Epidemiol. Infect. (2017), **145**, 1398–1408. © Cambridge University Press 2017
doi:10.1017/S0950268817000036

Legionella prevalence and risk of legionellosis in Japanese households

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Received 6 September 2016; Final revision 28 December 2016; Accepted 28 December 2016; first published online 7 February 2017

SUMMARY

This study determined the occurrence of legionellae in private houses for which there were no available data on aquatic environments other than the water supply system. From June 2013 to November 2014, we collected 138 water and 90 swab samples from aquatic environments in 19 houses. *Legionella* DNA was detected via a loop-mediated isothermal amplification assay in 66 (47·8%) water and 17 (18·9%) swab samples. High *Legionella* DNA detection rates were observed in water samples from washing machines and aquariums. *Legionella* spp. was isolated from 9 (6·5%) water and 3 (3·3%) swab samples. *Legionella pneumophila* SG 1 was detected from the outlet water of a bathtub spout and a bath sponge. Use of amoebic co-culture effectively increased legionellae and *Legionella* DNA detection rates from all sample types. A logistic regression analysis revealed that the heterotrophic plate count was significantly related to *Legionella* contamination. Our findings indicate that there is a risk of legionellosis from exposure to *Legionella* spp. in a variety of aquatic environments in residential houses. Control measures for legionellae in houses should include frequent cleaning and disinfecting to reduce heterotrophic bacteria in water and, where possible, preventing aerosolization from aquatic environments.

Key words: Legionella, household, logistic regression analysis, washing machine, aquarium.

INTRODUCTION

Legionellae, Gram-negative bacteria found in aquatic environments are causative agents of legionellosis [1]. This disease is acquired by inhalation or aspiration of aerosols containing legionellae from an environmental source. Legionellosis has two distinct forms: Legionnaires' disease and Pontiac fever [2]. Sporadic *Legionella* infections, rather than outbreak cases, account for most *Legionella* infections [3]. A substantial proportion of sporadic cases may be residentially acquired [4], and these infections have been documented worldwide. Several cases of legionellosis linked to home water supplies were previously reported [5, 6].

Previously, approximately 700–900 cases of *Legionella* infection were reported in Japan each year [7], but in 2014 the number of reported cases increased to 1248 (http://www.nih.go.jp/niid/ja/survei/2085-idwr/ydata/5672-report-ja2014-20.html).

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Sporadically occurring cases of community-acquired legionellosis accounted for most of these infections [7]. While most cases of *Legionella* infection are associated with spas in public bath facilities and accommodations [8], some sporadic cases associated with baths involved residential infections [9].

Control measures for preventing *Legionella* infection from water supply systems have been reported previously. Chlorination of potable water, increasing water heater temperatures, and limiting exposure to aerosols in private residences were recommended [4]. Regular flushing of showers decreased *Legionella* to below detectable levels [10]. Because *Legionella* requires an appropriate temperature and pH to grow, extreme water temperatures and pH can be used to prevent *Legionella* proliferation [11].

Despite the occurrence of *Legionella* in a wide range of aquatic environments, a limited number of studies have analyzed *Legionella* contamination in the water supply systems of houses [12], and, to our knowledge, no information on *Legionella* contamination in other aquatic environments within houses is available. The aim of this study was to investigate residential *Legionella* contamination. Additionally, the relationship between *Legionella* contamination and the characteristics of the water samples collected in households, including the number of heterotrophic bacteria, residual-free chlorine concentration, pH, and temperature, was investigated to determine whether these factors could be utilized to prevent the colonization of *Legionella* in household aquatic environments.

METHODS

Sample collection

Samples were collected from 19 private homes during the period between July 2013 and November 2014. The private homes were selected from those of colleagues of our Institute and associates. House members were asked to collaborate and provide consent for their house samples to be collected and tested for *Legionella*. Fourteen of the 19 homes, which were located in Kanagawa Prefecture, provided a variety of samples, while the remaining five homes provided only aquarium water samples. These 14 houses consisted of five condominium units and nine single-family houses. All houses studied received chlorinated tap water that met the Japanese water quality standard. The ages of the condominium units and houses ranged from 1 to 30 years old and from 4 to 55 years old, respectively. No members of any of these homes contracted Legionnaires' disease or Pontiac fever before and during the investigation period.

Overall, 138 water samples (Table 1) and 90 swab samples (Table 2) were analyzed in this study. Water samples (up to 1000 ml) were collected in sterile vessels containing 0·5 g sodium thiosulphate/1000 ml sample water to neutralize residual chlorine. The samples were collected as follows: water samples were taken from the tap outlet of cold water supplies immediately after the valve was opened. Residual water in shower hoses and showerheads or garden hoses in the house yard was collected. To collect a water sample from a washing machine, about 10 liters of tap water from a tap directly connected to the washing machine was added into a drum, the washing machine was run for 10 min, and then 1000 ml of water was collected in a sterile container. Tap water was used for this procedure instead of sterile water to analyze the contamination of washing machines under their normal use conditions. Bath sponges that were used for cleaning bathtubs were squeezed by hand with sterile gloves to collect water samples. The swab samples were taken using a sterile Dacron swab (JCB Industry Limited, Tokyo, Japan) at each sampling site and were each immersed in 1·0 ml of sterilized phosphate-buffered saline (PBS) that had been diluted 50-fold.

Physical and chemical analyses

The temperature and pH of water samples were measured on site. The residual-free chlorine concentrations were measured at our laboratory by the DPD (N, N-diethyl-p-phenylenediamine) method using AQUAB AQ-201 (Shibata Scientific Technology, Saitama, Japan) in accordance with the manufacturer's instructions.

Heterotrophic plate count

The number of heterotrophic bacteria was determined by heterotrophic plate count (HPC). Water samples were inoculated with 10-fold serial dilutions. Serially diluted samples (100 µl) were inoculated onto R2A agar plates (BD, Tokyo, Japan), and the colonies were counted after the plates had been incubated at 25 °C for 7 days.

Legionella detection

Water samples (500 ml) were filtered through a 0·2-µm porosity polycarbonate membrane filter (Advantec

https://doi.org/10.1017/S0950268817000036 Published online by Cambridge University Press

Table 1. *Household Legionella contamination in water samples*

Samples	No. of houses	No. of samples	Temperature (°C) Mean	Temperature (°C) Range	pH Mean	pH Range	Residual chlorine conc. (mg/l) Mean	Residual chlorine conc. (mg/l) Range	HPC (CFU/ml) Mean	HPC (CFU/ml) Range	LAMP-positive (%)	Culture-positive (%)	(CFU/100 ml)	Amoeba co-culture LAMP-positive (%)	Amoeba co-culture Culture-positive (%)	Combined LAMP-positive (%)	Combined Culture-positive (%)
Samples from water systems																	
Kitchen tap	10	14	26·4	11·0–42·0	7·3	7·1–7·8	0·27	0–0·80	353	0–1·1 × 10^5	2 (14·3)	0		3 (21·4)	1* (7·1)	4 (28·6)	1 (7·1)
Bathroom tap	11	12	25·9	18·5–36·0	7·4	7·1–7·8	0·26	0–0·80	205	1–1·0 × 10^4	1 (8·3)	0		1 (8·3)	0	1 (8·3)	0
Bathtub spout	12	17	33·7	28·0–39·0	7·4	7·1–7·8	0·17	0–0·80	2432	0–1·7 × 10^6	8 (47·1)	1† (5·9)	15	7 (41·2)	0	9 (52·9)	1 (5·9)
Shower	7	8	25·9	23·0–31·5	7·4	7·1–7·8	0·15	0–0·49	7759	0–4·7 × 10^6	1 (12·5)	0		1 (12·5)	0	2 (25·0)	0
Hand-wash basin tap	7	8	25·7	22·0–30·0	7·3	7·2–7·4	0·27	0·02–0·80	302	4–4·6 × 10^5	1 (12·5)	1‡ (12·5)	370	1 (12·5)	0	2 (25·0)	1 (12·5)
House yard tap	1	2	24·8	24·8–24·8	7·4	7·4–7·4	0·60	0·6–0·6	0	0	0	0		0	0	0	0
Subtotal	14	61									13 (21·3)	2 (3·3)		13 (21·3)	1 (1·6)	18 (29·5)	3 (4·9)
Other samples																	
Kitchen tap-attached water filter	3	3	24·4	22·5–26·2	7·1	7·0–7·2	0	0–0	8413	1540–1·6 × 10^4	0	0		0	0	0	0
Bathtub water	6	8	32·8	26·5–42·0	7·3	7·2–7·5	0·09	0–0·45	1·9 × 10^5	35–4·7 × 10^6	3 (37·5)	1§ (12·5)	150	ND		3 (37·5)	1 (12·5)
Bath sponge	3	3	ND	–	ND	–	ND	–	ND	–	2 (66·7)	1† (33·3)	ND††	2 (66·7)	0	2 (66·7)	1 (33·3)
Toilet low tank	6	7	24·5	22·0–27·0	7·4	7·1–7·6	0·25	0–0·44	2983	65–1·8 × 10^4	1 (14·3)	0		2 (28·6)	0	3 (42·9)	0
Tank in a warm water-equipped washing toilet seat	1	2	29	29·0–29·0	7·5	7·5–7·5	0	0–0	5147	815–1·7 × 10^4	0	0		0	0	0	0
Washing machine	8	11	23·2	16·0–27·0	7·5	7·3–7·9	0·04	0–0·20	6210	150–5·0 × 10^5	7 (63·6)	0		8 (72·7)	0	10 (90·9)	0
Garden hose in a house yard	5	10	28·5	21·0–33·0	7·3	6·7–7·6	0·03	0–0·20	8·6 × 10^4	895–6·0 × 10^6	1 (10·0)	1¶ (10·0)	20	1 (10·0)	0	1 (10·0)	1 (10·0)
Pond in a house yard	3	3	23·8	23·6–24·0	4·3	3·8–4·8	0	0	ND	–	1 (33·3)	0		2 (66·7)	0	3 (100)	0
Aquarium	9	30	26·4	22·0–34·0	6·7	3·3–8·1	0	0–0	ND	–	16 (53·3)	3** (10·0)	20 20 25	17 (56·7)	0	26 (86·7)	3 (10·0)
Subtotal	19	77									31 (40·3)	6 (7·8)		32 (41·6)	0	48 (62·3)	6 (9·8)
Total	19	138									44 (31·9)	8 (5·8)		45 (32·6)	1 (0·7)	66 (47·8)	9 (6·5)

* *L. rowbothamii*.
†*L. pneumophila* SG1, ST22.
‡ *Legionella* sp. L-29.
§ *L. anisa*.
¶ *L. busanensis*.
***L. sainthelensi* was from two samples and *L. anisa* was from one sample.
†† Not determined. Bath spout is defined as a pipe and liplike projection at the side of a bathtub by which hot water enters the bath.

Table 2. *Household Legionella contamination in swab samples*

Samples	No. of houses	No. of samples	LAMP-positive (%)	Culture-positive (%)	Amoeba co-culture		Combined	
					LAMP-positive (%)	Culture-positive (%)	LAMP-positive (%)	Culture-positive (%)
Inner surface								
Kitchen tap	10	13	0	0	0	0	0	0
Kitchen tap-mount water filter	2	2	1 (50·0)	0	0	0	1 (50·0)	0
Hand-wash basin tap	6	8	0	0	1 (12·5)	1* (12·5)	1 (12·5)	1 (12·5)
Bathroom tap	12	14	1 (7·1)	0	2 (14·3)	1† (7·1)	3 (21·4)	1 (7·1)
Bathtub spout	13	13	6 (46·2)‡	0	5 (38·5)	0	7 (53·8)	0
Showerhead	6	7	0	0	0	0	0	0
Washing machine tap	1	1	0	0	0	0	0	0
Pump hose¶	1	2	1 (50·0)	1 (50·0)§	1 (50·0)	0	2 (100)	1 (50·0)
Toilet tap	3	4	0	0	1 (25·0)	0	1 (25·0)	0
Toilet low tank	3	3	0	0	0	0	0	0
House yard tap	2	3	0	0	0	0	0	0
Garden hose in a house yard	4	10	0	0	0	0	0	0
Aquarium	1	4	0	0	0	0	0	0
Outer surface of washing machine drum	3	4	0	0	1 (25·0)	0	1 (25·0)	0
Bathroom floor	1	2	1 (50·0)	0	0	0	1 (50·0)	0
Total	19	90	10 (11·1)	1 (1.1)	11 (12·2)	2 (2·2)	17 (18·9)	3 (3·3)

* *Legionella* sp. L-29.

† *Legionella* sp.

‡ One sample contained the DNA of *L. pneumophila* ST1966.

§ *L. anisa*.

¶ A pump to transfer leftover bathwater from a bathtub to a washing machine for laundry.

Toyo Kaisha, Tokyo, Japan). The membranes were then placed in 10 ml of 50-fold-diluted PBS, and any bacteria were dislodged by vortexing. Swab samples were suspended in 5 ml of 50-fold-diluted PBS. The suspension was then subjected to heat treatment at 50 °C for 20 min according to the methods of Kasuga *et al*. [13] followed by acid buffer treatment in HCl–KCl buffer (pH 2·2) for 4 min according to the methods of Bopp *et al*. [14]. The samples were subsequently inoculated onto GVPC agar (Oxoid, Hampshire, UK) plates by the spread plate method, and the plates were incubated at 36 °C for at least 7 days. The resulting suspected colonies that were smooth and grayish white or gray–blue–purple were subcultured on BCYE-α plates (Oxoid) and on horse blood agar plates, all of which were incubated at 36 °C for 5 days. The detection limit of the culture method was 100 colony-forming units (CFU)/l. Colonies that grew only on BCYE-α were verified as *Legionella* by polymerase chain reaction (PCR) with specific primers for the *Legionella* 5S rRNA gene (Cycleave PCR *Legionella* Detection Kit, Takara Bio, Shiga, Japan). The sequence type of *Legionella pneumophila* was determined in accordance with the European Working Group for *Legionella* Infections Sequence-Based Typing (SBT) protocol (http://www.hpa-bioinformatics.org.uk/legionella/legionella_sbt/php/sbt_homepage.php), as described previously [15, 16]. Serotyping was performed using a slide agglutination test with commercial antiserum (Denka Seiken, Tokyo, Japan). Non-*L. pneumophila* isolates were identified by sequencing the 16S rRNA and *mip* genes [17, 18]. Obtained sequences of 16S rRNA and *mip* genes were queried against the DNA Data Bank of Japan (DDBJ) database and the *Legionella mip* gene sequence database (http://www.hpa-bioinformatics.org.uk/cgi-bin/legionella/mip/mip_id.cgi), respectively, using BLAST. Enumeration of *Legionella* was determined by counting colonies that were identified as *Legionella* bacteria.

Amoeba co-culture

An *Acanthamoeba* sp. strain (CT1812-19) that was originally isolated from a cooling tower was grown in a 50-ml cell culture flask with 3 ml of peptone-yeast extract-glucose broth at 30 °C [19]. The amoebae were harvested and pelleted by centrifugation at 1000 ***g*** for 10 min. The supernatant was removed, and the amoebae were resuspended in a 50-ml cell culture flask with 3 ml of 50-fold-diluted PBS. After the study samples were heat treated, 1·5 ml of each sample was added to a suspension of amoeba in a 50-ml cell culture flask and incubated at 25 °C for 3 days. After incubation, the culture was subjected to acid treatment and then inoculated onto selective agar plates as described above. Plates were incubated at 36 °C for at least 7 days, and suspected colonies were tested for *Legionella*.

Detection of *Legionella* DNA by LAMP and direct DNA analysis

To detect *Legionella* DNA in samples, a loop-mediated isothermal amplification (LAMP) assay (Eiken Chemical, Tokyo, Japan) was used [20]. Re-suspensions of water samples, suspensions of swab samples, and amoebal co-culture solutions were subjected to DNA extraction using the alkali heat extraction method [21], in accordance with the manufacturer's instructions for LAMP assay. Negative (TE buffer) and positive controls (*Legionella* DNA) are provided with the kit. Every run of LAMP was performed with both control samples. The reactions in LAMP assays for *Legionella* were performed in a Loopamp Real-time Turbidimeter LA-320C (Eiken Chemical) at 65 °C for 60 min followed by 80 °C for 5 min to terminate the reaction. Turbidity was monitored spectrophotometrically at 650 nm with the LA-320C. The results were analyzed with the LA-320C software package. The detection limit of the assay was guaranteed 60 CFU per test-tube [20], but we detected 1 CFU of *L. pneumophila* per tube in the LAMP reaction. All LAMP-positive DNA samples were tested using conventional PCR of the *L. pneumophila*-specific *mip* gene [9]. For *mip*-positive DNAs, nested-SBT was performed (http://www.hpa-bioinformatics.org.uk/legionella/legionella_sbt/php/sbt_homepage.php).

Statistical analysis

Data were analyzed using SPSS for Windows release 17·0 (SPSS Inc., Chicago, IL, USA). Logarithmic transformation of HPCs was carried out to normalize the non-normal distributions. Univariate and stepwise multivariate logistic regressions to establish independent predictors of contamination were conducted. Logistic regression analyses were used to determine whether *Legionella* contamination was associated with a particular set of microbial and physical characteristics. Data were excluded from analysis if any single value was missing.

https://doi.org/10.1017/S0950268817000036 Published online by Cambridge University Press

RESULTS

Detection of *Legionella* DNA

The detection rates of *Legionella* DNA were high among water samples from washing machines or aquariums and moderately high among water and swab samples from bathtub spouts (Tables 1 and 2). The duration of washing machine use ranged from 3 months to 14 years (mean, 5·88 years). Regardless of the duration of use, *Legionella* DNA was detected in samples from almost all washing machines.

Among water samples from water systems in homes, *Legionella* DNA was detected in 18 out of 61 samples (29·5%) collected from seven houses. *Legionella* DNA was detected in outlet water samples collected from two 30-year-old condominium units and five single-family houses that were each over 11 years old. A portion of the LAMP-positive samples was analyzed by SBT, sequencing both the 16S rRNA and *mip* genes. These analyses revealed that a swab sample from a bathtub spout contained *L. pneumophila* ST1966, and that an outlet water sample from a bathtub spout contained another *mip*-positive DNA with partial alleles (0, 0, 0, 0, 2, 0, 0) for SBT, although *L. pneumophila* SG1, ST22 was isolated from the same water sample.

Combining the amoeba co-culture method with a conventional culture method increased the LAMP detection rates from 31·9% (44 samples) to 47·8% (66 samples) in water samples and from 11·1% (10 samples) to 18·9% (17 samples) in swab samples (Tables 1 and 2).

Isolation of *Legionella*

The isolation rates of *Legionella* spp. in the samples were low. The combination of the amoeba co-culture method and conventional culture method increased the isolation rates of *Legionella* spp. from 5·8% (eight samples) to 6·5% (nine samples) in water samples and from 1·1% (one sample) to 3·3% (three samples) in swab samples (Tables 1 and 2). The number of *Legionella* in water samples detected by the culture method ranged from 15 to 370 CFU/100 ml (Table 1). *L. pneumophila* SG 1, ST22 was detected in outlet water samples from a bathtub spout and a bath sponge collected at the same house. *Legionella* DNA (16S rRNA gene) was also detected from a washing machine water sample collected in this house, but the *L. pneumophila mip* gene was not detected in this sample. *Legionella* DNA was not detected in other samples collected from this house, including outlet water samples from a shower, a hand-wash basin tap, and kitchen and bathroom taps.

Legionella sainthelensi and *Legionella anisa* were isolated from two aquarium water samples (20 and 25 CFU/100 ml) and one aquarium water sample (20 CFU/100 ml), respectively. Of the 30 total aquarium samples, two were collected from separate marine water aquariums. *Legionella* DNA was detected from both marine aquariums, and *L. sainthelensi* was isolated from one of the two marine water aquariums.

L. anisa was isolated from water samples from a bathtub (150 CFU/100 ml), an aquarium, and a pump hose from one house. In this house, the water leftover in the bathtub was used for changing the aquarium water and for washing in the washing machine. The leftover water was usually transferred from the bathtub to the washing machine using a water pump through a pump hose. *Legionella* DNA was detected in a washing machine water sample and a washing machine drum swab sample from this house, while *L. anisa* was not isolated from these samples.

Association between water characteristics and the detection of *Legionella* DNA

The associations of HPC, residual chlorine concentration, pH, and water temperature with the presence of *Legionella* DNA were evaluated by applying a univariate logistic regression analysis (Table 3). HPC and pH were positively associated with the presence of *Legionella* DNA, while the residual-free chlorine concentration showed an inverse association with the presence of *Legionella* DNA. Based on this initial finding, water sample HPC, pH, and residual chlorine concentration were introduced into a multivariate logistic regression model. HPC (odds ratio (OR) 1·443, 95% confidence interval (CI) 1·102–1·891, $P < 0·01$) and pH (OR 10·891, 95% CI 1·314–90·249, $P < 0·05$) were independently associated with the presence of *Legionella* DNA.

DISCUSSION

Our study shows that *Legionella* is found in aquatic environments at multiple sites in homes. These aquatic environments may become potential sources of *Legionella* infection.

The risk of *Legionella* infection from the home water supply was initially reported by Stout *et al.* [5] and later reviewed by Pedro-Botet *et al.* [6]. They

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Table 3. *Univariate logistic regression of parameters associated with Legionella contamination*

	LAMP for *Legionella* spp.						
	+ ($n = 33$)			− ($n = 40$)			
Factor	Mean	Range	S.D.	Mean	Range	S.D.	OR (95% CI)
Heterotrophic plate count (logarithmic value of CFU/ml)	4·13	0–7·25	1·89	2·81	0–6·78	1·81	1·467 (1·121–1·921)*
Residual chlorine conc. (mg/l)	0·09	0–0·50	0·15	0·23	0–0·80	0·28	0·050 (0·004–0·615)†
pH	7·55	7·2–8·2	0·29	7·40	7·1–7·8	0·22	11·321 (1·553–82·536)†
Temperature (°C)	26·8	16·0–39·5	6·30	27·5	18·5–39·0	4·50	0·974 (0·892–1·063)

S.D., standard deviation; OR, odds ratio; CI, confidence interval.
*$P < 0·01$; †$P < 0·05$.
The numbers of $LAMP^+$ and $LAMP^-$ samples from each sampling site used in the tests were as follows: kitchen tap: 3, 7; bathroom tap: 1, 9; bathtub spout: 6, 6; shower: 1, 4; hand-wash basin tap: 4, 6; bathtub water: 6, 2; toilet low tank: 1, 2; tank from a warm water washing toilet seat: 0, 1; washing machine: 10, 0; and garden hose in a house yard: 1, 3, respectively. Seven water samples that were collected from one house and were not included in Table 1, were used in Table 3.

emphasized the importance of recognizing the home water supply as a source of *Legionella* transmission. However, those studies were based on a culture method and, therefore, could not detect bacteria that were in a viable-but-non-culturable state. In this study, we performed both culture and DNA detection. *Legionella* DNA was detected in many water system samples; specifically, *Legionella* DNA was identified in water samples from taps and showers collected in seven out of 14 houses. The *Legionella* DNA detection rates in outlet water samples from kitchen, bathroom, hand-wash basin, and house yard taps and from showers ranged from 0% to 52·9%, with an average of 29·5%, which was much higher than the bacterial isolation rates.

We determined the sequence type of *L. pneumophila* in LAMP-positive samples, when *Legionella* was not isolated. A swab DNA from a bathtub spout contained ST1966, which is very rare, but we registered ST1966 as a new ST from *L. pneumophila* SG4 clinical isolate in Kanagawa prefecture in 2014. There was no relationship between this survey and the patient, although the area and the period were synchronized.

Combining the results of culture and DNA detection provides an improved understanding of the occurrence of *Legionella* spp. in domestic environments, and although the LAMP method is not quantitative, it can detect dead cells as well as viable cells. In this study, the *Legionella* detection rates determined by the culture method were 6·5% in water samples and 3·3% in swab samples, which are quite low compared with the *Legionella* DNA detection rates. However, this is not surprising because the detection of *Legionella* DNA is reported to be more sensitive than that of *Legionella* by the conventional culture method. Wang *et al*. and Furuhata *et al*. similarly observed a low prevalence of *Legionella* by the culture method but a much higher prevalence by quantitative PCR in drinking-water distribution systems [22] and by LAMP in hot spring water [23], respectively.

The combination of DNA amplification and the amoeba co-culture method increased the sensitivity of *Legionella* DNA detection in environmental samples. The amoeba co-culture method has been previously reported as a means of isolating *Legionella* spp. from environmental and clinical samples. Conza *et al*. [24] observed that a combination of the conventional culture method and the amoeba co-culture method yielded a three-log-higher sensitivity for detecting *L. pneumophila* from spiked compost. In this study, this combination also increased the positive rates of *Legionella* detection, especially at the DNA level.

The present study shows that *Legionella* is ubiquitous in homes and that there are multiple water environments in homes, where *Legionella* can colonize. While the risk of legionellae transmission from each of these aquatic environments is unclear, any aquatic environment contaminated with *Legionella* could be a potential source of *Legionella* dissemination. Redd and Cohen [25] reported that despite the widespread presence of *Legionella* in the environment, sporadic legionellosis is relatively infrequent. This conclusion is partly the result of the low sensitivity of earlier diagnostic methods. The development of improved diagnostic methods, such as the urinary antigen test and

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DNA detection methods, now make it possible to detect previously undetected *Legionella* cases.

Legionellae were detected from a garden hose in our study. Because watering with a garden hose produces aerosols, which can transmit legionellosis, this poses a risk of transmitting Legionnaires' disease. Cases of Legionnaires' disease and Pontiac fever linked to garden hoses have been previously reported [26].

Legionella DNA was also detected in showers and a toilet tank in this study. The presence of *Legionella* in showers has been previously described [27]. Notably, toilet flushing and showers both generate aerosols, which may result in the inhalation of legionellae if the water in these sources is contaminated with *Legionella* [28]. *Legionella* DNA was also detected with a high frequency in washing machines and aquariums in the present study. Risk of exposure to microbial pathogens may occur through the inhalation of aerosols generated during the washing machine cycle and/or discharge of spent water from the washing machine [29]. Contamination of *Legionella* sp. in a washing machine may be related to the occurrence of legionellosis, through the generation and spreading of aerosols containing *Legionella* sp. in the household. *Legionella* DNA was found not only in samples from fresh water aquariums but also in those from marine water aquariums. Gast *et al*. [30] observed that amoeba were capable of harboring legionellae in marine water. Importantly, aeration devices for fish in aquariums and washing machines both generate aerosols. Legionellae can persist in aquariums and washing machines, and these devices may pose a potential risk of causing *Legionella* infection, especially among people in high-risk groups. Smith *et al*. [31] reported that *L. pneumophila* and *L. birminghamensis* DNA were detected in one each of 14 aquarium water samples. They stated that owners of ornamental fish and the pet industry should take responsibility for the health of the people who care for fish.

L. pneumophila SG1 is a major cause of legionellosis and accounts for approximately 80% of isolates from patients with community-acquired pneumonia both worldwide [32] and in Japan [33]. In this study, a bath spout and a bath sponge from one house were each contaminated with *L. pneumophila* SG1, ST22, which is widely detected in patients as well as environments in many countries, but neither *L. pneumophila* nor *Legionella* DNA was detected from the water system of this house. These results suggest that *L. pneumophila* did not proliferate in the water system of this house and that bath spouts and bath sponges can persistently harbor and disseminate *L. pneumophila*. These environmental habitats could be reservoirs of *L. pneumophila* SG1.

The presence of *Legionella* spp. suggests the existence of biofilms and amoeba that might provide an appropriate environment for the proliferation of *Legionella*. In one of the houses from this study, *L. anisa* was detected in samples from bathtub water, an aquarium, and a pump hose. Based on a report by van der Mee-Marquet *et al*. [34] that *L. anisa* could be an indicator of water contamination by *L. pneumophila*, our results additionally suggest that *L. pneumophila* may also be able to grow in these environments. Leftover water in the bathtub was reused in the washing machine to wash clothes or to change aquarium water in the house. In typical Japanese houses, washing machines are usually positioned outside a bathroom, and many Japanese washing machines are equipped with a water pump and a hose to transfer leftover bathtub water to the washing machine. Japanese typically soak in the bathtub but wash their bodies outside the bathtub, so the leftover water is thought to be clean enough to be reused. The results of this study suggest that *L. anisa* was disseminated from the bathtub into the washing machine through a pump hose and also into the aquarium.

In water systems, the detection rates of *Legionella* DNA were moderately high in samples from bathtub spouts (52·9%) and showers (25·0%). Additionally, bathtub water accounted for 37·5% of positive *Legionella* DNA samples. These results suggested that equipment in bathrooms provide an environment for *Legionella* colonization. *Legionella* DNA was also highly detected in kitchen taps and hand-wash basin taps. Samples from bath spouts, showers, bathtubs, kitchen taps, and hand-wash basin taps were characterized as having mean temperatures over 25 °C, although the results of logistic regression analysis in this study did not suggest that warm temperatures promoted *Legionella* growth.

L. sainthelensi was detected in two aquarium samples in this study. *L. sainthelensi* was originally isolated from a natural water environment [35] and is rarely isolated from human cases. However, this species was reported as the causative agent of an outbreak of legionellosis in nursing homes with a contaminated water system [36]. *Legionella busanensis* and *Legionella rowbothamii*, which were detected in samples from a home garden hose and from the outlet water of a kitchen tap, respectively, in this study, are

https://doi.org/10.1017/S0950268817000036 Published online by Cambridge University Press

also rarely isolated species. *L. busanensis* was previously isolated from cooling towers [37]. To our knowledge, there have been no reports of *L. busanensis* or *L. rowbothamii* being isolated from legionellosis patients.

Controlling biofilm formation for *Legionella* prevention is difficult, especially in pipes. The results of our logistic regression analysis show that HPC is associated with the presence of *Legionella*, suggesting that a reduction of HPC may be effective for *Legionella* control. Our results also suggest that pH is positively associated with the presence of *Legionella*. Leoni *et al*. [38] and Marrie *et al*. [39] reported similar results. Results of logistic regression analysis did not indicate the association of *Legionella* colonization and temperature possibly because the temperature of the water samples collected in this study ranged from 16·0 to 39·5 °C, and these temperatures were too low to eradicate *Legionella*. Although multivariate logistic regression analysis using data collected in the present study did not show that residual chlorine concentrations independently affected *Legionella* contamination, effective concentrations of residual chlorine can generally disinfect HPC and *Legionella* in aquatic environments.

Measures for preventing *Legionella* contamination in houses should include frequent flushing of taps and showers to expel water harboring heterotrophic bacteria, such as stagnant water, and increasing daily water consumption [6]. Additional measures include frequently cleaning and disinfecting hoses, washing machines, bathtubs, and sponges with a disinfectant, such as chlorine, to prevent biofilm formation. Furthermore, where possible preventing aerosols from aquatic environments that could potentially be contaminated with *Legionella*, such as toilets [28], washing machines, and aquariums, may also be effective.

There were some limitations in this study. First, those were the small number, location and selection of houses investigated. Therefore, the results should not be considered representative of typical Japanese households. Second, the LAMP assay detects *Legionella* that is dead or alive with high sensitivity, resulting in the possibility of overestimation of *Legionella* risk. Despite these limitations, we believe our study provides important information regarding domestic *Legionella* infection.

In conclusion, our results reveal that aquatic environments in Japanese houses are highly contaminated with *Legionella*, and they suggest that exposure to *Legionella* spp. in these aquatic environments could increase the risk of legionellosis. Water systems can be a source of transmission of *Legionella* spp. Washing machines and aquariums should also be considered as potential sources of legionellosis. This study additionally reveals that *Legionella* contamination is associated with HPC and pH. Among these characteristics, reduction of HPC may be particularly important for preventing *Legionella* contamination. To minimize such contamination, control measures should include frequently flushing taps and showers to expel water harboring heterotrophic bacteria, frequently cleaning and disinfecting surfaces to remove biofilms, and preventing aerosols from aquatic environments as much as possible. The effects of the preventive measures recommended herein should be investigated in future studies.

ACKNOWLEDGEMENTS

This study was partially supported by Health and Labour Sciences Research Grants (H25-kenki-ippan-007, H25-kenki-ippan-009, H28-kenki-ippan-005, and H28-kenki-ippan-006) from the Ministry of Health, Labour and Welfare of Japan. We thank Dr K. Yagita, Department of Parasitology, National Institute of Infectious Diseases, for kindly providing an *Acanthamoeba* strain.

DECLARATION OF INTEREST

None.

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