**INTRODUCTION**

*Legionella* species are Gram-negative bacteria that are widely present in both natural and artificial water bodies (Fields et al., 2002). Infection with *Legionella* spp. leads to clinical manifestations, including Legionnaires’ disease and Pontiac fever. *Legionella* spp. proliferates in aquatic environments within free-living protozoal hosts (Greub et al., 2004). Infection is passed on to humans via inhalation, aspiration, or micro-aspiration of *Legionella*-carrying aerosols. Legionnaires’ disease is prevented mainly by monitoring and controlling the contamination of environmental waters with *Legionella* spp. Also, accurately identifying the source of *Legionella* infection in environmental waters, especially during a disease outbreak, is important for public health.

The culture method is routinely used to test *Legionella* spp. in environmental samples (Totaro et al., 2017; Furuhat et al., 2013). However, this method has certain limitations associated with the culture characteristics of *Legionella* spp. These bacteria exhibit a slow growth rate, and growth can be inhibited in the presence of other microorganisms, thereby transforming the culture into a viable but non-culturable (VBNC) form under certain environmental conditions. Recently, quantitative real-time polymerase chain reaction (real-time qPCR) specific for *Legionella* 16S rRNA or macrophage infectivity potentiator genes have been widely applied to overcome the limitations of standard culture methods. (Tourn-Bodilis et al., 2011; Bonetta et al., 2010; Edagawa et al., 2008). Real-time qPCR can detect
VBNC Legionella types; however, it cannot distinguish between viable and dead cells. Further, although real-time qPCR is significantly more sensitive in detecting lower levels of contamination than the culture method, it cannot detect low levels of Legionella contamination in a natural environment.

Recently, the amoebic co-culture technique has been used for to detect bacterial species from clinical and environment samples (Inoue et al., 2019; Edagawa et al., 2015; La Scola et al., 2001). Acanthamoeba, a genus of amoeba, serves as a host for Legionella pneumophila. Amoebic co-culture using Acanthamoeba is used to detect Legionella spp., including VBNC and Legionella-like amoebal pathogen (LLAP) types, after their co-proliferation within the amoebae.

In this study, we used the culture method, real-time qPCR, and real-time qPCR combined with amoebic co-culture to analyze the contamination of various environmental water samples with Legionella spp. Samples were collected from cooling towers, water-amenity facilities, and tap water sources in Kansai area of Japan.

**MATERIALS AND METHODS**

In total, 110 water samples were collected from 19 cooling towers, 31 water-amenity facilities, and 60 river water sources of tap water from the Osaka Prefecture of the Kansai area in Japan. All samples (400 mL each) were collected in sterile bottles containing 0.01% sodium thiosulfate. Samples were immediately transported to a laboratory for further processing.

Each sample was concentrated by filtration through a 0.22-μm-pore size polycarbonate filter (Advantec Tokyo Co. Ltd., Tokyo, Japan). The membrane was then immersed in 4 mL of sterile deionized water, vortexed for 1 min, and shaken vigorously 50 times. From this suspension, 1 mL was stored at −20°C for DNA extraction and 1.5 mL was used for amoebic co-culture. A 0.2-mL aliquot of the suspension was used for acid treatment culture method, while the remaining suspension was heated in a water bath at 50°C for 30 min and used for the heat treatment culture method.

A volume of 1 mL of the above sample suspension was mixed with 1 mL of 0.2M acid-phosphate buffer (pH2.2), and 0.2 mL of this acid-treated sample was inoculated after 5 min onto Wadowsky-Yee-Okuda agar plates (WYO-alpha plates; Eiken Chemical Co. Ltd., Tokyo, Japan). From the heat-treated sample, 0.1 mL was inoculated onto an WYO-α plate. After incubation for 5–7 days at 37°C, 1–5 colonies resembling Legionella spp. were selected and cultured on blood agar plates (Eiken Chemical Co. Ltd.) and on buffered charcoal–yeast extract agar plates supplemented with alpha-ketoglutarate (BCYE-alpha; Eiken Chemical Co. Ltd.). After culture for 3 days at 37°C, the isolates that grew on BCYE-alpha but not on blood agar were examined by Gram staining. A final Gram-negative staining was considered suggestive of the presence of Legionella spp. Such colonies were observed under UV light and 1–5 colonies were next randomly selected for a latex agglutination test (Kanto Chemical Co., Tokyo, Japan) and an immune serum agglutination test (Denka Seiken Co. Ltd., Tokyo, Japan) to identify the serogroups of L. pneumophila, L. bozemanii, L. dumoffii, L. gormanii, and L. micdadeii. For Legionella strains not identified by these tests, Legionella spp. were identified by 16S rRNA PCR and nucleotide sequencing as described below. Acanthamoeba castellanii strain ATCC 30234 was grown in 75-cm² culture flasks at 30°C for 4 days on 50 mL of peptone/yeast extract/glucose (PYGC) medium (10 g proteose peptone, 10 g yeast extract, 10.1 g glucose, 5 g NaCl, 0.95 g L-cysteine hydrochloride, 1.74 g Na₂HPO₄, and 1.36 g KH₂PO₄ in 1 L of distilled water; the pH was adjusted to 6.8). Cells were harvested by centrifugation and re-suspended in PYGC medium at a density of approximately 1 x 10⁶ cells/mL. The amoebal suspension was distributed into each well of 12-well micro-plates at 30°C until the cells formed monolayers. Just before processing the water samples, the PYGC medium was removed from each well, and the cells were washed twice with 1 mL of Neff’s amoeba saline (120 mg NaCl, 3 mg MgCl₂, 3 mg CaCl₂, 142 mg Na₂HPO₄, and 136 mg KH₂PO₄ in 1 L of distilled water).

A volume of 1.5 mL of the processed environmental water sample was inoculated into the amoeba micro-plate wells (amoebic co-culture). After incubating for 7 days at 30°C, 1 mL of the suspension was stored at −20°C for DNA extraction. DNA was extracted using a QIAamp DNA Mini Kit (Qiagen K.K., Tokyo, Japan) and Legionella spp. were detected using real-time qPCR. The supernatant was stored at −20°C until use.

Real-time qPCR was performed according to the manufacturer’s instructions as using a Cycleave PCR Legionella (16S rRNA) Detection Kit (Takara Bio Inc., Shiga, Japan). PCR reactions were performed using an ABI PRISM 7900HT Real-time qPCR System (Applied Biosystems) according to the manufacturer’s instructions. The PCR mixtures (20 μL) were prepared according to the manufacturer’s protocol and reactions were performed under the following amplification conditions: 95°C for 10 s, then 45 cycles of 95°C for 5 s, 55°C for 10 s, and 72°C for 25 s. DNA amplification was detected by monitoring the fluorescence at two wavelengths, viz., FAM and ROX. The amplified 16S rRNA of Legionella and the internal control gene were detected by FAM and ROX, respectively. The samples whose DNA was not amplified due to the presence of inhibitors were
The detection of *Legionella* spp., using amoebic co-culture qPCR, qPCR, and culture methods from environmental water samples are presented in Table 1. Overall, $1.1 \times 10^7$ – $5.4 \times 10^7$, and $1.6 \times 10^2$ – $4.4 \times 10^2$ *Legionella* spp. were detected using amoebic co-culture qPCR and qPCR, respectively. When bath water samples were analyzed (Edagawa et al., 2015), the bacterial detection rate increased from 67.6% to 83.8% using the amoebic co-culture method, and 71.4% of the samples wherein *Legionella* spp. were not detected (ND) using qPCR, tested positive. Detection rates for environmental water samples were 74.5% using amoebic co-culture with qPCR and 75.5% for qPCR, which were comparable to earlier results. Of the 27 ND samples using qPCR alone, four samples (4.6%) tested positive after analysis using the amoebic co-culture method. Using the culture method, various *Legionella* spp. were detected in three water samples collected from amenity water facilities, viz., sample no. 82, *L. pneumophila* serogroup 7 and *L. anisa* at 90 CFU/100 mL; sample no. 81, *L. pneumophila* serogroup 7 and *L. anisa* at 70 CFU/100 mL; and sample no. 75, *L. pneumophila* serogroup 8 at 10 CFU/100 mL. *Legionella* spp. were ND by the culture method in samples collected from cooling towers and natural river water.

Of all the tested samples, 87 (79.1%, 18 samples from the cooling tower, 22 samples from water-amenity facilities, and 47 samples from river water sources of tap water) tested positive using either qPCR with amoebic co-culture or culture method (Tables 2–4). Using the amoebic co-culture method, 19 samples (15.8%, 1 sample from the cooling tower, 8 samples from amenity water, and 10 river water samples) were confirmed to contain a 10-fold higher number of bacteria compared to qPCR analysis without amoebic co-culture. Thirteen PCR products from the above 19 PCR-positive samples were successfully sequenced for species identification. A BLAST homology search indicated a 99–100% sequence identity with *L. pneumophila* (sample no. 82) and *L. anisa* (sample nos. 81 and 75).

### RESULTS AND DISCUSSION

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the same samples without amoebic co-culture are observed; more than 10-fold higher bacterial numbers compared with water from hotels in Portugal. An environmental strain that was isolated from shower L. quateirensis is usually ND by culture methods. 

With amoebic co-culture, more than 10% of samples were positive for Legionella spp. that could not be detected by the culture method. Overall, 98–100% concordance was achieved with unculturable species such as L. lytica (sample no. 35, 616/616, 100%), L. rowbothamii (LLAP6; sample no. 31, 612/616, 99.4%), LLAP (sample no. 56, 608/615, 98.9%), and L. quateirensis (sample no. 62, 607/617, 98.5%) and L. feeleii (sample no. 32, 583/583, 100%). A resulting molecular phylogenetic tree is shown in Figure 1. L. lytica (Hockey et al., 1996) and L. rowbothamii (Adeleke et al., 2001) are usually ND by culture methods. L. quateirensis is an environmental strain that was isolated from shower water from hotels in Portugal (Dennis et al., 1993). L. feeleii isolated from the environment is closely related to L. tunisiensis, which is isolated from patients with pneumonia and is pathogenic to humans (Thacker et al., 1985). To our knowledge, these species except L. feeleii have not yet been detected in Japan, probably since the culture method is still predominantly used to detect Legionella. In this study, Legionella spp. that could not be detected by the culture method could be detected after using the amoebic co-culture method. In this study, Legionella spp. were detected not only in samples from artificial water bodies, such as cooling towers and amenity points, but also in river water samples, such as tap water sources. In Japan, the source of infection was identified in less than half of the cases, but 75% of the cases where the source of infection was identified were in bath water of hot springs and bathing facilities (National Institute of Infectious Diseases 2000), and there are many

| Sample No. | Real-Time qPCR Method (copies/100mL) | With Amoebic Co-Culture | Without Amoebic Co-Culture |
|------------|-----------------------------------|-------------------------|----------------------------|
| 13         | 5.1 ×10^5                         | 1.3 ×10^3               |
| 14         | 1.4 ×10^3                         | 7.8 ×10^3               |
| 15         | 4.0 ×10^6                         | 2.1 ×10^6               |
| 16         | 1.1 ×10^4                         | 2.8 ×10^3               |
| 17         | 1.2 ×10^4                         | 4.8 ×10^4               |
| 18         | 4.4 ×10^4                         | 8.7 ×10^4               |
| 19         | 6.5 ×10^4                         | 8.6 ×10^5               |
| 20         | 3.1 ×10^5                         | 2.0 ×10^3               |
| 21         | 7.2 ×10^5                         | 1.3 ×10^6               |
| 22         | 9.9 ×10^5                         | 3.4 ×10^4               |
| 61         | 9.6 ×10^4                         | 5.2 ×10^4               |
| 62         | 7.8 ×10^5                         | 4.5 ×10^5               |
| 63         | 5.7 ×10^4                         | 4.0 ×10^6               |
| 65         | 3.8 ×10^4                         | 2.7 ×10^4               |
| 66         | 2.2 ×10^5                         | 2.4 ×10^5               |
| 67         | 2.1 ×10^5                         | 5.1 ×10^6               |
| 69         | 1.1 ×10^6                         | 1.7 ×10^6               |
| 64         | ND a)                            | 2.4 ×10^3               |

Notes: *: Using real-time qPCR with amoebic co-culture, more than 10-fold higher bacterial numbers compared with the same samples without amoebic co-culture are observed; a) ND: not detected (less than 1.0×10^3 copies/100mL).

### Table 3: Results of the real-time qPCR in the 22 water samples from amenity water facilities

| Sample No. | Real-Time qPCR Method (copies/100mL) | With Amoebic Co-Culture | Without Amoebic Co-Culture |
|------------|-----------------------------------|-------------------------|----------------------------|
| 53         | 3.9 ×10^3                         | 8.2 ×10^3               |
| 55         | 1.7 ×10^4                         | 1.5 ×10^6               |
| 56         | 1.0 ×10^6                         | 9.6 ×10^3               |
| 71         | 5.4 ×10^4                         | 4.9 ×10^4               |
| 72         | 1.5 ×10^4                         | 3.5 ×10^5               |
| 75         | 1.7 ×10^4                         | 2.8 ×10^5               |
| 81         | 1.1 ×10^4                         | 3.6 ×10^4               |
| 82         | 5.0 ×10^4                         | 3.0 ×10^4               |
| 83         | 5.9 ×10^4                         | 8.7 ×10^5               |
| 85         | 1.9 ×10^4                         | 2.2 ×10^6               |
| 86         | 5.0 ×10^3                         | 2.2 ×10^4               |
| 87         | 1.0 ×10^4                         | 4.6 ×10^5               |
| 88         | 5.9 ×10^6                         | 8.1 ×10^4               |
| 89         | 3.0 ×10^4                         | 8.6 ×10^5               |
| 90         | 2.5 ×10^5                         | 1.6 ×10^6               |
| 91         | 3.8 ×10^5                         | 4.4 ×10^7               |
| 92         | 3.2 ×10^6                         | 7.5 ×10^3               |
| 95         | 2.6 ×10^5                         | 4.6 ×10^4               |
| 54         | ND a)                            | 2.7 ×10^4               |
| 84         | ND                               | 5.1 ×10^3               |
| 96         | ND                               | 5.5 ×10^4               |
| 97         | ND                               | 5.2 ×10^5               |

Notes: *: Using real-time qPCR with amoebic co-culture, more than 10-fold higher bacterial numbers compared with the same samples without amoebic co-culture are observed; a) ND: not detected (less than 1.0×10^3 copies/100mL).
investigation reports on bath water (Sasahara et al., 2004; Karasudani et al., 2009; Furuhata et al., 2004). Countries apart from Japan have reported Legionella contamination and resulting human infection due to tap water systems. In the United States, a switch from water sources to rivers that show the presence of Legionella has led to an increase in the number of Legionella cases in distribution areas (Davis et al., 2016; Zahran et al., 2003). Once engulfed by amoebae using phagocytosis, L. pneumophila invades fusion of the phagosome and the lysosome, and replicates a mechanism similar to one that enables it to survive within macrophages (Horwitz et al., 1987; Miyamoto et al., 1997). This ability to invade and multiply within host cells is considered to correlate with its pathogenicity in humans. However, the pathogenicity of Legionella in humans is not well defined. The amoebic co-culture method used in this study utilizes the ability of Legionella to grow in amoebae and human macrophages. L. pneumophila uses the same genes to multiply within the same samples without amoebic co-culture are observed; more than 10-fold higher bacterial numbers compared with the same samples without amoebic co-culture are observed. In this study, we observed that 78.3% of river water samples tested Legionella positive by qPCR with amoebic co-culture. In Japan, the detection rate of amoebae including pathogenic species in river water as tap water sources was reported to be 68.7% (Edagawa et al., 2009), it is highly likely that Legionella spp. parasitize and proliferate within these amoebae. The monitoring of river waters, especially potable sources, may be necessary to prevent the outbreak of Legionnaires’ disease associated with tap water distribution.

To date, around 60 species of Legionella have been identified, and less than 50% of these species are suspected to cause infections in humans. The amoebic co-culture method used in this study utilizes the ability of Legionella to grow in amoebae, A. castellanii and human macrophages (Segal et al., 1999). Additionally, the intracellular growth in

### Table 4. Results of the real-time qPCR in the 47 river water samples as a source of tap water

| Sample No. | Real-Time qPCR Method (copies/100mL) |
|------------|-------------------------------------|
|            | With Amoebic Co-Culture | Without Amoebic Co-Culture |
| 1          | 8.9 x 10^3                  | 3.8 x 10^3                  |
| 8          | 9.0 x 10^3                  | 1.6 x 10^3                  |
| 10         | 1.1 x 10^4                  | 8.1 x 10^3                  |
| 11         | 9.7 x 10^3                  | 1.7 x 10^3                  |
| 12         | 5.3 x 10^3                  | 5.5 x 10^3                  |
| 26         | 1.6 x 10^4                  | 1.0 x 10^3                  |
| 28         | 8.1 x 10^3                  | 1.3 x 10^4                  |
| 29         | 1.0 x 10^4                  | 2.0 x 10^4                  |
| 31         | 1.7 x 10^6*                 | 3.0 x 10^3                  |
| 35         | 2.0 x 10^6*                 | 7.2 x 10^3                  |
| 36         | 3.2 x 10^6*                 | 6.5 x 10^3                  |
| 37         | 1.0 x 10^4                  | 1.4 x 10^4                  |
| 38         | 3.1 x 10^4                  | 4.9 x 10^4                  |
| 39         | 1.0 x 10^4                  | 1.8 x 10^4                  |
| 40         | 3.3 x 10^4                  | 2.1 x 10^4                  |
| 41         | 5.9 x 10^5                  | 7.9 x 10^3                  |
| 42         | 2.2 x 10^6                  | 8.4 x 10^4                  |
| 43         | 2.6 x 10^4                  | 2.0 x 10^3                  |
| 44         | 9.1 x 10^4                  | 1.2 x 10^4                  |
| 46         | 5.1 x 10^5                  | 3.7 x 10^3                  |
| 48         | 9.2 x 10^4                  | 9.2 x 10^4                  |
| 49         | 8.8 x 10^3                  | 1.3 x 10^4                  |
| 50         | 1.7 x 10^4                  | 1.6 x 10^4                  |
| 51         | 7.3 x 10^4                  | 3.2 x 10^2                  |
| 57         | 6.8 x 10^4                  | 4.6 x 10^4                  |
| 58         | 1.3 x 10^5                  | 9.7 x 10^4                  |
| 59         | 1.9 x 10^6*                 | 1.0 x 10^5                  |
| 60         | 1.4 x 10^5                  | 1.1 x 10^5                  |
| 79         | 3.6 x 10^4                  | 1.7 x 10^5                  |
| 80         | 8.0 x 10^3                  | 1.9 x 10^4                  |
| 98         | 5.0 x 10^4                  | 2.8 x 10^5                  |
| 99         | 3.8 x 10^4                  | 4.7 x 10^4                  |
| 100        | 3.8 x 10^5                  | 2.7 x 10^5                  |
| 101        | 4.2 x 10^4                  | 3.6 x 10^5                  |
| 102        | 4.2 x 10^4*                 | 1.8 x 10^3                  |
| 103        | 6.8 x 10^4                  | 5.1 x 10^5                  |
| 104        | 9.8 x 10^4*                 | 2.2 x 10^3                  |
| 105        | 3.1 x 10^3                  | 4.7 x 10^4                  |
| 106        | 6.7 x 10^4                  | 2.2 x 10^6                  |
| 107        | 3.1 x 10^4                  | 2.4 x 10^5                  |
| 108        | 9.4 x 10^4                  | 9.0 x 10^5                  |
| 109        | 4.2 x 10^5                  | 1.2 x 10^7                  |
| 110        | 1.4 x 10^6                  | 5.7 x 10^6                  |
| 2          | 4.0 x 10^3*                 | ND (a)                      |
| 4          | 1.6 x 10^3*                 | ND                          |
| 32         | 3.3 x 10^5*                 | ND                          |
| 47         | 4.7 x 10^4*                 | ND                          |

Notes: *: Using real-time qPCR with amoebic co-culture, more than 10-fold higher bacterial numbers compared with the same samples without amoebic co-culture are observed; ND: not detected (less than 1.0 x 10^2 copies/100mL).
A. castellanii affects monocyte entry mechanisms and enhances the virulence of L. pneumophila (Cirillo et al., 1999). Therefore, positive qPCR signals following amoebic co-culture may indicate the existence of viable and virulent Legionella (Samrakandi et al., 2002). By performing amoebic co-cultures, “dead bacteria” or Legionella with no ability to multiply in amoebae may be possible to be engulfed and digested (Edagawa et al., 2016). Thus, amoebic co-cultures can aid the proliferation of Legionella, which are both viable and capable of growing in amoebae. This combined methodology can indicate the contamination with Legionella spp. with higher accuracy.

Legionella have reportedly been detected in the

![FIG. 1. Phylogenetic relationship between the sequences of partial 16S rRNA genes (616bp) from uncultured and cultured Legionella isolates obtained in this study and Legionella spp. or strains deposited in GenBank. The source of the sample is shown at the end of the sample name as follows: C, cooling tower; A, amenity water facility; R, river. Asterisk (*) is Legionella detected by culture method. Coxiella burnetii (HM208383) was used as an outgroup species. Numbers at nodes are bootstrap percentages (based on 1,000 resamplings) only values above 60% are shown.](image)
sputum of patients with pneumonia, who tested negative using common diagnostic tests for Legionnaires’ disease, including the culture method and the amoebic co-culture method. The latter method has been used to detect amoeba-resistant bacteria including Legionella, and a combination of molecular biological techniques has been used to discover new species of these bacteria (Thomas et al., 2010; Benamar et al., 2017). Although the number of confirmed Legionella infections is rising in Japan, the source of the infection has been identified only in a very few cases. Legionnaires’ disease is rarely proven by culture, and detection of urinary antigen is now common. The combinatorial method using qPCR followed by the amoebic co-culture method used in this study is a useful tool for sampling Legionella contamination at low concentrations, particularly since the conventional culture method does not detect Legionella under these conditions.

Amoebic co-cultures in combination with qPCR analyses revealed the presence of viable Legionella spp. in environmental waters collected from various sources in Japan. Gene sequencing indicated the presence of bacterial species responsible for infections in humans as well as unculturable bacterial species. We concluded that environmental waters are inhabited by Legionella spp., although these samples are not always detected by the traditional culture method. Applying the amoebic co-culture technique prior to real-time qPCR might be more useful to detect viable and virulent Legionella spp. since their ability to invade and multiply within Acanthamoeba can be correlated with their pathogenicity.

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