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# Distribution of Monoclonal Antibody Subgroups and Sequence-Based Types among *Legionella pneumophila* Serogroup 1 Isolates Derived from Cooling Tower Water, Bathwater, and Soil in Japan

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***Legionella pneumophila* serogroup (SG) 1 is the most frequent cause of legionellosis. This study analyzed environmental isolates of *L. pneumophila* SG 1 in Japan using monoclonal antibody (MAb) typing and sequence-based typing (SBT). Samples were analyzed from bathwater (BW; $n = 50$), cooling tower water (CT; $n = 50$), and soil (SO; $n = 35$). The distribution of MAb types varied by source, with the most prevalent types being Bellingham (42%), Oxford (72%), and OLDA (51%) in BW, CT, and SO, respectively. The ratios of MAb 3/1 positive isolates were 26, 2, and 14% from BW, CT, and SO, respectively. The environmental isolates from BW, CT, and SO were divided into 34 sequence types (STs; index of discrimination [IOD] = 0.973), 8 STs (IOD = 0.448), and 11 STs (IOD = 0.879), respectively. Genetic variation among CT isolates was smaller than seen in BW and SO. ST1 accounted for 74% of the CT isolates. The only common STs between (i) BW and CT, (ii) BW and SO, and (iii) CT and SO were ST1, ST129, and ST48, respectively, suggesting that each environment constitutes an independent habitat.**

*Legionella pneumophila* serogroup (SG) 1 is the most common agent causing legionellosis found in patients; however, differences in SGs have been found both in isolates from patients and from soil and various freshwater environments (9), such as cooling towers and bathing facilities. In patients, most strains (80%) belonged to SG 1 in our previous study (2). In cooling tower water isolates of Japan, *L. pneumophila* SGs 1 and 7 accounted for 67 and 23%, respectively, with other SGs being rarely isolated. On the other hand, the isolates from bathwater and from soil were more serotypically diverse, but SG 1 was still dominant in both environments, at 31% (1) and 26% (10), respectively. *L. pneumophila* SG 1 can be divided based on having or not having the virulence-associated epitope recognized by monoclonal antibody (MAb) 3/1 (13). In England and Wales, of the clinical isolates, 91.6% were MAb 3/1 positive compared to only 8.3% of the environmental isolates (12).

*L. pneumophila* isolates can be characterized by sequence-based typing (SBT) using the seven loci (*flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, and *neuA*) proposed by the European Working Group on *Legionella* Infections (EWGLI; http://www.ewgli.org/ [11, 21]). This is a separate classifier from serogroup or MAb subtyping and is generally more precise due to the mutability of the latter factors. It allows for phylogenetic studies and identification of isolates that are closely related. The variation in STs of clinical and environmental isolates of *L. pneumophila* worldwide is very diverse. The indices of discrimination (IODs) (14) of environmental isolates and clinical isolates were determined to be 0.888 and 0.964, respectively, in Canada and 0.822 and 0.946, respectively, in the United States (15, 22). In England and Wales, however, environmental isolates are more variable than clinical ones (IODs of 0.933 and 0.901, respectively [12]), but the diversity is comparably great.

When 69 SG1 clinical isolates from Japan were subjected to SBT, they could be divided into 41 sequence types (STs). The IOD was 0.979. The ST with the most isolates ($n = 7$) was ST1. This is the most common ST occurring in the environment and among patients worldwide. Other major STs were ST306 ($n = 6$), ST120 ($n = 5$), and ST138 ($n = 5$). All ST306 and ST138 isolates, with one exception (ST306), were derived from bathwater (or suspected to be), suggesting that these strains readily adapt to bathwater habitats. The source of all ST1 and ST120 isolates remains unclear (2). In Japan, data from the National Epidemiological Surveillance of Infectious Diseases indicate that hot springs and public baths are primary sources of *L. pneumophila*, rather than cooling towers; however, in most cases the source of the bacteria is unknown (19).

We analyzed here environmental isolates of *L. pneumophila* SG 1, which is the principal cause of legionellosis in bathwater (the main source of infection in Japan), soil (a potential source of contamination for various water systems), and cooling tower water (another major source of legionellosis). Isolates were identified using MAb typing and SBT and then compared to previous clinical isolates (2) to determine relations between isolates from different environments and from patients.

Received 15 September 2011 Accepted 26 March 2012
Published ahead of print 6 April 2012
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doi:10.1128/AEM.06869-11

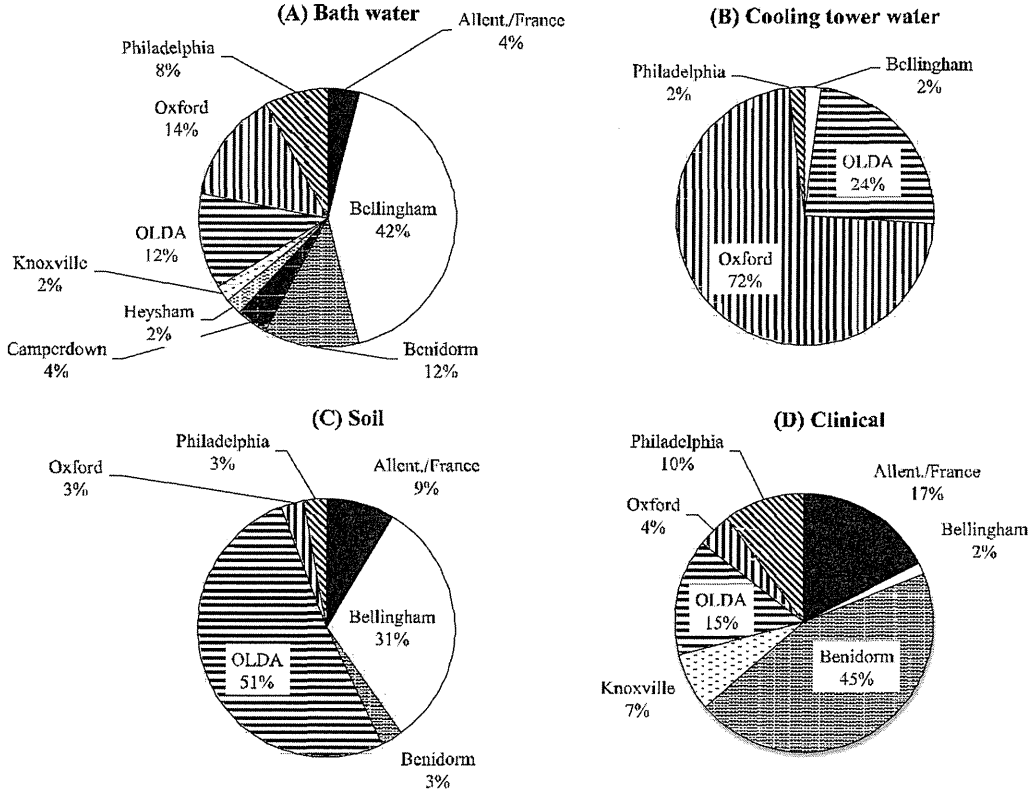


FIG 1 Distributions of MAb types. (A) Isolates from bathwater ($n$ = 50); (B) isolates from cooling tower water ($n$ = 50); (C) isolates from soil ($n$ = 35); (D) isolates from patients of legionellosis ($n$ = 69 [2]). Allentown/France, Benidorm, Knoxville, and Philadelphia are MAb 3/1-positive subgroups. Bellingham, Camperdown, Heysham, OLDA, and Oxford are MAb 3/1-negative subgroups. MAb 3/1 indicates the virulence-associated epitope.

## MATERIALS AND METHODS

***L. pneumophila* strains.** A total of 135 environmental strains of *L. pneumophila* SG 1, which were independently isolated and unrelated to cases of infection, were analyzed, including isolates from bathwater (BW; $n$ = 50), cooling tower water (CT; $n$ = 50), and soil (SO; $n$ = 35). All of the CT and BW isolates were obtained from different facilities: 66% of the CT isolates and 42% of the BW isolates originated from the Kanto region in central Japan. The SO isolates, which were independently collected from across Japan, were obtained from topsoil samples from roadsides, farmlands, gardens, etc. (10).

**MAb subgrouping.** A total of 135 environmental strains of *L. pneumophila* SG 1 were subtyped serologically, using MAbs as described previously, into nine subgroups named Allentown/France, Bellingham, Benidorm, Camperdown, Heysham, Knoxville, OLDA, Oxford, and Philadelphia (13).

**SBT.** SBT was performed according to the EWGLI SBT protocol (http://www.ewgli.org/) as described previously (11, 21). The isolates that failed amplification of *neuA* (whose indicated allele number was "0") were not given ST numbers but were allocated arbitrary numbers prefixed by J (2). A minimum-spanning tree that had categorical coefficients of similarity and the priority rule of the highest number of single-locus variants as parameters was used to indicate differences in the number of loci among operational taxonomic units (OTU). The neighbor-joining method was then used to find pairs of OTU that minimized the total branch lengths by number of base substitutions on *flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, and *neuA* concatenated sequences (2,501 bp) at each stage of OTU clustering. Both trees were constructed using BioNumerics software (version 6.5; Applied Maths, Sint-Martens-Latem, Belgium).

## RESULTS

**MAb subgrouping.** The isolates examined here were comprised of nine MAb types in all. The BW isolates were comprised of all nine MAb types, the CT isolates were comprised only of four, and the SO isolates were comprised of six. The distributions of MAb subgroups in the environmental isolates differed from one another, and from that found in clinical isolates (Fig. 1). The most common MAb subgroup in BW isolates was the Bellingham subgroup (42%), whereas the Oxford subgroup was the most common in CT (72%) and the OLDA subgroup was the most common in SO (51%). Bellingham, Oxford, and OLDA are MAb 3/1-negative subgroups. On the other hand, the most common subgroup observed in Japanese clinical isolates was Benidorm (45%), which is MAb 3/1 positive (2). Benidorm was detected in 12% of isolates from bathwater and 3% of isolates from soil. Of the 135 environmental isolates, only 14% had the virulence-associated epitope recognized by MAb 3/1: Benidorm, Allentown/France, Philadelphia, and Knoxville (13). In BW, 26% of the isolates were MAb 3/1 positive, compared to 14% in SO and a mere 2% in CT.

**SBT.** The 135 environmental isolates (with the exception of one SO isolate in which amplification of the *neuA* target failed) could be divided into 50 STs, including 33 singletons (IOD = 0.886; Tables 1 and 2 ). The ST with the largest number of isolates was ST1 ($n$ = 43, 29%), followed by ST48 ($n$ = 10, 6.7%), ST129 ($n$ = 7, 4.7%), ST739 ($n$ = 6, 4.0%), and ST22 ($n$ = 5, 3.3%). Strains with indigenous STs were isolated from each environment. The only common STs across environments were ST1 (37 from

TABLE 1 STs of Japanese environmental isolates of *L. pneumophila* serogroup 1

| Source environment and ST | No. (%) of isolates | MAb(s) (no. of isolates) |
|---|---|---|
| Cooling tower water | | |
| 1 | 37 (74) | Oxford (27), OLDA (10) |
| 154 | 4 (8) | Oxford (2), OLDA (1), Philadelphia (1) |
| 598 | 3 (6) | Oxford |
| 150 | 2 (4) | Oxford |
| Others | 4 (8) | |
| Total | 50 (100) | |
| Bathwater | | |
| 1 | 6 (12) | OLDA (4), Oxford (2) |
| 129 | 5 (10) | Bellingham |
| 599 | 3 (6) | Bellingham |
| 52 | 2 (4) | OLDA (1), Oxford (1) |
| 86 | 2 (4) | Bellingham |
| 127 | 2 (4) | Bellingham |
| 136 | 2 (4) | Oxford (1), Philadelphia (1) |
| 141 | 2 (4) | Philadelphia |
| Others | 26 (52) | |
| Total | 50 (100) | |
| Soil[a] | | |
| 48 | 9 (26) | Bellingham |
| 739 | 6 (18) | OLDA |
| 22 | 5 (15) | OLDA |
| 448 | 3 (9) | OLDA (1), Oxford (1), Benidorm (1) |
| 129 | 2 (6) | Bellingham |
| 352 | 2 (6) | Allentown/France |
| 445 | 2 (6) | OLDA |
| 593 | 2 (6) | OLDA |
| Others | 3 (9) | |
| Total | 34 (100)[b] | |

[a] Excluding an isolate with failed *neuA* amplification.
[b] The sum of percentages is not 100% because each percentage was rounded.

CT and 6 from BW), ST48 (9 from SO and 1 from CT), and ST129 (5 from BW and 2 from SO).

There were no regional differences in the distribution of ST1 in either the CT or the BW isolates (76% [22/29] of CT isolates from the Kanto region and 71% [15/21] from other regions; 14% [3/21] of BW isolates from the Kanto region and 10% [3/29] from other regions). ST1 was not detected among the SO isolates.

The 50 CT isolates were divided into eight STs (IOD = 0.448). The 50 BW isolates were divided into 34 STs (IOD = 0.973). The 35 SO isolates (with one exception that failed *neuA* amplification) were divided into 11 STs (IOD = 0.879).

The minimum-spanning tree illustrates the distribution of the STs (Fig. 2). Thirty of the fifty STs obtained in this analysis were unique to Japan, according to data submitted to the EWGLI SBT database as of March 2012. Twenty of the fifty STs had already also been detected in clinical isolates in Japan and/or abroad, according to the same database. Most SO isolates formed three distinct groups (groups S1, S2, and S3 in Fig. 2). Group S2 had no linkage with other STs. CT isolates formed group C1 and group C2. The two groups were adjacent in the minimum-spanning tree, but even the most related STs (ST161 and ST150) that belonged to group C1 and group C2, respectively, differed in four loci. The BW isolates were dispersed, forming one major group (group B1) and two smaller groups. This finding was supported by neighbor-joining analysis based on a nucleotide sequence comparison of seven concatenated loci of SBT (2,501 bp) of the same isolates as in Fig. 2 (Fig. 3). Figure 3 shows that isolates belonging to each group found in the minimum-spanning tree were also clustered. However, the relationships observed between groups in the dendrogram were different from the minimum-spanning analysis, except for the cluster of group S1 and group C1. Two groups of isolates from cooling tower water (group C1 and group C2) were located distally (unlike Fig. 2). Groups C2, B2, and B3 shared many informative sites between groups, compared to groups C1, S1, S2, S3, and B1, as shown by the bootstrap support value of 74%.

**Combining the sequence typing and the MAb subgrouping.** Some STs were composed of isolates belonging to different MAb subgroups (and *vice versa*). Thus, ST1 ($n = 43$) was composed of isolates belonging to the Oxford ($n = 29$) and OLDA ($n = 14$) subgroups. ST154 ($n = 4$) contained the Oxford ($n = 2$), OLDA ($n = 1$), and Philadelphia ($n = 1$) subgroups. ST448 ($n = 3$) consisted of OLDA, Oxford, and Benidorm isolates. In contrast, all ST48 ($n = 9$) and all ST129 ($n = 5$) isolates were Bellingham. By combining the data of SBT and MAb subgrouping, we could divide the 135 isolates into 58 types (IOD = 0.933; Tables 1 and 2).

## DISCUSSION

We analyzed *L. pneumophila* SG 1 isolates from three distinct environments using MAb typing and SBT in Japan: cooling tower water, bathwater, and soil. The distributions of MAbs and STs of isolates differed both between the environments and from previous clinical isolates (2).

Of the SG 1 clinical isolates from Japan, 80% had the virulence-associated epitope recognized by MAb 3/1 (2). As for the analyzed 135 environmental isolates, MAb 3/1-positive isolates accounted for only 14%. Similar observations have also been made in studies conducted in other countries (i.e., Germany [3], England and Wales [12], and the United States [15]). Although these data indicated MAb 3/1 as the virulence-associated epitope, our study's MAb 3/1-positive isolates dispersed on the dendrogram by SBT (Fig. 3) in the three kinds of analyzed environments, suggesting the MAb 3/1 epitope is easily lost or gained during adaptation to environments when there is no pressure to retain human pathogenicity. Loss of the MAb 3/1 epitope may bring some advantage for fitness, as MAb 3/1-negative isolates dominated in each environment.

Although 30 of the 50 STs obtained in this analysis were unique to Japan, the EWGLI SBT database indicated that the majority of unique STs have single-locus variants abroad. Among the unique STs, only ST138 and ST162 in group B3, and ST141 have neither single-locus variants nor double-locus variants abroad. ST138 of the Benidorm subgroup is the primary clinical isolate associated with bathwater in Japan (2; unpublished results). Thus, a few STs might be unique to Japan, which is isolated by water.

All of the Japanese ST1 strains were of the MAb 3/1-negative OLDA or Oxford subgroups, whereas the ST1 strains in the EWGLI database are divided into nine MAb types. This distribution of MAb types within ST1 may be a regional difference. On the other hand, a regional difference did not always apply. All nine ST48 from our results were of the Bellingham subgroup, and according to the EWGLI SBT database prior to May 2011, all of the MAb-typed ST48 strains submitted thus far were Bellingham. Since May 2011, however, Camperdown and OLDA strains containing ST48 have been deposited. If more strains could be ana-

TABLE 2 STs and MAb subtypes of 135 Japanese environmental isolates of *L. pneumophila* serogroup 1[a]

| Strain | Origin | MAb subgroup | MAb 3/1 | Allele no. | | | | | | | ST[b] | Yr |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *flaA* | *pilE* | *asd* | *mip* | *mompS* | *proA* | *neuA* | | |
| NIIB 267 | B | OLDA | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 2000 |
| NIIB 273 | B | OLDA | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 2000 |
| NIIB 277 | B | Oxford | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 2000 |
| NIIB 720 | B | Oxford | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 2005 |
| NIIB 766 | B | OLDA | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 2005 |
| NIIB 772 | B | OLDA | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 2005 |
| NIIB 270 | B | OLDA | Neg. | 1 | 10 | 3 | 1 | 1 | 1 | 1 | 52 | 2000 |
| NIIB 271 | B | Oxford | Neg. | 1 | 10 | 3 | 1 | 1 | 1 | 1 | 52 | 2000 |
| NIIB 275 | B | Oxford | Neg. | 2 | 3 | 18 | 5 | 5 | 1 | 2 | 92 | 2000 |
| NIIB 886 | B | Allentown/France | Pos. | 2 | 6 | 17 | 14 | 12 | 8 | 11 | 788 | 2001 |
| NIIB 1090 | B | Bellingham | Neg. | 2 | 10 | 3 | 3 | 9 | 4 | 11 | 545 | 2005 |
| NIIB 729 | B | Bellingham | Neg. | 2 | 10 | 3 | 6 | 9 | 4 | 11 | **614** | 2005 |
| NIIB 370 | B | Philadelphia | Pos. | 2 | 12 | 3 | 6 | 8 | 14 | 9 | **141** | 2002 |
| NIIB 885 | B | Philadelphia | Pos. | 2 | 12 | 3 | 6 | 8 | 14 | 9 | **141** | 2004 |
| NIIB 158 | B | Bellingham | Neg. | 3 | 13 | 1 | 10 | 14 | 9 | 11 | **127** | |
| NIIB 925 | B | Bellingham | Neg. | 3 | 13 | 1 | 10 | 14 | 9 | 11 | **127** | 2004 |
| NIIB 160 | B | Bellingham | Neg. | 6 | 6 | 15 | 28 | 4 | 14 | 11 | 129 | |
| NIIB 231 | B | Bellingham | Neg. | 6 | 6 | 15 | 28 | 4 | 14 | 11 | 129 | |
| NIIB 887 | B | Bellingham | Neg. | 6 | 6 | 15 | 28 | 4 | 14 | 11 | 129 | 2002 |
| NIIB 889 | B | Bellingham | Neg. | 6 | 6 | 15 | 28 | 4 | 14 | 11 | 129 | 2004 |
| NIIB 890 | B | Bellingham | Neg. | 6 | 6 | 15 | 28 | 4 | 14 | 11 | 129 | 2005 |
| NIIB 888 | B | Camperdown | Neg. | 6 | 6 | 15 | 28 | 4 | 4 | 11 | **164** | 2002 |
| NIIB 712 | B | Camperdown | Neg. | 6 | 6 | 15 | 28 | 9 | 14 | 11 | **601** | 2005 |
| NIIB 929 | B | Benidorm | Pos. | 6 | 7 | 15 | 3 | 4 | 14 | 2 | **165** | 2004 |
| NIIB 715 | B | Benidorm | Pos. | 6 | 10 | 11 | 28 | 4 | 14 | 9 | **602** | 2005 |
| NIIB 1073 | B | Knoxville | Pos. | 6 | 10 | 15 | 3 | 19 | 4 | 11 | **604** | 2005 |
| NIIB 1197 | B | Heysham | Neg. | 6 | 10 | 15 | 3 | 21 | 4 | 3 | **606** | 2005 |
| NIIB 707 | B | Benidorm | Pos. | 6 | 10 | 15 | 13 | 17 | 14 | 11 | **122** | 2005 |
| NIIB 128 | B | Bellingham | Neg. | 6 | 10 | 15 | 28 | 4 | 4 | 11 | **125** | |
| NIIB 743 | B | Bellingham | Neg. | 6 | 10 | 15 | 28 | 44 | 14 | 11 | **201** | 2005 |
| NIIB 1213 | B | Bellingham | Neg. | 6 | 10 | 15 | 28 | 4 | 14 | 11 | 278 | 2005 |
| NIIB 229 | B | Philadelphia | Pos. | 6 | 10 | 17 | 6 | 9 | 4 | 9 | **136** | |
| NIIB 295 | B | Oxford | Neg. | 6 | 10 | 17 | 6 | 9 | 4 | 9 | **136** | 2000 |
| NIIB 278 | B | Bellingham | Neg. | 6 | 10 | 17 | 28 | 19 | 4 | 6 | **599** | |
| NIIB 696 | B | Bellingham | Neg. | 6 | 10 | 17 | 28 | 19 | 4 | 6 | **599** | 2005 |
| NIIB 699 | B | Bellingham | Neg. | 6 | 10 | 17 | 28 | 19 | 4 | 6 | **599** | 2005 |
| NIIB 710 | B | Bellingham | Neg. | 6 | 10 | 19 | 28 | 19 | 14 | 11 | **600** | 2005 |
| NIIB 230 | B | Philadelphia | Pos. | 6 | 10 | 21 | 6 | 9 | 4 | 9 | **137** | |
| NIIB 733 | B | Benidorm | Pos. | 6 | 10 | 21 | 13 | 17 | 14 | 11 | **131** | 2005 |
| NIIB 126 | B | Oxford | Neg. | 6 | 16 | 14 | 3 | 21 | 14 | 3 | 124 | |
| NIIB 159 | B | Bellingham | Neg. | 7 | 6 | 17 | 3 | 14 | 11 | 11 | 128 | |
| NIIB 1109 | B | Bellingham | Neg. | 7 | 6 | 17 | 28 | 36 | 11 | 11 | **86** | 2005 |
| NIIB 1115 | B | Bellingham | Neg. | 7 | 6 | 17 | 28 | 36 | 11 | 11 | **86** | 2005 |
| NIIB 805 | B | Bellingham | Neg. | 7 | 8 | 17 | 3 | 14 | 11 | 11 | **603** | 2005 |
| NIIB 1044 | B | Bellingham | Neg. | 7 | 10 | 17 | 3 | 13 | 9 | 11 | **605** | 2005 |
| NIIB 268 | B | Allentown/France | Pos. | 8 | 10 | 3 | 10 | 2 | 1 | 6 | **610** | 2000 |
| NIIB 1206 | B | Benidorm | Pos. | 10 | 12 | 7 | 3 | 16 | 18 | 6 | **138** | 2005 |
| NIIB 594 | B | Benidorm | Pos. | 10 | 22 | 7 | 3 | 16 | 9 | 6 | **162** | 2001 |
| NIIB 891 | B | Oxford | Neg. | 11 | 14 | 16 | 1 | 15 | 13 | 2 | 159 | 2005 |
| NIIB 1099 | B | OLDA | Neg. | 12 | 8 | 11 | 23 | 29 | 26 | 2 | 260 | 2005 |
| NIIB 65 | C | Oxford | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 1996 |
| NIIB 121 | C | OLDA | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | |
| NIIB 122 | C | OLDA | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | |
| NIIB 124 | C | OLDA | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | |
| NIIB 182 | C | Oxford | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 1997 |
| NIIB 217 | C | OLDA | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 1986 |
| NIIB 223 | C | OLDA | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 1986 |
| NIIB 224 | C | Oxford | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 1986 |
| NIIB 225 | C | OLDA | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 1986 |
| NIIB 226 | C | OLDA | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 1986 |
| NIIB 228 | C | OLDA | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 1986 |
| NIIB 237 | C | Oxford | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 1996 |
| NIIB 239 | C | Oxford | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 1993 |
| NIIB 418 | C | Oxford | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 2004 |
| NIIB 547 | C | OLDA | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 2001 |
| NIIB 563 | C | Oxford | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 2001 |
| NIIB 568 | C | Oxford | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 2001 |
| NIIB 586 | C | Oxford | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 2001 |
| NIIB 597 | C | Oxford | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 2001 |
| NIIB 697 | C | Oxford | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 2005 |
| NIIB 717 | C | Oxford | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 2005 |
| NIIB 722 | C | Oxford | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 2005 |
| NIIB 725 | C | Oxford | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 2005 |
| NIIB 732 | C | Oxford | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 2005 |
| NIIB 739 | C | Oxford | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 2005 |
| NIIB 742 | C | Oxford | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 2005 |
| NIIB 744 | C | Oxford | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 2005 |
| NIIB 758 | C | Oxford | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 2005 |
| NIIB 764 | C | Oxford | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 2005 |
| NIIB 802 | C | Oxford | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 2005 |

(Continued on following page)

TABLE 2 (Continued)

| Strain | Origin | MAb subgroup | MAb 3/1 | Allele no. | | | | | | | ST[b] | Yr |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *flaA* | *pilE* | *asd* | *mip* | *mompS* | *proA* | *neuA* | | |
| NIIB 1048 | C | Oxford | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 2005 |
| NIIB 1050 | C | Oxford | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 2005 |
| NIIB 1052 | C | OLDA | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 2005 |
| NIIB 1057 | C | Oxford | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 2005 |
| NIIB 1082 | C | Oxford | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 2005 |
| NIIB 1201 | C | Oxford | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 2005 |
| NIIB 1592 | C | Oxford | Neg. | 1 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 2006 |
| NIIB 946 | C | Bellingham | Neg. | 5 | 2 | 22 | 27 | 6 | 10 | 12 | 48 | 2005 |
| NIIB 552 | C | Oxford | Neg. | 11 | 4 | 3 | 1 | 1 | 1 | 1 | **161** | 2001 |
| NIIB 595 | C | Oxford | Neg. | 11 | 14 | 16 | 1 | 15 | 13 | 1 | 150 | 2001 |
| NIIB 1207 | C | Oxford | Neg. | 11 | 14 | 16 | 1 | 15 | 13 | 1 | 150 | 2005 |
| NIIB 694 | C | Oxford | Neg. | 11 | 14 | 16 | 10 | 15 | 13 | 11 | **598** | 2005 |
| NIIB 746 | C | Oxford | Neg. | 11 | 14 | 16 | 10 | 15 | 13 | 11 | **598** | 2005 |
| NIIB 1098 | C | Oxford | Neg. | 11 | 14 | 16 | 10 | 15 | 13 | 11 | **598** | 2005 |
| NIIB 590 | C | Oxford | Neg. | 11 | 14 | 16 | 16 | 15 | 13 | 2 | 154 | 2001 |
| NIIB 591 | C | Oxford | Neg. | 11 | 14 | 16 | 16 | 15 | 13 | 2 | 154 | 2001 |
| NIIB 778 | C | OLDA | Neg. | 11 | 14 | 16 | 16 | 15 | 13 | 2 | 154 | 2005 |
| NIIB 1077 | C | Philadelphia | Pos. | 11 | 14 | 16 | 16 | 15 | 13 | 2 | 154 | 2005 |
| NIIB 1269 | C | Oxford | Neg. | 11 | 14 | 16 | 16 | 15 | 13 | 1 | **607** | 2005 |
| NIIB 611 | C | OLDA | Neg. | 18 | 4 | 3 | 1 | 1 | 1 | 1 | **163** | 2001 |
| NIIB 2366 | S | OLDA | Neg. | 2 | 3 | 6 | 10 | 2 | 1 | 6 | 22 | 2001 |
| NIIB 2388 | S | OLDA | Neg. | 2 | 3 | 6 | 10 | 2 | 1 | 6 | 22 | 2001 |
| NIIB 2403 | S | OLDA | Neg. | 2 | 3 | 6 | 10 | 2 | 1 | 6 | 22 | 2001 |
| NIIB 2404 | S | OLDA | Neg. | 2 | 3 | 6 | 10 | 2 | 1 | 6 | 22 | 2001 |
| NIIB 2409 | S | OLDA | Neg. | 2 | 3 | 6 | 10 | 2 | 1 | 6 | 22 | 2001 |
| NIIB 2375 | S | Oxford | Neg. | 2 | 3 | 18 | 10 | 2 | 1 | 6 | 448 | 2001 |
| NIIB 2395 | S | OLDA | Neg. | 2 | 3 | 18 | 10 | 2 | 1 | 6 | 448 | 2001 |
| NIIB 2338 | S | Benidorm | Neg. | 2 | 3 | 18 | 10 | 2 | 1 | 6 | 448 | 2001 |
| NIIB 2380 | S | Allentown/France | Pos. | 2 | 3 | 18 | 10 | 25 | 5 | 6 | **740** | 2001 |
| NIIB 2363 | S | OLDA | Neg. | 2 | 3 | 18 | 13 | 2 | 1 | 6 | 445 | 2001 |
| NIIB 2392 | S | OLDA | Neg. | 2 | 3 | 18 | 13 | 2 | 1 | 6 | 445 | 2001 |
| NIIB 2339 | S | OLDA | Neg. | 2 | 3 | 40 | 13 | 2 | 1 | 6 | 593 | 2001 |
| NIIB 2373 | S | OLDA | Neg. | 2 | 3 | 40 | 13 | 2 | 1 | 6 | 593 | 2001 |
| NIIB 2355 | S | OLDA | Neg. | 5 | 1 | 22 | 26 | 6 | 10 | 12 | 45 | 2001 |
| NIIB 2332 | S | Bellingham | Neg. | 5 | 2 | 22 | 27 | 6 | 10 | 12 | 48 | 2001 |
| NIIB 2335 | S | Bellingham | Neg. | 5 | 2 | 22 | 27 | 6 | 10 | 12 | 48 | 2001 |
| NIIB 2346 | S | Bellingham | Neg. | 5 | 2 | 22 | 27 | 6 | 10 | 12 | 48 | 2001 |
| NIIB 2383 | S | Bellingham | Neg. | 5 | 2 | 22 | 27 | 6 | 10 | 12 | 48 | 2001 |
| NIIB 2398 | S | Bellingham | Neg. | 5 | 2 | 22 | 27 | 6 | 10 | 12 | 48 | 2001 |
| NIIB 2399 | S | Bellingham | Neg. | 5 | 2 | 22 | 27 | 6 | 10 | 12 | 48 | 2001 |
| NIIB 2405 | S | Bellingham | Neg. | 5 | 2 | 22 | 27 | 6 | 10 | 12 | 48 | 2001 |
| NIIB 2406 | S | Bellingham | Neg. | 5 | 2 | 22 | 27 | 6 | 10 | 12 | 48 | 2001 |
| NIIB 2408 | S | Bellingham | Neg. | 5 | 2 | 22 | 27 | 6 | 10 | 12 | 48 | 2001 |
| NIIB 2371 | S | Bellingham | Neg. | 6 | 6 | 15 | 28 | 4 | 14 | 11 | 129 | 2001 |
| NIIB 2411 | S | Bellingham | Neg. | 6 | 6 | 15 | 28 | 4 | 14 | 11 | 129 | 2001 |
| NIIB 2370 | S | OLDA | Neg. | 6 | 10 | 23 | 10 | 18 | 14 | 0[c] | J6[d] | 2001 |
| NIIB 2342 | S | OLDA | Neg. | 12 | 8 | 11 | 2 | 10 | 12 | 2 | 739 | 2001 |
| NIIB 2356 | S | OLDA | Neg. | 12 | 8 | 11 | 2 | 10 | 12 | 2 | 739 | 2001 |
| NIIB 2381 | S | OLDA | Neg. | 12 | 8 | 11 | 2 | 10 | 12 | 2 | 739 | 2001 |
| NIIB 2386 | S | OLDA | Neg. | 12 | 8 | 11 | 2 | 10 | 12 | 2 | 739 | 2001 |
| NIIB 2390 | S | OLDA | Neg. | 12 | 8 | 11 | 2 | 10 | 12 | 2 | 739 | 2001 |
| NIIB 2394 | S | OLDA | Neg. | 12 | 8 | 11 | 2 | 10 | 12 | 2 | 739 | 2001 |
| NIIB 2327 | S | Allentown/France | Pos. | 12 | 8 | 11 | 13 | 10 | 12 | 2 | **352** | 2001 |
| NIIB 2353 | S | Allentown/France | Pos. | 12 | 8 | 11 | 13 | 10 | 12 | 2 | **352** | 2001 |
| NIIB 2343 | S | Philadelphia | Pos. | 21 | 14 | 29 | 30 | 15 | 29 | 6 | **741** | 2001 |

[a] Origins: B, bathwater; C, cooling tower water; S, soil. MAb 3/1: Pos., positive; Neg., negative.

[b] Underlining indicates an ST first reported from Japan and later reported from other countries. Bold facing indicates STs unique to Japan as of 8 March 2012.

[c] Allele number "0" means a failed amplification.

[d] J6, an arbitrary number allocated to unique six-allele profile without *neuA*.

lyzed, we predict that different MAb types within many ST groups would be detected.

The isolates from soil were divided into three groups (Fig. 2) by the spanning tree analysis and only had two common STs that were detected in different environments: ST48 with an isolate from cooling tower water and ST129 with an isolate from bathwater. These findings indicate that these bacteria generally inhabit the soil but are able to contaminate water sources. Further investigations of more isolates from soil may identify STs that link the three groups or that have more corresponding STs with isolates from water environments. Nine of the 11 STs of soil isolates were also detected in clinical isolates, in contrast to only 11 of 34 in bathwater and 3 of 8 from cooling tower water. These findings support the possibility that soil is one of the infectious sources of legionellosis.

In Canada, the distribution of STs in strains from natural water sources was noted as significantly different compared to strains from a manufactured environment (22). We note a similar finding in the present study. The water of Japanese public baths is often derived from hot springs. The characteristics of hot spring water, namely, chemical features such as pH and temperature, are highly variable, whereas the water from hot or cold water systems and cooling towers tend to have rather similar characteristics due to similar water treatment procedures. In our results, STs and MAb types of isolates from bathwater both differed from and were more varied than those of cooling tower water. These features might be

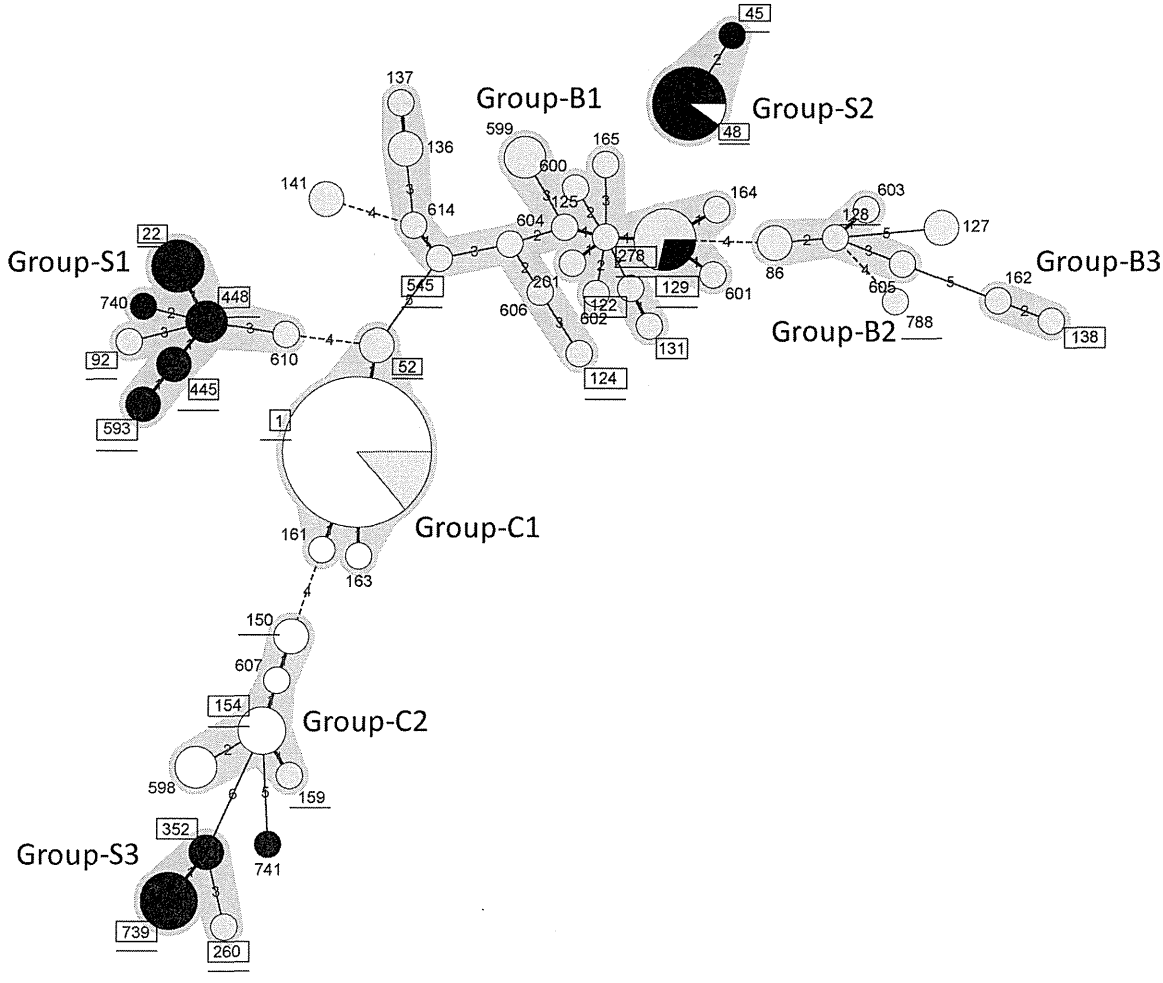


FIG 2 Minimum-spanning tree showing how the *L. pneumophila* isolates, with seven determined alleles, are distributed in terms of their STs. The ST number is shown beside the circle. An underlined ST number indicates that the ST also has been reported abroad, and a boxed ST number indicates that clinical isolates with the same STs were detected. The size of the circle indicates the number of isolates. The white parts of the circles (pie charts) indicate isolates derived from cooling tower water ($n = 50$), the gray indicates isolates derived from bathwater ($n = 50$), and the black indicates isolates derived from soil ($n = 34$). Short thick branches connect single-locus variants, thin branches connect double- or triple-locus variants, broken branches connect four-locus variants, and thinner branches connect five- or six-locus variants. The numbers of locus variants are proportional to the length of branches. Groups that were generated with single-, double-, and triple-locus variants are indicated by differently shaded backgrounds. Groups C1 and C2 had their major isolates derived from cooling tower water, groups B1, B2, and B3 had their major isolates derived from bathwater, and groups S1, S2, and S3 had their major isolates derived from soil.

related to the kind of host amoebae, which adapt to and inhabit various environments (20, 23). It has been shown that the growth of *L. pneumophila* in some species of host amoebae depends on bacterial genetic background (4, 8). Some isolates with particular STs adapted for amoebae that live in bathwater may be infectious to humans.

*L. pneumophila* SG 1 isolates in cooling tower water in Japan were divided into two genetic groups (group C1 and group C2; Fig. 3). Recombination events may have occurred between members of group C1 and group C2. For example, ST161 (*flaA11*, *pilE4*, *asd-3*, *mip-1*, *mompS1*, *proA1*, and *neuA1*) was a recombinant between ST1 (*flaA1*, *pilE4*, *asd-3*, *mip-1*, *mompS1*, *proA1*, and *neuA1*) and ST154 (*flaA11*, *pilE14*, *asd-16*, *mip-16*, *mompS15*, *proA13*, and *neuA2*), which was a predicted primary founder, and ST150 (*flaA11*, *pilE14*, *asd-16*, *mip-1*, *mompS15*, *proA13*, and *neuA1*) was also a recombinant between ST1 (with adjacent alleles, *mip-1* and *neuA1*) and ST154, shortening the distance between the two groups on the minimum-spanning tree (Fig. 2).

The IOD (0.886) of the 135 environmental isolates was lower than described in our previous report based on clinical isolates (0.979 [2]). These findings were similar to those reported in Canada and the United States (15, 22). The lower diversity observed among environmental isolates compared to clinical isolates may be due to the high prevalence of ST1 (22). ST1 is the most prevalent ST in the world (3, 6–7, 12, 15–16, 22, 26). We have also shown that the majority of environmental isolates, especially from cooling tower waters in Japan (37/50, or 74%), are ST1. Similar results were shown in South Korea (46/68 [67.6%] of SG 1 isolates from cooling tower water were ST1), which is adjacent to Japan (16). In a Canadian study, 34.2% of *L. pneumophila* strains from manufactured environments and 7.7% of *L. pneumophila* isolates from natural water sources (lakes and hot springs) were SG 1 and ST1. Among the Canadian strains, five of six SG 1 isolates from cooling tower waters were ST1 (22). In a U.S. study, ST1 accounted for 40% of the *L. pneumophila* SG 1 environmental isolates; however, the types of environments were not indicated. In

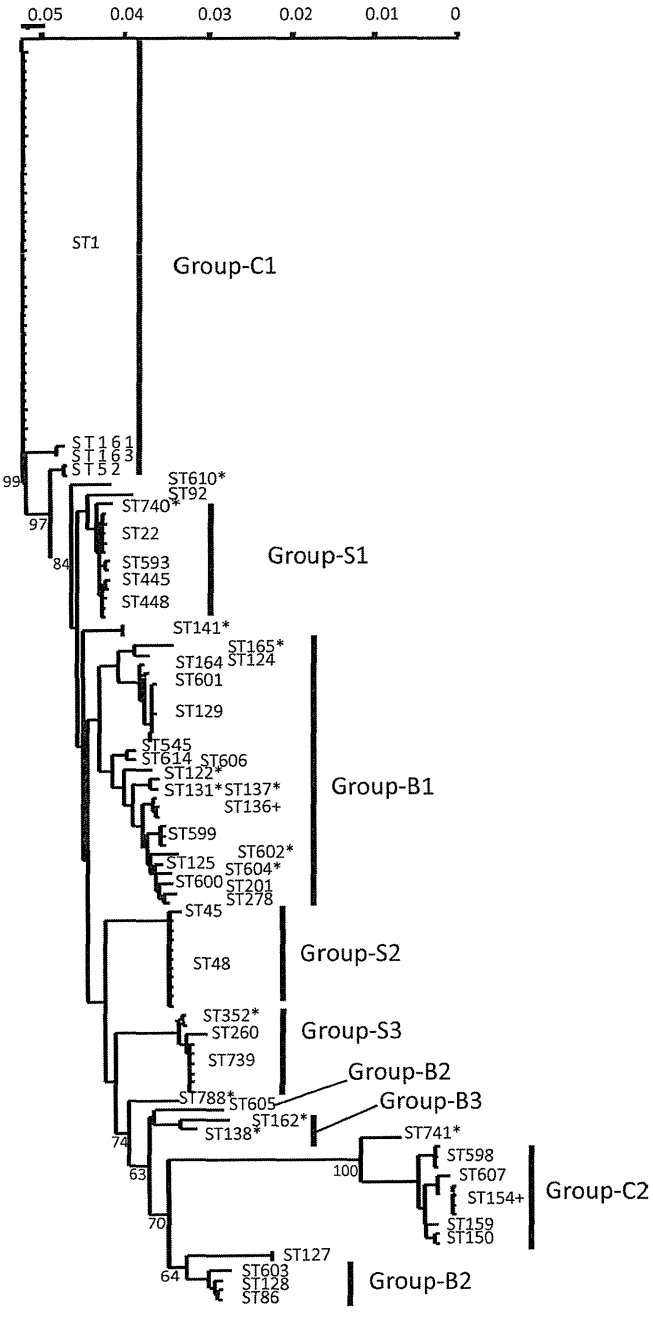


FIG 3 Phylogenetic tree of *flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, and *neuA* concatenated sequences from *L. pneumophila* serogroup 1 isolates determined by the neighbor-joining method. Bootstrap support values for nodes outside groups higher than 50% are shown. The scale bar indicates genetic distances between sequences. The groups correspond to those in Fig. 2. STs of isolates that are MAb 3/1 positive are denoted by asterisks (*), and STs of a part of isolates that are MAb 3/1 positive are denoted by plus symbols (+).

Singapore in tropical southeast Asia, the IOD of environmental *L. pneumophila* isolates was found to be 0.970, and three (two from a cooling tower and one from a water tank) of 16 SG 1 isolates were ST1 (17). In a study conducted in England and Wales, 154 of 276 *L. pneumophila* isolates, including 29 isolates derived from cooling tower water, were SG 1, and 54/154 (35%) SG 1 environmental isolates were ST1 (12). In our study, ST1 accounted for 74 and 12% of the environmental SG 1 isolates from cooling tower water and bathwater, respectively, whereas no ST1 was found in isolates from soil. Isolates with ST1 have adapted to water environments, especially in manufactured water systems such as cooling towers, and have been detected around the world. The ability of ST1 isolates to adapt to natural water sources such as lakes and hot springs might be rather low. Moreover, they might be unfit to survive well in soil environments. The predominant ST, ST1, of isolates from cooling tower water induced an insufficient IOD, 0.448, whereas the discrimination powers for isolates from bathwater and soil were sufficiently significant (0.973 and 0.879, respectively).

Handling of potting soil could be considered a risk factor for legionellosis. Surveys in several countries have detailed various *Legionella* species, including *L. pneumophila* SG1, that were isolated from potting soil or composted materials (5, 18, 25). SBT analysis on composted material isolates in United Kingdom revealed that their *L. pneumophila* SG1 isolates belonged to ST84 (18). Seven alleles belonging to ST84 were unshared by soil isolates in our study (except for one allele, *flaA12*, which was), although ST84 has been detected in clinical isolates in Japan (2) and other countries, according to the EWGLI database. Groups S1, S2, and S3, mainly formed by isolates derived from soil, were distant phylogenetically from groups of isolates derived from water environments. Only ST129 soil isolates shared the B1 group with isolates from a water environment. Although some isolates from cooling tower water and bathwater were included in groups S1, S2, and S3, this might imply that some part of these *L. pneumophila* subpopulations primarily inhabits soil, occasionally mutating and becoming fit to contaminate water environments. Recently, indigenous soil samples were collected in Thailand, and 115 *Legionella* isolates, including 2 *L. pneumophila* SG1 isolates, were identified (24; EWLGI database). One ST identified from the *L. pneumophila* SG1 soil sample isolates related to group S1 and the other to group S2, supporting the idea that most soil isolates belong to particular groups. It is also interesting that the most prevalent ST1 isolates from water samples were not isolated from soil in our study, suggesting the possibility of habitat segregation of *L. pneumophila*. To elucidate this possibility, we need to investigate more environmental isolates from both soil and water.

## ACKNOWLEDGMENTS

We thank Norman K. Fry (Respiratory and Systemic Infection Laboratory, Health Protection Agency) for assigning the newly identified alleles.

This study was supported by the Health and Labor Sciences research grant H22-kenki-014 (to F.K.) and partially supported by Ministry of Education, Culture, Sports, Science, and Technology grant KAKENHI 23590530 (to J.A.-M).

The Working Group for *Legionella* in Japan included Mie Sasaki, Miyagi Prefectural Institute of Public Health and Environment; Mikako Hosoya, Niigata Prefectural Institute of Public Health and Environmental Sciences; Yuko Watanabe, Toshiro Kuroki, Kanagawa Prefectural Institute of Public Health; Masamichi Wada, Nagano Environmental Conservation Research Institute; Hitoshi Doi, Osaka Prefectural Institute of Public Health; and Koichi Murakami, Fukuoka Institute of Health and Environmental Sciences.

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Journal of Microbiological Methods 86 (2011) 25–32

Contents lists available at ScienceDirect

Journal of Microbiological Methods

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

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ARTICLE INFO

*Article history:*
Received 12 October 2010
Received in revised form 17 March 2011
Accepted 21 March 2011
Available online 2 April 2011

*Keywords:*
Chlorination
Hot tub
*Legionella* control
Rapid detection method

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 $3.0\times10^3$ (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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0167-7012/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.mimet.2011.03.012

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

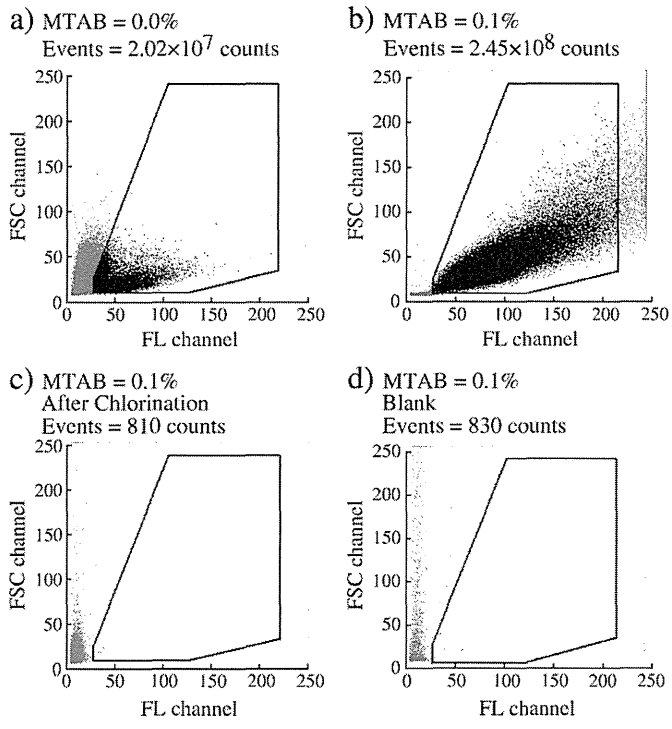


**Fig. 1.** Fluorescence light (FL) vs. forward scattered light (FSC) in the rapid detection method system, showing specific gating clusters for treatment with (a) 0.0% and (b) 0.1% myristyl trimethyl ammonium bromide (MTAB) at pH 7.0, for disinfection with (c) 20 mg $L^{-1}$ of the free residual chlorine (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 $\chi$ 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 $(3.0 \times 10^3)$ 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

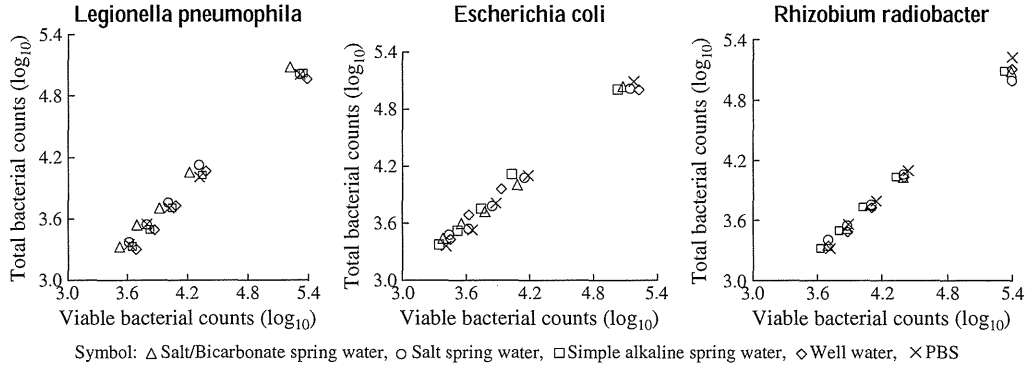


**Fig. 2.** The limit of determination of the total bacterial counts in the spiked samples prepared from different spring water sources. Each sample was adjusted to approximately 5.00, 4.00, 3.70, 3.48, and 3.30 log bacterial counts per milliliter by the rapid detection method, and compared with each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

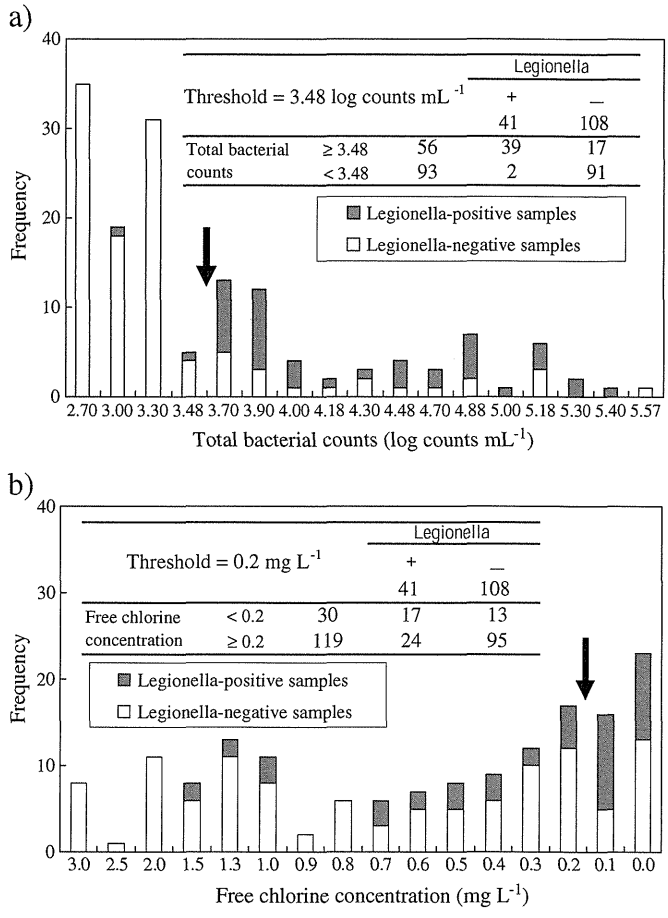


**Fig. 3.** Comparison of the frequency of distribution of water samples positive or negative for culturable *Legionella*. (a) Bacterial counts were measured by the rapid detection method, and (b) the free residual chlorine concentration was measured by the *N,N*-diethyl-*p*-phenylenediamine method.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

## 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.

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

This work was supported by the H19-20 Nagasaki Prefectural Specific Research Project, Health and Labour Sciences Research Grants (H19-kenki-014 to F. K. and H19-kenki-015 to T. E.), and by grants from the Japan Science and Technology Agency.

## 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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*Journal of Medical Microbiology* (2010), 59, 1242–1246 DOI 10.1099/jmm.0.016089-0

Case Report

# Isolation of *Legionella rubrilucens* from a pneumonia patient co-infected with *Legionella pneumophila*

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Received 7 October 2009
Accepted 15 June 2010

We report what we believe to be the first clinical isolation of *Legionella rubrilucens* from a pneumonia patient co-infected with *Legionella pneumophila*. *L. rubrilucens* strains were found in both a patient's sputum and the water of a hot spring in which the patient bathed, and DNA analysis by PFGE showed that they were indistinguishable.

## Introduction

Since Brenner *et al.* (1979) isolated the bacterium that caused pneumonia in patients with Legionnaires' disease and named it *Legionella pneumophila*, more than 20 species of *Legionella* have been implicated in human diseases (Stout *et al.*, 2003). In 1980, an environmental strain of *Legionella rubrilucens* was isolated from tap water by G. W. Gorman (Brenner *et al.*, 1985). However, to date, isolation of *L. rubrilucens* has not been described from patients co-infected with *L. pneumophila*.

Here we report that *L. rubrilucens* strains were found in both a patient's sputum and the water of a hot spring in which the patient bathed, and DNA analysis performed by PFGE (Amemura-Maekawa *et al.*, 2005) showed that they were indistinguishable. To our knowledge, this is the first report suggesting that *L. rubrilucens* can co-infect humans infected with *L. pneumophila*. Since the progress of *Legionella* pneumonia is very rapid in general, it can be fatal without early diagnosis and treatment. In this study, due to the early diagnosis, *Legionella* pneumonia was treated successfully.

## Case report

A 54-year-old Japanese man, height 165 cm, weight 72 kg, presented with a high fever, a feeling of weariness and pain in the joints. He had smoked 20 cigarettes a day for 34 years and had been drinking 350 ml beer and about 360 ml shouchu (Japanese liquor, alcohol concentration 25–30 %) daily for the past 10 years. He had been working as a caretaker for the elderly.

After continuous high fever for 2 days, he was admitted for treatment and examination of the cause of the fever. He still complained of weariness throughout the body without signs of pains in the joints, cough or sputum. His vital signs were stable with blood pressure 120/70 mmHg, pulse rate 120 beats $min^{-1}$ and body temperature 39.2 °C. No rale was audible in either of the lungs. The Influenza A and B Antigen test was negative. Laboratory data showed a white blood cell count of 17 100 cells $\mu l^{-1}$ (neutrophils 87.7 %) and a C-reactive protein value of 15.86 mg $dl^{-1}$ in the serum. Urine analysis revealed that both a protein test and occult blood test were strongly positive (3+), assumingly due to an inflammatory response of the urinary tract from a bacterial infection. On the X-ray film, we observed minimal infiltrative shadows on the left middle and lower lobes.

After admission, his febrile state did not change despite the administration of flomoxef at 2 g per day. On the 3rd day, he started to have a dry cough and fine crackles were audible in the left lower lobe. We found extended infiltrative shadows on the left middle and lower lobes in the chest X-ray (Fig. 1). The oxygen saturation by pulse oximetry ($SpO_2$) had declined to 93 % at this point.

During admission, it was reported that he had bathed in a hot spring twice, 11 days and 2 days before admission, and

Abbreviation: CAM, clarithromycin.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of the clinical *Legionella rubrilucens* isolate is AB537503.

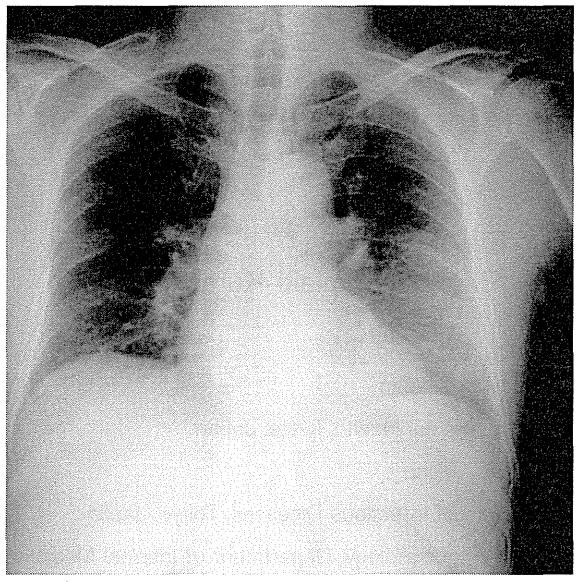

**Fig. 1.** Chest X-ray film taken on the 3rd day of admission showing ground glass shadows in the left middle and lower lung fields.

it was suspected that he might be infected with *Legionella* from the hot-spring water. A urine antigen test for *Legionella* (Biotest AG) was positive. Furthermore, we observed two *Legionella*-like colonies on GVPC plates (BCYE supplemented with glycine, vancomycin, polymyxin B and cycloheximide; Oxoid) from sputum obtained on the 4th day after admission. The *Legionella*-like colonies demonstrated red autofluorescence under 365 nm UV light and were identified as *Legionella* species (non-*L. pneumophila*) by a standard block-based PCR using LEG primers targeting the 16S rRNA gene (for the detection of members of the *Legionella* genus; Yamamoto *et al.*, 1993) and *Lmip* primers targeting the *mip* gene (for detection of *L. pneumophila*; Mahbubani *et al.*, 1990). The presence or absence of amplified products was determined following gel electrophoresis and the above colonies yielded a band of the expected size with the LEG primers but no band with the *Lmip* primers. Subsequently, the colonies were identified as *L. rubrilucens* using a DNA–DNA hybridization kit (Kyokuto Pharmaceutical Industrial) at the Iwate Prefecture Research Center for Environmental Health and by sequencing of the 5′-region of the 16S rRNA gene (100 % identity with the type strain WA-270A-C2; accession no. of the clinical isolate is AB537503; 488 bp in length) at the National Institute of Infectious Diseases in Japan. Unfortunately, no *L. pneumophila* strain was isolated from sputa. Real-time PCR of the remaining sputum targeting both the 5S rRNA gene for detection of the *Legionella* genus and the *L. pneumophila*-specific *mip* gene (CycleavePCR *Legionella* Detection kit; Takara Bio) was performed according to the supplier's instruction, using the SmartCycler System (Cepheid). For DNA extraction, the sputum was treated with 2 % Triton X-100 and 1 mg proteinase K $ml^{-1}$ and purified using a MonoFas Column for the *Legionella* genome (GL Sciences). The detection limits for *L. pneumophila* were 1.2 c.f.u. per reaction when targeting the 5S rRNA gene, and 12 c.f.u. per reaction when targeting the *mip* gene, using the purified DNA of *L. pneumophila* 80-045 (Saito *et al.*, 1981). It was also confirmed that the detection limit for *L. rubrilucens* was 320 c.f.u. per reaction when targeting the 5S rRNA gene. About 200 µl sputum was used for DNA extraction. Each reaction sample (25 µl) contained 5 µl purified DNA. All reaction samples were negative except for one sample in which 5S rRNA DNA equivalent to 2 c.f.u. *L. pneumophila* was detected although *mip* DNA was negative. No inhibition was found throughout the test. The results suggested that DNA from *Legionella* species was present in the sputum sample at very low levels.

By environmental investigation conducted on the 9th day of admission, a sample from the bath water of the hot spring was collected. The chlorine concentration of the water was 0.1 mg $l^{-1}$, and *L. rubrilucens* and *L. pneumophila* were isolated from the sample at 60 c.f.u. per 100 ml and 40 c.f.u. per 100 ml, respectively. Bathwater was drained and changed every day and some bathtubs were equipped with hydrotherapy jet circulation. The *L. pneumophila* strain was identified by PCR and was determined as serogroup 15 by the Dresden panel of monoclonal antibodies (Helbig *et al.*, 1997). Monovalent antisera specific for *L. pneumophila* serogroups 1–15 (Denka Seiken) were non-reactive. Using the antisera, some strains of *L. pneumophila* assigned to serogroups 4, 10 or 15 by the Dresden panel have been grouped into non-reactive strains (unpublished data). The sequence type of the *L. pneumophila* was *flaA* (10), *pile* (10), *asd* (7), *mip* (28), *mompS* (16), *proA* (18), *neuA* (6) (Gaia *et al.*, 2005; Ratzow *et al.*, 2007). Because this was a new profile, it was sent to the EWGLI-SBT database (http://www.hpa-bioinformatics.org.uk/legionella/legionella_sbt/php/sbt_homepage.php) and assigned the number ST768. The DNA restriction profile of *L. rubrilucens* from the patient's sputum and the hot-spring water revealed that the isolates were indistinguishable (Fig. 2a), while epidemiologically unrelated *L. rubrilucens* strains showed different profiles (Fig. 2b).

Subsequently, using a Biotest enzyme immunoassay, we examined the reactivity of *Legionella* soluble antigens (Okada *et al.*, 2002) from heat-killed McFarland 4 cell suspensions of the clinical and environmental *L. rubrilucens* strains and an environmental *L. pneumophila* strain. *L. pneumophila* serogroup 15 soluble antigen, but not *L. rubrilucens* soluble antigen, showed a positive enzyme immunoassay reaction, suggesting that the positivity of the urine *Legionella* antigen test might be due to the infection with the serogroup 15 *L. pneumophila* strain. Indirect fluorescence antibody titres against *L. rubrilucens* and *L. pneumophila* isolates of serum obtained 3 months after infection were examined, but they were very low ($\leqslant$1 : 16, interpreted as a negative response). The in-house formalin-killed *L. pneumophila* serogroup 1 Philadelphia-1 (type strain) was used as a control antigen. A titre of 1 : 256 positive antiserum was used as a control.

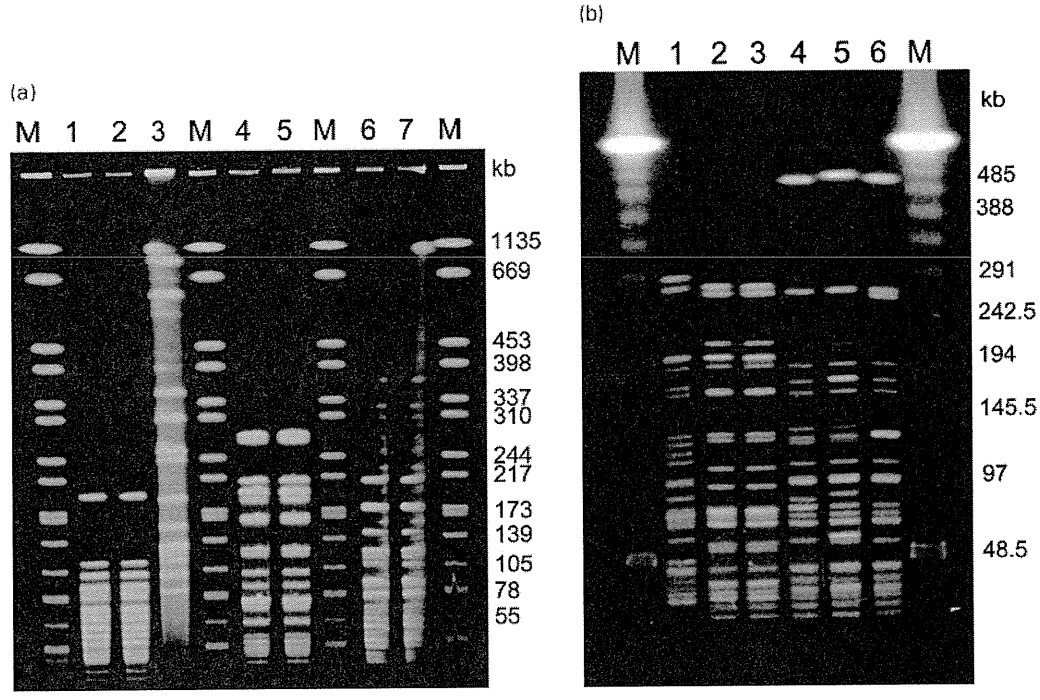


**Fig. 2.** (a) Restriction profiles of *L. rubrilucens* digested with *Sfi*I, *Xba*I and *Not*I using PFGE. The *L. rubrilucens* isolates from the patient's sputum and the hot-spring water were analysed. For the electrophoresis, a linearly ramped switching time from 5 to 50 s was applied for 19 h at 6 V $cm^{-1}$ and 14 °C. Lanes: M, molecular marker (*Salmonella* Braenderup, *Xba*I digestion); 1, 4 and 6, *L. rubrilucens* from the patient's sputum; 2, 5 and 7, *L. rubrilucens* from the hot-spring water; 3, *L. pneumophila* serogroup 15. Lanes 1, 2 and 3, *Sfi*I digestion; lanes 4 and 5, *Xba*I digestion; lanes 6 and 7, *Not*I digestion. (b) Restriction profiles of *L. rubrilucens* isolates from the patient, the hot-spring water and unlinked environments digested with *Xba*I. A linearly ramped switching time from 0.3 to 30 s was applied for 16 h at 6 V $cm^{-1}$ and 14 °C. Lanes: M, molecular marker (lambda ladder); 1, *L. rubrilucens* WA-270A-C2 (ATCC 35304); 2, *L. rubrilucens* from the patient; 3, *L. rubrilucens* from the hot-spring water; 4, *L. rubrilucens* NIIB 0335 (Thai NIH 8211) from cooling tower water; 5, *L. rubrilucens* NIIB 0337 (Thai NIH 8383) from cooling tower water; 6, *L. rubrilucens* NIIB 0345 (Thai NIH 8568) from cooling tower water.

From the 4th day of admission, we administered clarithromycin (CAM) at 400 mg per day and meropenem at 1 g per day. After 3 days of treatment, since the fever alleviated, we stopped meropenem but continued CAM. Finally, by the 10th day of administration, the patient's clinical symptoms had improved, and the laboratory data such as white blood cell count and C-reactive protein value, and the shadows in chest X-ray, returned to normal on the 13th day of administration.

## Discussion

The causative agent of *Legionella* pneumonia is the genus *Legionella*, which is commonly found in hot springs, soil and water supply facilities. *Legionella* species accounted for 3–10 % of causative pathogens for all community-acquired pneumonia cases in a worldwide survey (Miyashita *et al.*, 2006).

Since a new infectious diseases control law in Japan was enacted in April 1999, legionellosis cases classified as Category-4-notifiable infectious diseases must be notified to the proper agencies as well as to the prefecture governor (Ministry of Health, Labour and Welfare in Japan, 1999). In the present case, since the urine *Legionella* antigen was positive, we reported this legionellosis case to the local health centre. In order to elucidate the source of the infection, *Legionella* was cultivated from the patient's sputum and the hot-spring water and DNA analysis of the *L. rubrilucens* strains by PFGE was performed. The clinical and environmental *L. rubrilucens* strains were indistinguishable. A final diagnosis is recommended to be made by criteria that include (a) isolation of *Legionella* from clinical specimens, (b) detection of *Legionella* DNA by PCR in clinical specimens, (c) a positive response for urine *Legionella* antigen and (d) an increase in serum anti-*Legionella* antibody titres. The final diagnosis was made by (a), (b) and (c) in our case. Urinary antigen detection is a rapid and easy test and can detect most cases of legionellosis caused by *L. pneumophila* serogroup 1. However, without isolation of clinical strains, the source of infection cannot be definitely confirmed and, as found in our study, the possibility of mixed infection remains unrecognized. Therefore, clinical specimens for *Legionella* isolation should always be cultured.

There has only been one report of *L. rubrilucens* infection as far as we know (Berger *et al.*, 2006). In that study, two patients had increased serum titres for the bacterium: one patient with seroconversion (high level of evidence, fourfold increase in antibody titre between acute and convalescent-phase serum samples or seroconversion from 0 to 1 : 100) and one patient with single high titre (low level of evidence, >1 : 400) for *L. rubrilucens* among 18 patients where infectious amoeba-associated agents were identified in a study of 157 intensive-care units patients with 210 episodes of pneumonia. However, to our knowledge, a culture-positive case of *L. rubrilucens* has never been reported.

In the present case, the patient was a healthy middle-aged man with no underlying disease. Since at first the patient had fever, shivering, weariness all over the body and muscle pain but no respiratory symptoms, we suspected that he was suffering from influenza. On the 3rd day of hospitalization, we heard a fine crackle sound in the left middle and lower lobes, and then we decided to reconsider the cause of his symptoms. After learning that the patient had been bathing in a hot spring, we examined the possibility of *Legionella* infection. This case emphasizes the importance of getting precise information about the patient's current history to detect unknown causes of pneumonia.

It is not easy to prove the pathological role of *L. rubrilucens* in the pneumonia in this case. The patient was thought to be apparently healthy; however, he had smoked for 34 years and had a habit of alcohol drinking. Both smoking and drinking alcohol are thought to be risk factors for infection. In this regard, it can be suggested that the patient was at risk of developing legionellosis when he was exposed to *Legionella* in the hot-spring water. *L. rubrilucens* was detected from the sputum obtained at least 6 days after bathing in the hot-spring water, suggesting that *L. rubrilucens* in the sputum was not merely exhibiting colonization and that this was a dual infection with *L. pneumophila* and *L. rubrilucens*. It is likely that the *L. pneumophila* was the primary infecting agent in the patient. Alternatively, *L. pneumophila* infection and temporal colonization by *L. rubrilucens* from spa bath water was possible.

In the early stages of *Legionella* pneumonia, some patients present various symptoms such as fever, cough, sputum, diarrhoea, impaired level of consciousness, etc., without respiratory symptoms (Yagyu *et al.*, 2003). In the present case, the patient complained only of fever and joint pain without respiratory symptoms. In two large outbreaks of legionellosis in Japan, the patients presented light or no respiratory symptoms at the first visit (Yagyu *et al.*, 2003; Sasaki *et al.*, 2008). In *Legionella* pneumonia, infiltrative shadows often appear 3 days after the first visit (Kirby *et al.*, 1979; Kroboth *et al.*, 1983). Taking into account these findings, it is important to note that in the early stages of *Legionella* pneumonia, some patients may have no respiratory symptoms.

Concerning the shadows in the chest X-rays of patients with *Legionella* pneumonia, interstitial shadows, infiltration and consolidation have been reported (Kirby *et al.*, 1979; Kroboth *et al.*, 1983). Pulmonary infiltrates are predominantly seen in the lower lobe, sometimes bilaterally, and there is rapid progression and pleural effusion (Kroboth *et al.*, 1983; Dietrich *et al.*, 1978; Tan *et al.*, 2000). High-resolution computed tomography also revealed that air-space consolidation, ground-glass opacity and pleural effusion were common features in patients with Legionnaires' disease and that the shadows show a peripheral and bilateral distribution in multiple segments (Yagyu *et al.*, 2003; Matsumoto *et al.*, 2008). In our case, we found ground-glass or light infiltrative shadows in the left middle and lower lobes.

Treatments for *Legionella* pneumonia have been described in the guidelines for the management of adults with community-acquired pneumonia (Miyashita *et al.*, 2006; Niederman *et al.*, 2001; Bartlett *et al.*, 1998; Committee for The Japanese Respiratory Society Guidelines for the Management of Respiratory Infections, 2006). Macrolides including erythromycin, CAM, etc., have been recommended for the treatment of *Legionella* pneumonia. In our case, the patient recovered completely with 13 days of CAM at 400 mg per day. Although *Legionella* pneumonia tends to progress rapidly to severe pneumonia, many patients can be cured if given appropriate early therapy.

In conclusion, in the present case, *L. rubrilucens* was isolated from the patient's sputum and from hot-spring water by culturing, and PFGE revealed that the isolates from these two sources were indistinguishable. To our knowledge, this is the first case in which human airway infection or colonization by environmental *L. rubrilucens* has been confirmed.

## Acknowledgements

We thank Wantana Paveenkittiporn (National Institute of Health, Thailand) for providing the *L. rubrilucens* isolates, Michio Koide (Department of Medicine and Therapeutics, University of the Ryukyus) for providing human antiserum, Shinji Izumiyama (Department of Parasitology, National Institute of Infectious Diseases) for his technical advice, Jürgen Herbert Helbig (Institute for Medical Microbiology and Hygiene, Technical University Dresden) for monoclonal antibody typing and Kaori Iwabuchi (Iwate Prefecture Research Center for Environmental Health) for helpful advice.

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