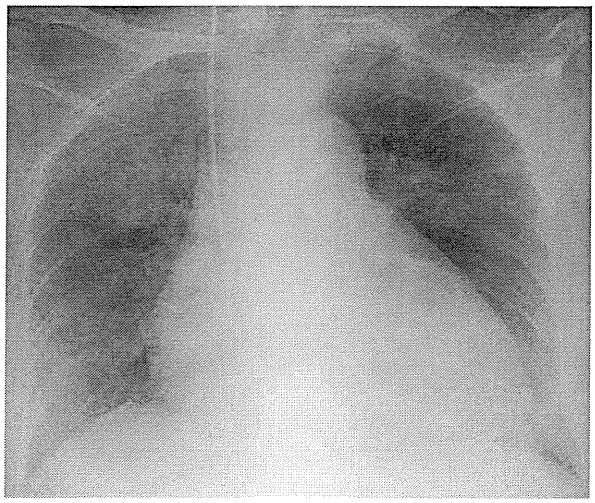
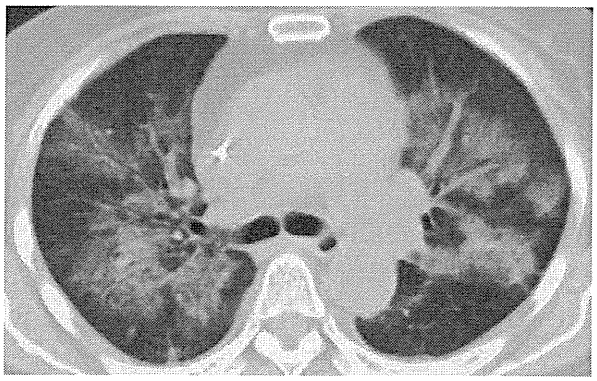


Fig. 8 Chest CT scan showing diffuse bilateral groud-glass opacities with sparing of peripheral regions.



軽度認め，その他有意な所見はなかった

気管挿管時検査所見：白血球 5,900/μL (Seg 80.0%, Band 14.0%, Eos 0.0%, Baso 0.0%, Lym 5.0%, Mono 1.0%), CRP 17.47mg/dL, LDH 421IU/L, Na 142mEq/L，胸部単純レントゲン写真（Fig. 7）では，右下肺野に有意な濃度上昇と心陰影の拡大を認めた．胸部CT（Fig. 8）では肺野末梢を除きびまん性に高吸収域となった．

治療経過：挿管直後に気管支洗浄を行い，検体を細菌検査に提出していたが，6日後に SG3 が培養された．出血性膀胱炎・BK ウィルス腎症に対してシプロフロキサシン（以下 CPFX）400mg/日が投与されていたが，AZM 500mg/日を追加した．しかしながら胸部レントゲン上，肺炎像の改善はなく呼吸状態は徐々に増悪し 38℃ 台の発熱も遷延し，集中治療室に入室してから2週間で死亡された．

考　察

迅速診断法の普及と全国的にサーベイランスが実施されるようになったことにより，本邦でのレジオネラ症の届出数は増加し，2008 年には 884 例の届出があった[2]．当院でも，2007 年以前に経験したレジオネラ症は年間 1〜3 例であり，肺炎例では市中肺炎入院患者の 2% 前後であったが，2008 年は年間 8 例に増加し，全国的な傾向と一致していた．このうち3例が今回報告した SG3 による軽症例である．

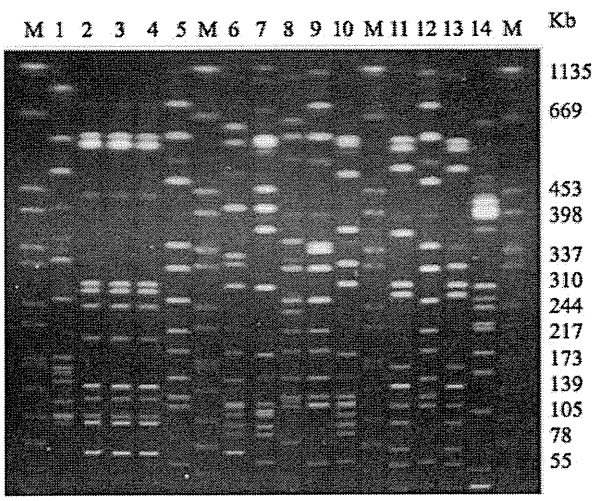
レジオネラ症は急速に進行に進展し，重症化する可能性のある致死率の最も高い呼吸器感染症の一つであるが，最近の循環式給湯式公衆浴場での集団発生の検討から，軽症レジオネラ症例も比較的多いことが報告されている[3]．その報告では，軽症レジオネラ肺炎を8例検討し，全例において 38℃ 以上の発熱を認めている．呼吸器症状を呈したのは半数の4例，基礎疾患を有するのは2例であり，使用抗菌薬はエリスロマイシンとリファンピシン，レボフロキサシンの組合せが大半であった．またレジオネラ肺炎の早期において 10% に胸部異常陰影が認められなかったという報告[4]，39 例のレジオネラ肺炎のうち8名は重症肺炎ではなかったという報告[5]がある．

今回経験した4症例のうち，症例1は市中肺炎であった．インフルエンザ桿菌の有意な菌量が培養されているので細菌感染が続発したものと考えられるが，画像所見は非定型肺炎に合致したものであった．他の2症例は慢性の気道感染症を疑わせるもので，特に症例3は肺 MAC 症と診断されている．呼吸器症状も乏しいので，呼吸器検体からレジオネラ属菌が検出されたからといってレジオネラ急性感染症とすることには疑問があるかもしれない．一般には臨床検体からレジオネラ属菌が検出されたら，少量であっても起炎菌とされているが，慢性の気道感染症で colonization のような状態があるのか今後検討する必要がある[6]．症例4は免疫抑制状態の患者に発症した院内肺炎症例であった．サイトメガロウィルス肺炎やニューモシスチス肺炎の関与を除外する必要はある．当初からこれら病原体に対する予防策は施行され，また血清マーカーが陰性であることから，それらの関与を否定的とした．気道に colonization していたレジオネラ属菌は宿主の免疫系が進行性に障害された時点で病原性を増すという報告もあり[7]，症例4の病態を示唆しているかもしれない．尚，院内環境調査を実施したが，同菌の検出はみられなかった．

レジオネラ肺炎の原因菌の多くは *L. pneumophila* SG1 であり，全体の 84.2% と報告されている[8]．その他の菌種では，*L. pneumophila longbeachae* 等が続き，SG3 の頻度は 1.0% にしかすぎない．

本邦における SG3 の報告はこれまでに 1995 年と

Fig. 9　Restriction profiles of *Legionella pneumophila* serogroup 3 digested by *Sfi*I using pulsed-field gel electrophoresis. Electrophoresis condition was as follows: a linearly ramped switching time from 5 to 50 s was applied for 19 hr at 6 v/cm and 14℃. Isolates of lane 1 and lanes 5-14 are epidemiologically unrelated with isolates of lanes 2-4 in Okayama. Lane 1, NIIB 0167, a clinical isolate; lane 2, NIIB 2503 (K76), a clinical isolate; lane 3, NIIB 2504 (K89), a clinical isolate; lane 4, NIIB 2505 (K95), a clinical isolate; lane 5, NIIB 2535, a clinical isolate; lane 6, NIIB 0818, an environmental isolate from bath water; lane 7, NIIB 1150, an environmental isolate from bath water; lane 8, NIIB 1245, an environmental isolate from bath water; lane 9, NIIB 1623, an environmental isolate from bath water; lane 10, NIIB 1686, an environmental isolate from bath water; lane 11, NIIB 2064, an environmental isolate from bath water; lane 12, NIIB 2100, an environmental isolate from bath water, lane 13: NIIB 2423, an environmental isolate from hot water supplier; lane 14, NIIB 1612, an environmental isolate from cooling tower water; M, marker, *Salmonella* Braenderup H9812/*Xba*I.



2005 年に報告された 2 例[9][10]のみであり，症例数が少なく，SG3 の臨床像については検討されていない．また海外においては，4 名の患者が造血幹細胞移植後に SG3 が院内肺炎の起炎菌となったことが報告されている[11]．

レジオネラ肺炎では早期診断・早期治療が救命率を上げる重要なポイントであり，ガイドラインでは迅速診断キットの使用を推奨している[1]．レジオネラ尿中抗原迅速診断キットの特異度は 97.1% と報告しているものもあるが，注意すべきは *L. pneumophila* serogroup1 しか検出しないことであり，すべてのレジオネラを含めるとその感度は 80% まで下がる[12]．またレジオネラ肺炎を臨床像から鑑別する Winthrop-University Hospital criteria があり，有効性が報告されている[13]．

今回経験した 4 症例のうち 3 症例は当初，レジオネラ肺炎を念頭に治療介入する必要があるような緊急性はなく，うち 2 症例は胸部異常陰影を主訴に無症状で来院した．レジオネラ属菌はルーチンの細菌検査では培養されないので，見逃されている症例が多いと危惧されているが，当院の細菌検査室では肺炎が疑われる呼吸器感染症の検体は全例 WYO-α 培地でレジオネラ属菌の培養を試みており，このため今回の 4 症例の診断しえた．

レジオネラ属菌は細胞内寄生菌であり，マクロファージやアメーバ内で増殖する．レジオネラ属菌のライフサイクルは鞭毛形成発現と大きく関り，鞭毛形成の無いタイプは wild type と比べて感染性が減弱する[14]．この 4 症例の臨床分離菌株を国立感染症研究所で Sequence-Based Typing（SBT）に従って遺伝子解析を行い，いずれも同一の型（ST93）であることが判明した．鞭毛形成に関しては位相差電子顕微鏡を用いればその有無を確認できるが，この 4 症例に関しての鞭毛形成の有無は不明である．また症例 4 を除く，3 症例の菌株の同一性を検討するべくパルスフィールド電気泳動を行い，3 株とも同一パターンを示しており，同一の汚染環境を疑うところではあるが，感染源の同定には至らなかった（Fig. 9）．

診断に至る一助として，画像所見がある．レジオネラ肺炎の胸部画像所見に関しては Yagyu らによる検討にて軽症レジオネラ肺炎の CT 所見として，consolidation とすりガラス陰影の混在などを挙げ[3]，Sakai らは HRCT 所見を検討し，consolidation intermingled with GGO としている[15]．症例 1 は非定型肺炎パターンであり，レジオネラ肺炎を鑑別疾患として十分考慮できるものであるが，症例 1 はインフルエンザ桿菌肺炎が続発しているので，インフルエンザ桿菌による修飾は受けた可能性はある．症例 2，3 の画像所見は非結核性抗酸菌症等の慢性気道疾患に類似したものであり，レジオネラ症の陰影とは考えにくい．治療介入にて陰影の改善を認めている事は，非結核性抗酸菌症が AZM 内服で改善したとも考えられ，一概にレジオネラ症が気管支肺炎像をとるとは言えない．前述の Yagyu らの報告[3]では軽症レジオネラ肺炎の画像所見で慢性気道感染，気管支肺炎像を呈したものはなかった．症例 4 は，心不全の合併が疑われるので画像上正確な評価は困難と思われる．

外来治療が可能であった 3 症例には AZM を使用し，院内肺炎の 1 症例には AZM と CPFX を使用した．また軽症例に関しては，内服抗菌薬テリスロマイシンが有効であった報告もある[16]．

最近では，レジオネラ症発症頻度は，夏（6月～8月）と秋（9月～11月）に比較的高い傾向がある[17]とされており，その他に月間降水量の増加[18]やレジオネラ肺炎が発症する6日から10日前に認められる高い湿度[19]との関連も取り上げられている．今回報告した3例の症状発現の時期は6月から8月の夏場であった可能性がある．病歴・画像所見だけではなく，軽症例の存在や特定の季節発症の存在を知ることで，ルーチンにレジオネラ属菌を狙った培養検査をしない施設においても，レジオネラ症を疑い特殊検査を施行することが診断向上につながるかもしれない．

外来治療が可能であったSG3による軽症の呼吸器感染症3症例とSG3による院内肺炎の1例を報告した．日常臨床でこのような軽症のレジオネラ症が存在すること，さらにレジオネラ属菌は院内肺炎の重要な起炎菌であることを認識し，レジオネラ症も指向した検査を行う必要があると思われる．

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Four Cases of Respiratory Infections Caused by *Legionella Pneumophila* Serogroup 3

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Legionella pneumonia tends to be severe and is known to be fatal. Introduction of the urinary *Legionella* antigen test and changes in the Infectious Disease Law have led to increased numbers of reports, and milder cases are now occasionally seen. We experienced three cases demonstrating mild respiratory infections and one case demonstrating nosocomial pneumonia associated by *Legionella pneumophila* serogroup 3. Case 1 showed multiple ground-glass opacities on HRCT and productive cough. Cases 2 and 3 showed abnormal findings on chest X-ray, and chest CT findings in both cases suggested chronic respiratory infection. Case 4 experienced fever and hypoxia, and pulmonary edema was noted on X-ray. All of them four cases were diagnosed with respiratory infections isolated *L. pneumophila* serogroup 3 by culture results, and three of them cases were treated in the outpatient clinic. Thus, milder cases of *Legionella* pneumonia may be encountered during routine care, and tests for *Legionella* should be performed in such cases.

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