Isolation of *Legionella* Species from *Noyu* (Unattended Natural Hot Springs in Mountains and Fields) Samples in Japan

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In order to understand the habitation conditions of the bacteria of the genus *Legionella* in *Noyu* (unattended natural hot springs in mountains and fields) in Japan, isolation of *Legionella* spp. was attempted in the *Noyu* samples from 11 prefectures nationwide between May and September 2012, and the following results were obtained. Overall, *Legionella* spp. was isolated from 16 of 43 samples (37.2%). The species was isolated from the Hokkaido region to the Chugoku region but not from the Shikoku region to the Kyushu region. The number of bacteria detected was usually small, less than $5.0 \times 10^1$ CFU/100 ml, as found in 11 samples (68.8%), while counts of $10^2$ or more to $10^3$ or less CFU/100 ml were found in two samples (12.5%). *Legionella pneumophila* was the most commonly found strain, with 19 strains (90.5%) found, and was the dominant species. Regarding the serogrouping, four strains (21.1%) fell under group 1, the most common grouping, followed by three strains (15.8%) in group 3, two strains (10.5%) in group 5, etc. Moreover, the detected bacterial strains other than *L. pneumophila* included two strains (9.5%) of *L. Iondeniensis*. The temperature of the *Noyu* from which *Legionella* spp. was isolated was between 33.1°C and 41.5°C with a pH ranging from 5.2 to 8.1. The present report is the first report to clarify the habitation conditions of strains of *Legionella* spp. isolated from *Noyu* in Japan.

Key words: *Legionella pneumophila* / Hot spring water / *Noyu* (unattended natural hot spring water).

There are hot springs in Japan called “*Noyu,*” some of which are naturally flowing hot springs in mountains, rivers and lakes that are used for bathing by hot spring lovers. However, no accommodation facilities are available in the vicinity of these hot springs. Furthermore, some *Noyu* do not have bath tubs or circulation facilities, and water from these hot springs flows constantly, resulting in a lack of water sanitization, as these springs are not subject to the Public Bath House Act.

In 2002, a mass outbreak of legionellosis transmitted through hot spring water in Miyazaki Prefecture, Japan (Yabuuchi and Agata, 2004) highlighted the importance of hygienic management of bath water (Kawano et al., 2007). Many hot spring bathing facilities attached to accommodation facilities have circulation facilities in which sanitization with chlorine agents at a concentration of 0.2-0.4 mg/l free residual chlorine is used, in accordance with the Manual for Countermeasures to Legionellosis, 3rd. ed. (Building Management Education Center, 2009).

We have been investigating the contamination of the water of hot springs with *Legionella* spp. in Japan for several years (Furuhata et al., 2009; 2010; 2011a; 2011b; 2012a; 2012b). In a recent investigation, isolation of *Legionella* spp. from hot spring bath water...
samples was achieved in 89 of 366 (24.3%) cases (Furuhata et al., 2011a).

In this study, to investigate the distribution of Legionella spp. in Noyu (unattended natural hot springs in mountains and fields) nationwide, we attempted to isolate Legionella spp. from Noyu samples obtained from various regions.

A total of 43 Noyu samples were collected from 11 prefectures in Japan using sterile polyethylene containers (500 ml) between May and September 2012. The samples were stored in a refrigerator until tested as follows.

The Noyu samples (200 ml) were concentrated to 1 ml using centrifugation at 6,000 rpm for 30 min. The concentrate was mixed with an equal volume of a 0.2-M HCl-KCl solution (pH 2.2), and the mixture was kept at room temperature for 15 min followed by inoculation of a 100-μl sample onto GVPCα medium (Nikken Bio Medical Laboratory Inc., Kyoto, Japan) with a Conradi stick. Following incubation for seven days at 36°C, several colonies of each isolate suggestive of the genus Legionella were removed, smeared onto a two-compartment agar plate (Nikken Bio Medical Laboratory Inc., Kyoto, Japan), consisting of BCYEα and blood agar medium, and subjected to pure culture and testing for cysteine requirements. After five days of culture at 36°C, the isolates that did not grow on the blood agar medium but grew on the BCYEα medium alone were presumed to belong to the genus Legionella. Next, the isolates were identified using an immunochromatographic assay (Duopath® Legionella; Merck Ltd., Japan, Tokyo) and an immune serum agglutination test (Denka Seiken Co. Ltd., Tokyo, Japan). In addition, genetic identification was performed according to a previous article (Edagawa et al., 2008).

Each sample (1 ml) was mixed with Desoxycholate agar (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) to count red colonies after 20 h of incubation at 36°C.

First, coliform groups and Escherichia coli were examined in 100 ml of the sample using the Colilert (IDEXX Laboratories, Inc., USA) according to the manufacturer’s method. Subsequently, one loopful was removed from the container with positive coliform groups and smeared in streaks onto EMB agar medium (Nissui Pharmaceutical Co. Ltd., Tokyo). After 24 h of incubation at 36°C, bacteria in different forms were extracted for incubation in nutrient agar media (Nissui Pharmaceutical Co. Ltd., Tokyo) at 36°C for 24 h to obtain pure cultures of the various strains. The API20E (SYSMEX bioMérieux Co. Ltd., Tokyo) was used for the examinations to identify the bacterial strains, allowing the bacterial strains to be confirmed at an identification probability of more than 85%.

The isolation of Legionella spp. from Noyu nationwide is shown in TABLE 1. Isolates were obtained in 16 out of 43 samples (37.2%). When assessed according to individual prefectures, the isolation rates in Nagano and Shimane prefectures were the highest at 100%, followed by Yamagata prefecture at 66.7%, Niigata and Wakayama prefectures at 50%, Gunma prefecture at 33.3%, Hokkaido prefecture at 22.2% and Toyama prefecture at 16.7%. No Legionella spp. were isolated from Gifu, Tokushima or Kagoshima prefectures.

TABLE 2 shows the counts of Legionella spp. in 100 ml of Noyu among the 16 samples from which they were isolated. Less than 5.0 × 10⁵ CFU were found in 11 samples (68.8%), followed by 6.0 × 10⁴ CFU in three samples (18.8%) and over 10⁵ CFU (1.1 × 10⁶ CFU and 3.0 × 10⁵ CFU) in two samples (12.5%). As mentioned above, although the counts were low, we were able to verify that Legionella spp. do in fact widely inhabit Noyu throughout Japan.

The composition of the bacterial species of the 21 strains isolated from the 16 samples of Noyu collected throughout the country that were positive for Legionella species is shown in TABLE 2. Of these strains, L. pneumophila was most frequently identified, accounting for 19 strains (90.5%). Among these 19 strains, according to the serogroup, group 1 was the most common, with four strains (21.1%), followed by group 3 with three strains (15.8%), group 5 with two strains (10.5%) and groups 6 and 7, each with one strain (5.3%). With respect to the serogroup, the number of grouped strains was small, at 11 strains (57.9%), while the remaining eight strains (42.1%) could not be grouped. Moreover, of the bacterial strains identified other than L. pneumophila, L. londiniensis was the most common, at two strains (9.5%). Therefore, no correlations were observed between the serogroup and the prefecture in which the strains were isolated.

### TABLE 1. Isolation of Legionella spp. from Noyu (unattended natural hot springs) samples in Japan.

| Prefectures | No. of samples examined | No. of positive samples | (%) |
|-------------|-------------------------|-------------------------|-----|
| Hokkaido    | 9                       | 2                       | 22.2|
| Yamagata    | 6                       | 4                       | 66.7|
| Gunma       | 3                       | 1                       | 33.3|
| Nagano      | 3                       | 3                       | 100.0|
| Niigata     | 2                       | 1                       | 50.0|
| Toyama      | 6                       | 1                       | 16.7|
| Gifu        | 1                       | 0                       | 0.0 |
| Wakayama    | 2                       | 1                       | 50.0|
| Shimane     | 3                       | 3                       | 100.0|
| Tokushima   | 1                       | 0                       | 0.0 |
| Kagoshima   | 7                       | 0                       | 0.0 |
| **Total**   | **43**                  | **16**                  | **37.2** |
The temperature of the Noyu from which Legionella spp. was isolated was between 33.1°C and 41.5°C with a pH ranging from 5.2 to 8.1 (TABLE 2).

Among the 43 samples of Noyu, coliform groups were detected in 27 samples (62.8%). Although a number of strains had counts within the range of 1 CFU/ml to 8.1×10^5 CFU/ml, counts of less than 5.0×10^3 CFU/ml were found in 21 samples (77.8%), accounting for more than 50% (TABLE 3).

The samples were roughly classified according to the number of coliform groups, 0 CFU/ml or ≥1 CFU/ml, in association with the detection status of Legionella spp. as shown in TABLE 3. Specifically, there were 12 samples (27.9%) with ≥1 CFU/ml coliform groups in which Legionella spp. was found and another 12 samples (27.9%) with 0 CFU/ml coliform groups in which no Legionella spp. was found, yielding a concordance rate for both groups of 55.8%. On the other hand, there were 15 samples (34.9%) with ≥1 CFU/ml coliform groups in which no Legionella spp. was found, yielding a concordance rate for both groups of 44.2%. Based on the above findings, no correlations were found between the number of coliform groups and the detection status of Legionella spp. Furthermore, although correlations were examined among the 12 samples with ≥1 CFU/ml coliform groups in which Legionella spp. was found, no significant trends were observed.

Thirty-six of the 43 samples (83.7%) were found to be positive for coliform groups using the Colilert test. Among these, 31 samples (86.1%) were also positive for E. coli, displaying high positive rates for both species.

The identification results for the Enterobacteriaceae bacteria isolated from the samples of positive coliform groups, depending on the detection rate of Legionella spp., are shown in TABLE 4. The Enterobacteriaceae strain isolated with the highest frequency was E. coli, which was found in 17 strains (38.6%). This was followed by eight strains of Enterobacter aerogenes (18.2%), seven strains of Enterobacter cloacae (15.9%) and five strains of Klebsiella oxytoca (11.4%). With respect to the isolation status according to the detection rate of Legionella spp., comparable levels were displayed, showing no significant trends.

**TABLE 2. Noyu (unattended natural hot springs) samples with positive Legionella spp.**

| No. | Sample No. | Origin  | °C | pH | CFU/100ml | Species   | Serogroup |
|-----|------------|---------|----|----|-----------|-----------|-----------|
| 1   | N-35       | Hokkaido|    |    | 30        | L. pneumophila | 5        |
| 2   | N-36       | Hokkaido| 41.5| 5.3| 40        | L. pneumophila | 1, 5     |
| 3   | N-28       | Yamagata|    |    | 60        | L. pneumophila | UT*       |
| 4   | N-29       | Yamagata|    |    | 60        | L. londiniensis |          |
| 5   | N-30       | Yamagata| 38.6| 5.2| 60        | L. londiniensis |          |
| 6   | N-31       | Yamagata|    |    | 20        | L. pneumophila | 1        |
| 7   | N-11       | Gunma   | 40.0| 5.5| 40        | L. pneumophila | 3, 7      |
| 8   | N-20       | Nagano  | 35.4| 4.8| 110       | L. pneumophila | 3, UT     |
| 9   | N-21       | Nagano  | 33.1| 5.4| 30        | L. pneumophila | 1, UT     |
| 10  | N-39       | Nagano  |    |    | 40        | L. pneumophila | UT        |
| 11  | N-23       | Niigata | 41.3| 6.2| 30        | L. pneumophila | UT        |
| 12  | N-18       | Toyama  |    |    | 20        | L. pneumophila | 6, UT     |
| 13  | N-10       | Wakayama| 39.5| 8.1| 10        | L. pneumophila | 1        |
| 14  | N-25       | Shimane |    |    | 20        | L. pneumophila | UT        |
| 15  | N-26       | Shimane | 39.9| 6.7| 10        | L. pneumophila | 3        |
| 16  | N-27       | Shimane | 38.4| 5.4| 300       | L. pneumophila | UT        |

*: Untypable
*: No data

**TABLE 3. Number of coliforms in Noyu (unattended natural hot springs).**

| Legionella spp. | Number of coliforms (CFU/ml) | Total |
|-----------------|-----------------------------|-------|
| Positive (≥1CFU/100ml) | 4 (25.0) | 9 (56.2) | 0 | 2 (12.5) | 1 (6.3) | 16 (100) |
| Negative (<1CFU/100ml) | 12 (44.4) | 12 (44.4) | 2 (7.4) | 0 | 1 (3.7) | 27 (100) |
| Total | 16 (37.2) | 21 (48.7) | 2 (4.7) | 2 (4.7) | 2 (4.7) | 43 (100) |

*: Number of samples (%)
The important purpose of this study was to gain an understanding of whether Legionella spp. substantially inhabits natural hot springs in unattended areas in mountains and fields (Noyu). In this study, Legionella spp. was isolated from 16 (37.2%) of 43 Noyu samples obtained from various locations across Japan, clarifying for the first time that Legionella spp. is distributed in unattended naturally occurring hot springs. We have been researching the habitation of Legionella spp. in the hot spring waters of bathing facilities nationwide for some time (Furuhata et al., 2004), isolating Legionella spp. from 89 of 366 specimens (24.3%) in a study carried out from 2009 to 2010 (Furuhata et al., 2011a).

Interestingly, when comparing the isolation rates of our different studies, the isolation rate of Legionella spp. from unattended natural hot springs was higher in the present study. In our study carried out in 2001 investigating the presence of Legionella spp. in the soil in various locations in Japan, Legionella spp. was isolated from 86 of 1,362 samples (6.3%), thus indicating that this bacteria is widely distributed in the soil throughout Japan (Furuhata et al., 2002). From these findings, it can be easily surmised that soil-derived Legionella spp. has invaded and colonized the natural hot springs found in fields and mountains. Meanwhile, the use of chlorination to control Legionella spp. in order to prevent Legionnaire’s disease has spread in bathing facilities across Japan.

It is well known that Legionnaire’s disease is caused by Legionella spp. in hot spring water; however, there are no reported cases of Legionnaire’s disease caused by Legionella spp. in unattended hot springs in fields and mountains. That said, in this study, L. pneumophila was the dominant species among the Legionella spp. isolated from Noyu samples, although the number of these bacteria was small and the bacteria frequently corresponded to serogroup 1. Accordingly, the possibility that Legionnaire’s disease can be generated from unattended natural hot springs cannot be ruled out, and users must therefore be wary. Moreover, L. londiniensis was observed in addition to L. pneumophila among the bacterial strains isolated from Noyu samples. This strain was also the strain isolated most frequently from the hot spring water of bathing institutes, after L. pneumophila (Furuhata et al., 2011a), and it is interesting to note the commonality between these findings.

In this study, the number of coliform group bacteria, a pollution index of water, was also simultaneously measured and the relationship between the degree of detection of Legionella spp. and the number of coliform group bacteria was investigated. However, no prominent trends were observed. This finding is similar to the previous results of our investigation of the degree of detection of Legionella spp. in footbaths (Furuhata et al., 2012a). Moreover, although relationships between the identified Enterobacteriaceae bacteria isolated from Noyu samples, including E. coli were investigated, no particular trends were observed. As mentioned above, it is believed that there are no relationships between the detection of Legionella spp. from unattended natural hot springs and the conventional pollution index.

In the future, we plan to analyze the genetic pattern of L. pneumophila isolated from Noyu and the hot spring water of bathing institutes and to investigate any similarities between the two.

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| TABLE 4. Main species of Enterobacteriaceae in Noyu (unattended natural hot springs). |
|-----------------|---------------|---------------|------------|
| Species            | Positive (≥10CFU/100ml) | Negative (<10CFU/100ml) | Total |
|-----------------|----------------|----------------|--------|
| Escherichia coli | 8 (47.1) *    | 9 (33.3)       | 17 (38.6) |
| Enterobacter aerogenes | 3 (17.6)     | 5 (18.5)       | 8 (18.2) |
| Enterobacter cloacae  | 3 (17.6)     | 4 (14.8)       | 7 (15.9) |
| Klebsiella oxytoca      | 2 (11.8)     | 3 (11.1)       | 5 (11.4) |
| Raoultella terrigena     | 0            | 3 (11.1)       | 3 ( 6.8) |
| Klebsiella pneumoniae   | 1 ( 5.9)     | 1 ( 3.7)       | 2 ( 4.5) |
| Citrobacter freundii    | 0            | 1 ( 3.7)       | 1 ( 2.3) |
| Serratia marcescens     | 0            | 1 ( 3.7)       | 1 ( 2.3) |
| Total                   | 17 (100)     | 27 (100)       | 44 (100) |

*: Number of strains (%)
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