Isolation of *Legionella* Species from Hot Spring Bath Water Samples in Japan, and the Antibiotic Susceptibility of the *L. pneumophila* Isolates

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Abstract: In order to obtain an understanding of the inhabitation of *Legionella* spp. in hot spring bath water, the isolation of *Legionella* spp. was attempted in 30 prefectures nationwide in 2009-2010, and the following results have been obtained. 1) Isolation of the bacteria was achieved for 89 out of 366 samples (24.3%). When this was broken down into individual regions, the isolation rate in Kyushu and Okinawa regions was the highest at 58.6%, followed by Hokkaido at 42.4%. With regard to isolation by facility, isolation was achieved at 49 out of 124 facilities (39.5%). 2) In the 89 samples form which genus *Legionella* were isolated, counts of *Legionella* spp. in 10 ml of hot spring water were less than 10⁵ CFU in 45 samples (50.6%), followed by 28 samples within the 10⁴ CFU range (31.5%). 3) Of the bacterial species of the 111 strains isolated from the 89 samples of hot spring water collected from all over the country, the bacterial strain that was most frequently identified was *Legionella pneumophila*, and it accounted for 78 strains (69.6%). Among these strains by serogroup, group 1 was the most common with 25 strains (32.1%), followed by group 4 with 17 strains (21.8%). Moreover, out of the bacterial strains that were identified other than *Legionella pneumophila*, *Legionella londiniensis* was the second most common, with 20 strains (17.9%), followed by *Legionella micdadei* with 5 strains (4.5%). 4) The 90% MIC rate (MIC₉₀) of *Legionella pneumophila* isolates (82 strains) was tested with 10 drugs. Out of the 10 drugs, rifampicin showed the best antimicrobial activity of 0.016 μg/ml, followed by imipenem at 0.5 μg/ml. However, isolates showed high resistance to minocycline and piperacillin with the MIC₉₀ of minocycline at 16 μg/ml, and that of piperacillin at >256 μg/ml.

Key words: *Legionella pneumophila* / Antibiotic susceptibility / Hot spring bath waters

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Legionella spp. are a group of pathogenic bacteria that infect the respiratory system\(^1\), and about 50 species belonging to this genus have been reported. It is well known fact that Legionella spp. inhabit the soil close to human habitations\(^2\), and it is presumed that they enter artificial water environments such as hot spring water. It is believed that Legionella spp. are ingested by ameba and that the resulting Legionella spp. are scattered as aerosols, which can infect humans and cause respiratory diseases such as pneumonia\(^1\).

In 2002, a mass outbreak of legionellosis transmitted through hot spring water in Miyazaki Prefecture, Japan, highlighted the importance of the hygienic management of bath water\(^3\),\(^4\). We have been investigating the distribution of Legionella spp. in the water of all of the hot springs in Japan for several years\(^5\),\(^6\). In our country, the measures for preventing contamination with Legionella spp. in hot spring bath water is performed by local government, and hygiene guidance is provided to each facility. Five years have passed under this system since the last nationwide investigation.

To investigate the current contamination of Legionella spp. in hot spring water nationwide, we attempted to isolate Legionella spp. from hot spring water samples from various regions, and investigated the drug sensitivity of the isolates.

**MATERIALS AND METHODS**

**Samples**

A total of 366 hot spring water samples were collected in sterile polyethylene containers (300 ml) in 30 prefectures in Japan between April 2009 and March 2011. The samples were stored in a refrigerator until they were cultured, and tested.

**Isolation and identification**

Two hundred milliliters of hot spring water samples were concentrated to 1 ml by centrifugation at 6,000 rpm for 30 min. This concentrate was mixed with an equal volume of 0.2 M HCl-KCl solution (pH 2.2), and the mixture was treated at room temperature for 15 min, followed by inoculation of 100 µl of sample onto GVPC\(\alpha\) medium (Nikken Bio Medical Laboratory Inc., Kyoto, Japan) with a Conradi stick. After incubation for 7 d at 36°C, several colonies of each isolate suggestive of the genus Legionella were picked up, smeared onto a 2-compartment agar plate (Nikken Bio Medical Laboratory Inc., Kyoto, Japan) consisting of BCYE\(\alpha\) and blood agar media, and subjected to pure culture and testing for cystine requirements. After 3 d of culture at 36°C, the
isolates that did not grow on blood agar medium but grew on BCYEα medium alone were presumed to belong to the genus *Legionella*. The isolates were identified using the immunochromatographic assay (Duopath® Legionella; Merck Ltd. · Japan, Tokyo) and the immune serum agglutination test (Denka Seiken Co. Ltd., Tokyo, Japan). In addition, genetic identification was performed as described in a previous article8).

**Susceptibility testing**

Antibiotic susceptibility was determined using the Etest (SYSMEX bioMérieux Co., Ltd., Tokyo) according to the manufacturer’s technical guide. The drugs tested were erythromycin (EM), clarithromycin (CAM), azithromycin (AZM), minocycline (MINO), levofloxacin (LVFX), ciprofloxacin (CPFX), pipercillin (PIPC), imipenem (IPM), gentamicin (GM) and rifampicin (RFP) (total 10 drugs). A bacterial cell suspension (0.5 ml) was dripped onto 60 ml of BCYEα agar in a 150-mm dish (Corning Inc., USA) and smeared over the surface using a Conradi stick, and Etest strips were securely attached to the medium. The plates were cultured at 36°C for 5 d, and the growth inhibition zone formed around the strip was read. The MIC was judged by macroscopically reading the graduation at which the end of the growth inhibition zone and the strip crossed.

**RESULTS**

**Isolation of Legionella spp.**

The state of isolation of *Legionella* spp. from hot spring water nationwide is shown in Table 1. Isolation was achieved from 89 out of 366 samples (24.3%). When this is broken down into individual regions, the isolation rate in the Kyushu and Okinawa regions was the highest at 58.6%, followed by Hokkaido at 42.4%. In the other regions, the order was the Tohoku region at 33.3%, Chubu region at 20.5%, Kanto region at 20.2%, Chugoku region at 15.8%, and Kinki region at 11.8%. There was no isolation of bacteria from the Shikoku region. With regard to the isolation form each facility, isolation was achieved at 49 out of 124 facilities (39.5%).

The collected water samples were classified into two groups, indoor spa and open-air bath, and the results of the isolation rates in each group are shown in Table 2. Isolation was achieved in 48 out of 239 samples (20.1%) from indoor spas, and 41 out of 127 samples (32.3%) from open-air baths; the isolation rate for the open-air baths was significantly higher.

Fig. 1 shows the count of *Legionella* spp. in 100 ml of hot spring water in the 89 samples from which *Legionella* spp. were isolated. Counts of less than 10² CFU were found in 45 samples (50.6%), followed by 28 samples
within the $10^2$ CFU range (31.5%), 13 samples within the $10^3$ CFU range (14.6%), and 3 samples with over $10^4$ CFU (3.4%). Although the counts for the genus were low, we were able to verify that *Legionella* spp. widely inhabit hot spring waters all over Japan.

Fig. 2 shows the bacterial species of the 112 strains isolated from the 89 samples of hot spring water collected from all over the country that were positive for *Legionella* species. Out of these strains, the bacterial strain that was most frequently identified was *L. pneumophila*, accounting for 78 strains (69.6%) (Fig. 2-A). Among these 78 strains, by serogroup, group 1 was the most common, at 25 strains (32.1%), followed by group 4 with 17 strains (21.8%), group 9 with 8 strains (10.3%), group 5 with 7 strains (9.0%), groups 6 and 8, each with 5 strains (6.4%), and group 3 with 4 strains (5.1%). Isolates of groups 7, 10, 11, 13 and 15 were also found, although the rates for these were low. However, there was not a single strain form groups 2, 12 or 14 (Fig. 2-B). Moreover, out of the bacterial strains that were identified other than *L. pneumophila*, *L. londiniensis* was the most common with 20 strains (17.9%), followed by *L. micdadei* with 5 strains (4.5%). Other than these, *L. oakridgensis* (3 strains, 2.7%), *L. bozemanii* and *L. israelensis* (each with 2 strains, 1.8%), *L. dumoffii* and *L. jamestowniensis* (each with 1 strain, 0.9%) were also isolated.

**Drug sensitivity of *L. pneumophila* isolates**

Table 3 shows the MIC distribution of the 10 drugs for the 82 strains of *L. pneumophila* (serogroup 1, 30 strains; serogroup 3, 5 strains; serogroup 4, 17 strains; serogroup 5, 8 strains; serogroup 6, 2 strains; serogroup 8, 7 strains; serogroup 9, 5 strains; serogroup 10, 4 strains; serogroup 11, 2 strains; serogroup 13, 1 strain; and serogroup 15, 1 strain). With regard to the MIC distribution, EM was 0.032-1 µg/ml, and the peak (mode) was 0.25 µg/ml, CAM was 0.125-1 µg/ml, and the peak was 0.5 µg/ml, AZM was 0.064-4 µg/ml, and the peak was 0.25 µg/ml, MINO was 0.5-64 µg/ml, and the peak was 8 µg/ml, LVFX was 0.25-2 µg/ml, and the peak was 0.5 µg/ml, CFIX was 0.25-4 µg/ml, and the peak was 1 µg/ml, IPM was 0.016-32 µg/ml, and the peak was 0.064 µg/ml, GM was 1-4 µg/ml, and the peak was 2 µg/ml, RFF was 0.008-0.032 µg/ml, and the peak was 0.016 µg/ml. However, PIPC was found to have a wide range, over 0.064-256 µg/ml, and gradual peaks were observed at 64 µg/ml.
and >256 µg/ml, indicating that it had a bimodal distribution of efficacy. There were 17 strains that showed a strong resistance of 256 µg/ml to PIPC. Furthermore, the MIC distribution by serogroup was also studied, but the result for the serogroups resembled the separation as a whole, and no marked characteristics based on serogroup were found.

Table 3 shows the 90% MIC rate (MIC_{90}) for each drug. When the MIC_{90} for each drug tested was compared, out of the 10 drugs, RFP showed the highest antimicrobial activity of 0.016 µg/ml, followed by IPM at 0.5 µg/ml, EM, CAM, AZM, and LVFX at 1 µg/ml, and CIPFX and GM at 2 µg/ml. However, isolates showed a high rate of resistance to MINO and PIPC, with MINO at 16 µg/ml, and PIPC at >256 µg/ml.

**DISCUSSION**

Japan has one of the highest densities of hot springs in the world. According to a 2009 report by the Ministry of the Environment, there are more than 28,000 wellsprings and about 15,000 accommodation facilities. Exhaustion of wellsprings and scale-up of facilities form the background to the incresce in *Legionella* spp. contaminations in facilities.

We performed a nationwide survey of *Legionella* species in hot spring bath water in 2003 through 2004. Although the isolation rate markedly varied among prefectures, the bacteria were isolated from 204 of a total of 710 samples (28.7%), clarifying that the bacteria inhabit hot spring water in all prefectures from northern Hokkaido through to southern Kyushu.

In this current survey, performed five years after the previous one, *Legionella* spp. were isolated from 89 of a total of 366 samples (24.3%). During this period, education to prevent legionellosis and instruction in environmental hygiene surveillance at actual sites have been strengthened, but the detection rate did not markedly change from that of 5 years ago, showing the difficulty in controlling *Legionella* spp. which inhabit the natural environment of below the detection limit.

When the *Legionella* spp. isolation rate was compared between hot spring water samples collected from indoor spas and open-air baths, the rate of isolation from indoor spas was slightly higher than was noted 5 years ago, but that from open-air baths was still higher in this survey. Although the reason for this difference was unclear, there may be more opportunities to transfer *Legionella* spp. into open-air baths, such as transfer by fine particles, and the thorough cleaning of rock-made baths is difficult. Therefore, we considere farther hygiene control is necessary.

The numbers of bacteria detected were less than 100 CFU/100 ml in 45 samples, accounting for 50.6% of the positive samples in this survey. The counts were 100-990 and 1,000-9,900 CFU/100 ml in 31.5% and 14.6%, respectively, showing that the number of samples containing the bacteria at a detectable level decreased as...
the bacterial count increased. However, samples containing the bacteria at 10,000 CFU/100 ml accounted for 3.4% of the total, a fact which should attract attention. These numbers of detected bacteria was very similar to that noted in the previous survey 5 years ago.

With regard to the identification of the isolated species, \textit{L. pneumophila} was dominant, as in the previous survey. However, by serotype, while the frequency of serogroup 1 was commonly high, those of serogroups 5 and 6 were higher in the previous survey, whereas those of serogroups 4 and 9 were higher in this survey. In previous reports, the relatively abundant isolates in hot spring water were serogroups 4, 5, and 6, whereas serogroup 1 was frequently isolated from cooling towers. However, in this survey, serogroup 1 was frequently isolated from hot spring water. The reason for this is unclear, but the findings indicate that inhabitation by serogroup 1 is not limited to cooling towers.

We introduced gene testing into the identification procedure, and species other than \textit{L. pneumophila} were isolated in 30.4% of positive samples, which was higher than in the previous survey. In particular, there were 20 strains of \textit{L. londiniensis}, and the isolation rate was 17.9%, showing its wide inhabitation of hot spring water in Japan. The pathogenicity of this species may be relatively weak, but it was isolated in the biggest outbreak of legionellosis in Japan which occurred in 2002. In addition, species not previously reported in Japan, such as \textit{L. israelensis}, \textit{L. jamestowniensis}, and \textit{L. oakridgensis}, were also identified, although the numbers of isolates were small.

In the drug sensitivity tests of \textit{L. pneumophila} from hot spring bath water, the MIC values of RFP and IPM were low, showing the high sensitivity, whereas those of PIPC and MINO were high, showing the high resistance. These findings were unchanged from those for isolates from hot spring water 5 years ago. Murakami et al. measured MIC of clinical isolates in Japan using the Etest, in which the MIC values of 9 drugs, excluding GM, were low for clinical isolates, thus showing the high sensitivity. The MIC distribution of hot spring-derived strains showed a slight shift toward resistance, compared to clinical isolates, but no rapid emergence of resistance was observed.

At present, no regulatory baseline count of \textit{Legionella} spp. has been established for hot spring water, so the baseline value set for bath water was applied. This baseline value is less than 10 CFU/100 ml, which actually represents no detection. However, clearing the baseline value does not rule out the presence of \textit{Legionella} spp. Since the quality of hot spring water varies greatly, and hot spring water supply systems vary among facilities, it is difficult to propose uniform countermeasures for contamination. Indiscriminate disinfection of bathtub water with high-concentration chlorine may not necessarily be the best way. For the hygienic maintenance and control of hot spring water, it is important to closely investigate the habitation state of \textit{Legionella} spp., and it is strongly desirable for each facility to take adequate actions in close consideration of the quality of host spring water and the characteristics of the supply system. Continuous hygiene control may prevent the occurrence of legionellosis.

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REFERENCES


