Close Genetic Relationship between *Legionella pneumophila* Serogroup 1 Isolates from Sputum Specimens and Puddles on Roads, as Determined by Sequence-Based Typing

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We investigated the prevalence of *Legionella* species isolated from puddles on asphalt roads. In addition, we carried out sequence-based typing (SBT) analysis on the genetic relationship between *L. pneumophila* serogroup 1 (SG 1) isolates from puddles and from stock strains previously obtained from sputum specimens and public baths. Sixty-nine water samples were collected from puddles on roads at 6 fixed locations. *Legionella* species were detected in 33 samples (47.8%) regardless of season. Among the 325 isolates from puddles, strains of *L. pneumophila* SG 1, a major causative agent of Legionnaires' disease, were the most frequently isolated ($n = 62$, 19.1%). Sixty-two isolates of *L. pneumophila* SG 1 from puddles were classified into 36 sequence types (STs) by SBT. ST120 and ST48 were identified as major STs. Environmental ST120 strains from puddles were found for the first time in this study. Among the 14 STs of the clinical isolates ($n = 19$), 4 STs ($n = 6$, 31.6%), including ST120, were also detected in isolates from puddles on roads, and the sources of infection in these cases remained unclear. The *lag-1* gene, a tentative marker for clinical isolates, was prevalent in puddle isolates (61.3%). Our findings suggest that puddles on asphalt roads serve as potential reservoirs for *L. pneumophila* in the environment.

Legionella pneumophila is a major agent causing Legionnaires' disease, which is a severe form of legionellosis and a potentially fatal pneumonia (1). *L. pneumophila* is a Gram-negative bacterium that is ubiquitous in natural environments. In addition, it has been found in anthropogenic environments, such as cooling towers, baths, showers, and decorative fountains (2–5). Legionellosis may be acquired through inhalation of aerosolized water contaminated with *Legionella* species (6). Therefore, aquatic facilities are potential sources of sporadic cases or outbreaks of infection. Although 58 species and more than 70 serogroups (SG) of *Legionella* species have been identified (7), more than 90% of legionellosis cases are caused by *L. pneumophila* (8). Among 15 serogroups of *L. pneumophila*, most clinical strains (80%) in Japan belonged to SG 1 (9). Recently, in the United States, Kozak et al. revealed that 75% of the *L. pneumophila* SG 1 clinical isolates but only 8% of environmental isolates harbored the *lag-1* gene, which is required for the expression of the virulence-associated epitope recognized by monoclonal antibody 2 of the international standard panel (10).

To identify the infection sources of legionellosis cases, sequence-based typing (SBT) was proposed by the European Working Group for *Legionella* Infections (EWGLI); SBT is a sequence-based scheme comprising defined regions of 7 genes (*flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, and *neuA*) for *L. pneumophila* (11–13). SBT has been used as a molecular typing method to characterize *L. pneumophila* SG 1 strains (14–16).

In Japan, public baths are a major source of infection, according to the National Epidemiological Surveillance of Infectious Diseases (17). Fatal cases of legionellosis from homes and spa pools have been reported (18, 19). Recently, several reports revealed that legionellosis could be acquired from puddles of rainwater on roads (20) and from air-conditioning systems of motor vehicles (21). These environments have thus been considered po-

tential new sources of infection. However, the genetic relationships between strains from clinical specimens and from these environmental sources have not been clearly analyzed by molecular typing techniques. Furthermore, our previous study reported that the comparative analysis of *L. pneumophila* SG 1 isolates from sputum specimens and public baths found a clonal group formed only by clinical isolates that were not associated with bath water (22). The short genetic distance between strains of the clonal group suggested that they were derived from a common and unrecognized type of source.

We hypothesize that clinical isolates that are not associated with bath water may be genetically close to isolates from puddles on asphalt roads. The main objective of this study was to characterize the genetic relationship between *L. pneumophila* SG 1 isolates from puddles and stock strains previously isolated from sputum specimens, public baths, and some other environmental sources.

MATERIALS AND METHODS

Bacterial strains. A total of 140 *L. pneumophila* SG 1 isolates were analyzed, including isolates from puddles on roads ($n = 62$), public baths ($n = 51$), cooling towers ($n = 5$), showers ($n = 3$), and sputum specimens ($n = 19$). Among these, 62 isolates from puddles and 5 isolates from other sources (1 isolate from a cooling tower, 2 isolates from 2 showers, and 2 isolates from 2 patients) obtained in this study were collected from 2010 to

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2011 and from 2010 to 2012, respectively, in Toyama Prefecture, Japan (Table 1); the remaining 73 isolates (51 isolates from 24 public baths, 4 isolates from 2 cooling towers, 1 isolate from a shower, and 17 isolates from 16 patients), obtained in a previous study, were collected from 2005 to 2012 in Toyama Prefecture, Japan (22).

Identification of *Legionella* species from puddles on a road. After rainfall, water samples were collected at 6 fixed locations along 3 main national roads once per month from November 2010 to October 2011 in Toyama Prefecture (Fig. 1). The eastern and southern parts of the prefecture were not selected for sampling because they are mountainous and sparsely populated. Samples from locations A to E were collected from November 2010 to October 2011; samples from location F were collected from February to October 2011.

Water samples (150 ml) from puddles on asphalt roads were filtered with a 0.22- μ m-pore-size polycarbonate membrane (catalog no. GTP04700; Millipore, Billerica, MA, USA) and resuspended in 3 ml of distilled water. After the concentrated samples were mixed with equal volumes of 0.2 mol/liter KCl-HCl buffer (pH 2.2) for 5 min at room temperature, they were spread onto glycine-vancomycin-polymyxin B-cycloheximide agar plates (bioMérieux, Lyon, France) and modified Wadowsky Yee agar plates (Oxoid, Basingstoke, Hampshire, United Kingdom). The agar plates were incubated at 35°C for 7 days in a moist chamber. Smooth gray colonies were subcultured onto buffered charcoal-yeast extract agar plates (bioMérieux) and blood agar plates (Eiken Chemical, Tokyo, Japan). The colonies growing only on buffered charcoal-yeast extract agar plates were presumed to belong to the genus *Legionella* by observation of the characteristic outward structures (cut-glass-like or mosaiclike appearance) under a stereo microscope with oblique illumination (23). The detection limit of the procedure was 10 CFU/100 ml. These colonies were tested using a latex agglutination test kit (catalog no. DR0800M; Oxoid, Hampshire, United Kingdom) and slide agglutination with commercial antisera (Denka Seiken, Tokyo, Japan) to determine the *Legionella* species and serogroups. The remaining colonies not identified by serological methods were examined by PCR with primers for *Legionella* genus-specific 16S rRNA genes and *L. pneumophila* species-specific *mip* genes (24, 25). The species of some isolates were determined by sequencing of 16S rRNA genes as described previously, with a slight modification (26). Sequencing was performed with an ABI Prism 3130xl genetic analyzer (Applied Biosystems, Foster City, CA, USA) and primers 27f (AGA GTTGATCCTGGCTCAG) and r1L (GTATTACCGCGGCTGCTGG).

SBT. SBT was performed according to the protocol of the EWGLI (http://www.hpa-bioinformatics.org.uk/legionella/legionella_sbt/php/sbt_homepage.php), as described previously (11, 12). Novel alleles and sequence types (STs) were submitted to the EWGLI SBT database for assignment. Clonal analyses were performed by using eBURST V3 (<http://eburst.mlst.net>). Groups that were generated with single-, double-, and triple-locus variants were defined as clonal groups (CGs).

PCR amplification of the *lag-1* gene. Genomic DNA was extracted by emulsifying several colonies of *L. pneumophila* in 100 μ l of 5% (wt/vol) Chelex-100 solution (Bio-Rad Laboratories, Tokyo, Japan). The suspension was boiled at 100°C for 10 min and then centrifuged at 20,000 $\times$ g for 5 min at room temperature. The supernatant was used as a DNA template. The primers lag-F and lag-R were used for amplification of the *lag-1* gene (10). The PCR was performed using GoTaq green master mix (Promega, Madison, WI, USA) under the following conditions: initial denaturation at 95°C for 2 min and 30 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 1 min.

Statistical analysis. The χ^2 test was performed to compare the proportions of *Legionella*-positive and -negative puddles, as well as *lag-1*-positive and -negative isolates, using Microsoft Excel (Microsoft, Tokyo, Japan). To test for significant differences in the cell concentrations of *Legionella* species, the Mann-Whitney U test was performed using R statistical software (version 2.15.1). A *P* value of <0.05 was considered statistically significant.

Nucleotide sequence accession numbers. The sequence data from this study have been submitted to DNA Data Bank of Japan (<http://www.ddbj.nig.ac.jp/>) under accession numbers AB811041, AB811042, AB811044, AB811045, AB811047 to AB811052, AB811054, AB811055, AB811057 to AB811064, AB811066 to AB811075, and AB811077.

RESULTS

Isolation of *Legionella* species from water samples of puddles on roads. *Legionella* species were detected in 33 samples (47.8%) from locations A (*n* = 4), B (*n* = 5), C (*n* = 7), D (*n* = 7), E (*n* = 7), and F (*n* = 3) (Fig. 1). *Legionella* species were isolated frequently, except in September and October 2011 (Table 2). Among the 33 positive samples, the concentrations of *Legionella* species ranged from 10 to 99 CFU/100 ml in 18 (54.5%) samples, 14 (42.4%) samples contained 100 to 999 CFU/100 ml, and 1 (3.0%) sample contained 7,520 CFU/100 ml. Even when the mean temperature was <0°C in January, *Legionella* species were isolated from 4 of 5 samples, and 3 samples contained 100 to 999 CFU/100 ml. Furthermore, the isolation rates of *Legionella* species at mean temperatures of $\geq 20^\circ\text{C}$ (50.0%, 12/24) and <20°C (46.7%, 21/45) were almost the same (*P* > 0.05; the χ^2 test), indicating that *Legionella* species were detected regardless of temperature. However, the concentrations of *Legionella* species ranged from 20 to 7,520 CFU/100 ml at mean temperatures of $\geq 20^\circ\text{C}$ and from 10 to 240 CFU/100 ml at mean temperatures of <20°C. The Mann-Whitney U test revealed that the concentrations of *Legionella* species at mean temperatures of $\geq 20^\circ\text{C}$ and <20°C were significantly different (*P* < 0.05): the geometric means $\pm$ standard deviations ($\log_{10}$ CFU/100 ml) in *Legionella*-positive puddles were 2.30 ± 0.68 and 1.63 ± 0.47 , respectively.

Among the 33 positive samples in which *Legionella* species were detected, *L. pneumophila* was detected in 26 samples (78.8%), including 4 samples at mean temperatures of <0°C in January. Thus, *L. pneumophila* was also frequently detected in samples from puddles regardless of temperature.

Serological distribution of *Legionella* species. We isolated 325 colonies from puddles from the 6 sampling locations. According to the serogroup typing, 234 isolates were identified as *L. pneumophila* strains. The remaining 91 isolates were examined by PCR, resulting in 11 additional *L. pneumophila* strains. Overall, the most-prevalent species was *L. pneumophila* (*n* = 245, 75.4%), whereas other *Legionella* species accounted for the remaining 24.6% (*n* = 80). Among the 245 *L. pneumophila* isolates, SG 1 accounted for 25.3% (*n* = 62), followed by SG 5 (*n* = 56, 22.9%), SG 8 (*n* = 50, 20.4%), and others (SG 2, SG 6, SG 9, SG 3, SG 11, SG 14, and untypeable; *n* = 77, 31.4%) (Fig. 2A). Among the 80 non-*L. pneumophila* isolates, 31 were randomly selected, and the species of these isolates were determined by 16S rRNA gene sequencing. *L. gresilensis* accounted for 71.0% (*n* = 22), followed by *L. longbeachae* (*n* = 6, 19.4%), *L. oakridgensis* (*n* = 1, 3.2%), *L. sainthelensi* (*n* = 1, 3.2%), and *L. waltersii* (*n* = 1, 3.2%) (Fig. 2B).

Distribution of STs and *lag-1* genes. We analyzed 62 *L. pneumophila* SG 1 isolates obtained from puddles on asphalt roads in comparison with 73 *L. pneumophila* SG 1 stock strains from a previous study (22) and 5 isolates obtained from sources other than puddles in this study (Table 1). The 19 (total) clinical isolates from sputum specimens were collected from 4 hospitals in Toyama Prefecture. Among these, 17 isolates were obtained from 17 patients; the remaining 2 isolates were obtained from the same patient but classified into different STs.

TABLE 1 Sequence types and *lag-1* gene results of *L. pneumophila* serogroup 1 isolates obtained in this study^a

Strain	Origin	Date of isolation		Allele no.							ST	Presence of <i>lag-1</i>
		Yr	Mo	<i>flaA</i>	<i>pilE</i>	<i>asd</i>	<i>mip</i>	<i>mompS</i>	<i>proA</i>	<i>neuA</i>		
LG1554	PU	2010	Nov	2	3	6	10	2	1	6	22	Negative
LG1555	PU	2010	Nov	2	3	9	10	2	1	6	23	Negative
LG1732	PU	2011	May	2	3	9	10	2	1	6	23	Negative
LG1743	PU	2011	May	2	3	9	10	2	1	6	23	Positive
LG1551	PU	2010	Nov	5	2	22	27	6	10	12	48	Negative
LG1552	PU	2010	Nov	5	2	22	27	6	10	12	48	Negative
LG1553	PU	2010	Nov	5	2	22	27	6	10	12	48	Negative
LG1625	PU	2011	Jan	5	2	22	27	6	10	12	48	Negative
LG1719	PU	2011	May	5	2	22	27	6	10	12	48	Negative
LG1723	PU	2011	May	5	2	22	27	6	10	12	48	Negative
LG1742	PU	2011	May	5	2	22	27	6	10	12	48	Negative
LG1737	PU	2011	May	2	3	18	13	25	5	6	75	Positive
LG1680	PU	2011	Mar	4	10	11	15	29	1	6	89	Positive
LG1729	PU	2011	May	12	8	11	20	5	12	6	118	Positive
LG1550	PU	2010	Nov	2	3	5	11	2	1	6	120	Positive
LG1674	PU	2011	Mar	2	3	5	11	2	1	6	120	Positive
LG1736	PU	2011	May	2	3	5	11	2	1	6	120	Positive
LG1746	PU	2011	May	2	3	5	11	2	1	6	120	Positive
LG1747	PU	2011	May	2	3	5	11	2	1	6	120	Positive
LG1771	PU	2011	Jun	2	3	5	11	2	1	6	120	Positive
LG1772	PU	2011	Jun	2	3	5	11	2	1	6	120	Positive
LG1805	PU	2011	Aug	3	13	1	10	14	9	11	127	Negative
LG1604	PU	2010	Dec	2	1	6	15	2	1	6	132	Positive
LG1749	PU	2011	May	2	1	6	15	2	1	6	132	Positive
LG1673	PU	2011	Mar	2	3	9	10	2	1	10	384	Positive
LG1766	PU	2011	Jun	2	3	9	10	2	1	10	384	Positive
LG1698	PU	2011	Mar	2	3	5	10	2	1	6	507	Negative
LG1740	PU	2011	May	2	3	5	10	2	1	6	507	Positive
LG1748	PU	2011	May	2	3	5	10	2	1	6	507	Positive
LG1546	PU	2010	Nov	8	10	3	10	2	1	6	610	Positive
LG1814	PU	2011	Aug	12	9	2	5	27	20	6	615	Positive
LG1679	PU	2011	Mar	12	8	11	10	5	12	6	624	Positive
LG1622	PU	2011	Jan	21	40	43	20	15	26	2	808	Negative
LG1632	PU	2011	Jan	21	40	43	20	15	26	2	808	Negative
LG1768	PU	2011	Jun	2	3	6	15	51	1	6	876	Positive
LG1733	PU	2011	May	2	3	5	3	2	1	9	1186	Positive
LG1735	PU	2011	May	2	3	5	3	2	1	9	1186	Positive
LG1631	PU	2011	Jan	2	3	5	13	2	1	6	1187	Positive
LG1767	PU	2011	Jun	2	3	5	13	2	1	6	1187	Positive
LG1638	PU	2011	Jan	2	3	5	28	2	1	6	1188	Positive
LG1799	PU	2011	Aug	2	3	5	58	2	1	6	1189	Positive
LG1683	PU	2011	Mar	2	3	18	12	35	1	6	1190	Positive
LG1797	PU	2011	Aug	2	3	58	10	2	1	6	1191	Positive
LG1753	PU	2011	May	2	10	5	10	2	1	6	1192	Negative
LG1739	PU	2011	May	2	10	5	10	2	1	9	1193	Negative
LG1741	PU	2011	May	2	10	5	10	2	1	9	1193	Negative
LG1678	PU	2011	Mar	2	10	5	47	18	5	9	1194	Negative
LG1681	PU	2011	Mar	2	10	9	12	2	5	6	1195	Positive
LG1540	PU	2010	Nov	2	10	14	3	18	4	11	1196	Negative
LG1730	PU	2011	May	2	10	57	10	18	5	9	1197	Negative
LG1630	PU	2011	Jan	2	23	13	25	18	22	9	1198	Positive
LG1697	PU	2011	Mar	2	23	13	25	18	22	9	1198	Positive
LG1813	PU	2011	Aug	2	31	5	21	18	12	6	1199	Positive
LG1811	PU	2011	Aug	4	8	11	10	11	12	10	1200	Positive
LG1745	PU	2011	May	12	8	11	56	2	12	34	1201	Positive
LG1686	PU	2011	Mar	12	15	11	56	11	12	34	1202	Positive
LG1541	PU	2010	Nov	21	14	29	1	15	29	6	1203	Positive
LG1543	PU	2010	Nov	34	27	56	57	72	29	44	1204	Negative
LG1544	PU	2010	Nov	34	27	56	57	72	29	44	1204	Negative

(Continued on following page)

TABLE 1 (Continued)

Strain	Origin	Date of isolation		Allele no.							ST	Presence of lag-1
		Yr	Mo	flaA	pilE	asd	mip	mompS	proA	neuA		
LG1691	PU	2011	Mar	34	27	56	57	72	29	44	1204	Negative
LG1804	PU	2011	Aug	34	27	56	57	72	29	44	1204	Negative
LG1689	PU	2011	Mar	34	27	55	54	71	44	44	1225	Negative
LG1975	CT	2011	Jun	1	4	3	1	1	1	1	1	Negative
LG2051	SH	2012	Nov	7	6	17	3	13	11	11	59	Negative
LG2055	SH	2012	Nov	5	2	22	27	6	10	12	48	Negative
LG1535	PA	2010	Nov	2	3	5	15	2	1	6	973	Positive
LG1757	PA	2011	May	10	12	7	3	16	18	6	138	Positive

^a PU, puddle; CT, cooling tower; SH, shower; PA, patient; ST, sequence type.

Sixty-two *L. pneumophila* SG 1 isolates from puddles were classified into 36 STs (Tables 1 and 3). Twenty of the 36 STs were identified for the first time in this study. The major STs were ST120 (*n* = 7, 11.3%) and ST48 (*n* = 7, 11.3%), followed by ST1204 (*n* = 4, 6.5%), ST23 (*n* = 3, 4.8%), and ST507 (*n* = 3, 4.8%). Twenty-four STs were represented by only a single isolate.

Puddle isolates of ST120 were detected in samples from locations B (*n* = 2), C (*n* = 1), and E (*n* = 4), and puddle isolates of ST48 in samples from locations A (*n* = 1), B (*n* = 1), C (*n* = 3), and D (*n* = 2) (Fig. 1). Furthermore, ST120 and ST48 strains were isolated during 4 (November 2010 and February, May, and June 2011) and 3 (November 2010 and January and May 2011) different months, respectively.

PCR amplification showed that 59.7% (37/62) of *L. pneumophila* SG 1 isolates from puddles harbored the *lag-1* gene (Table 3). Among the isolates belonging to ST120 and ST48, which were the major STs of puddle isolates, the *lag-1* gene was present in all ST120 isolates (7/7) and was missing in all ST48 isolates (0/7).

Clonal analysis. Clonal analyses were performed using *L. pneumophila* isolates obtained from puddles on roads (*n* = 62), public baths (*n* = 51), cooling towers (*n* = 5), showers (*n* = 3), and patients with legionellosis (*n* = 19) (Table 4). The puddle isolates formed 2 major CGs, CG1 and CG4, which included

58.1% and 8.1% of puddle isolates, respectively (Table 4). On the other hand, bath isolates formed other CGs, CG2 and CG3, which included 49.0% and 25.5% of bath isolates, respectively.

Among the 14 STs of clinical isolates (*n* = 19), 4 STs (ST120, ST132, ST384, and ST507; *n* = 6) and 3 STs (ST138, ST505, and ST644; *n* = 6) were also detected in isolates from puddles and public baths, respectively. The remaining 7 STs (*n* = 7) were detected in CGs that included environmental isolates, although the same ST was not observed in the environmental isolates. CG1, CG2, CG3, CG4, and CG5 included 7 STs (ST120, ST132, ST353, ST384, ST506, ST507, and ST973), 3 STs (ST2, ST502, and ST644), 2 STs (ST505 and ST682), ST42, and ST138, respectively.

DISCUSSION

We investigated the prevalence of *Legionella* species isolated from puddles on asphalt roads and the genetic relationships between isolates from sputum specimens and environmental sources. Sixty-two isolates of *L. pneumophila* SG 1 from puddles were classified into 36 STs by SBT, with ST120 and ST48 being the major STs; strains of these 2 STs were widely distributed in Toyama Prefecture regardless of the season. Although ST48 environmental strains were primarily isolated from soil and partially from cooling towers, as reported previously (27), ST120 environmental strains were not mentioned either in the previous report or in the EWGLI SBT database as of 27 February 2013. On the other hand, ST120 clinical strains were detected in 5.8% of isolates from patients with legionellosis in Japan, although the sources of infection remain unclear as determined by epidemiological investigation (9). It was not described whether isolation of *L. pneumophila* strains from environmental sources, including water from puddles on roads, was carried out or not in these cases. Our results showed that 1 clinical strain also belonged to ST120. This strain was not associated with bathwater as determined by epidemiological investigation. Furthermore, isolation of *L. pneumophila* strains from other environmental sources was not carried out. Thus, the source of infection in this case was not clarified. Hicks et al. reported that a 1-cm increase in rainfall was associated with a 2.6% increased risk of legionellosis (28). Furthermore, Fisman et al. identified an association between legionellosis and increased humidity (odds ratio per 1% increase in relative humidity of 1.08, and 95% confidence interval of 1.05 to 1.11) 6 to 10 days before the occurrence of legionellosis cases (29). Therefore, our findings suggest the possibility that patients with legionellosis caused by ST120 strains may have inhaled splashed aerosols from puddles contaminated with

FIG 1 Locations of the 6 fixed points for sampling from puddles on roads. Sixty-nine samples were collected from the 6 locations as follows: A (*n* = 12), B (*n* = 12), C (*n* = 12), D (*n* = 12), E (*n* = 12), and F (*n* = 9).

TABLE 2 Distribution of *Legionella* species from puddles on roads in Toyama Prefecture, Japan

			No. of samples (no. with <i>L. pneumophila</i>):					Geometric mean $\pm$ SD ($\log_{10}$ CFU/100 ml) in <i>Legionella</i> -positive puddles
Yr	Mo	Mean temp ($^{\circ}$ C)	Analyzed	<i>Legionella</i> positive	With CFU/100 ml of:			
					10–99	100–999	>1,000	
2010	Nov	11.4	5	4 (4)	4 (4)	0	0	1.3 $\pm$ 0.3
	Dec	10.4	5	3 (2)	3 (2)	0	0	1.4 $\pm$ 0.3
2011	Jan	−0.6	5	4 (4)	1 (1)	3 (3)	0	2.0 $\pm$ 0.1
	Feb	9.0	6	5 (5)	4 (4)	1 (1)	0	1.6 $\pm$ 0.5
	Mar	11.5	6	4 (1)	3 (1)	1 (0)	0	1.7 $\pm$ 0.5
	Apr	15.0	6	1 (0)	0	1 (0)	0	2.4
	May	21.2	6	4 (4)	1 (1)	3 (3)	0	1.9 $\pm$ 0.4
	Jun	32.2	6	2 (2)	1 (1)	1 (1)	0	1.8 $\pm$ 0.2
	Jul	31.0	6	3 (1)	0	2 (1)	1 (0)	3.2 $\pm$ 0.5
	Aug	26.2	6	3 (3)	1 (1)	2 (2)	0	2.2 $\pm$ 0.5
	Sep	18.0	6	0	0	0	0	Not measured
	Oct	16.4	6	0	0	0	0	Not measured
Total			69	33 (26)	18 (15)	14 (11)	1 (0)	

this strain. The fact that all ST120 isolates in this study harbored the *lag-1* gene, a tentative marker for clinical isolates, supports this hypothesis. Alternatively, patients with legionellosis may be infected indirectly through a puddle route. In this regard, driving an automobile using windshield wiper fluid without added windshield washes that usually contain biocidal agents like propranolol has been reported as a newly identified risk factor for the disease (30), and *L. pneumophila* strains were actually found in windshield wiper fluid without added windshield washes (31). So, automobiles may be contaminated with *L. pneumophila* strains originating from puddles due to splashing. To date, Palmer et al. have not reported the STs of the isolates from windshield wiper fluid.

The *lag-1* gene was prevalent in puddle isolates (59.7%). Among the *L. pneumophila* SG 1 isolates in Toyama Prefecture, 100% (17/17) of clinical isolates and 43.1% (22/51) of bath isolates harbored the *lag-1* gene (J. Kanatani, J. Isobe, K. Kimata, T. Shima,

M. Shimizu, F. Kura, T. Sata, and M. Watahiki, unpublished data). The frequencies of the isolates harboring the *lag-1* gene were not significantly different between isolates from puddles and public baths ($P > 0.05$, χ^2 test). In Japan, the number of legionellosis cases peaked in July, the second half of the rainy season (32, 33). To elucidate the possibility that splashed aerosols from puddles on a road, as well as public bath water, are a probable source of legionellosis, we need further investigation of the virulence of these strains by a combination of molecular typing profiles such as SBT, *lag-1* allele typing, and monoclonal antibody subgrouping (9).

Among the 14 STs of clinical isolates ($n = 19$), 4 STs (ST120, ST132, ST384, and ST507; $n = 6$) were also detected in isolates from puddles on roads, suggesting that 33.3% (6/18) of patients (6/19 of clinical isolates) may be infected directly or indirectly through a puddle route rather than a bath route. As with the case of the ST120 clinical isolate, the infection source of the remaining

FIG 2 Distribution of *Legionella* species from puddles. (A) Frequencies of serogroups among *L. pneumophila* isolates. (B) Frequencies of *Legionella* species among non-*L. pneumophila* isolates identified by 16S rRNA gene sequencing.

TABLE 3 Sequence types of *L. pneumophila* serogroup 1 isolates obtained from puddles on roads in Toyama Prefecture, Japan

ST ^a	No. (%) of isolates	No. with indicated result for <i>lag-1</i>	
		Positive	Negative
120	7 (11.3)	7	0
48	7 (11.3)	0	7
1204 ^b	4 (6.5)	0	4
23	3 (4.8)	3	0
507	3 (4.8)	2	1
132	2 (3.2)	1	1
384	2 (3.2)	2	0
808	2 (3.2)	0	2
1186 ^b	2 (3.2)	2	0
1187 ^b	2 (3.2)	2	0
1193 ^b	2 (3.2)	0	2
1198 ^b	2 (3.2)	2	0
Others ^c	24 (38.7)	16	8
Total	62 (100)	37	25

^a ST, sequence type.^b The ST was identified for the first time in this study.^c Fifteen of 24 STs were identified for the first time in this study, and each was presented by only a single isolate.

5 clinical isolates also remained unclear. For the identification of unrecognized sources of legionellosis, in our opinion, *L. pneumophila* strains should be isolated from clinical specimens and various environmental sources, including water from puddles on roads, and analyzed by molecular typing methods such as SBT and monoclonal antibody subgrouping (9).

In samples from puddles, *L. pneumophila* SG 1, SG 5, and SG 8 were frequently detected. In those from soil, among 87 *L. pneumophila* isolates, SG 1 accounted for 42.5% ($n = 37$), followed by SG 8 (18.4%, $n = 16$) and SG 3 (16.1%, $n = 14$), although SG 5 was not detected (J. Amemura-Maekawa, K. Kikukawa, J. H. Helbig, S. Kaneko, A. Suzuki-Hashimoto, K. Furuhashi, B. Chang, M. Murai, M. Ichinose, M. Ohnishi, F. Kura, and the Working Group for *Legionella* in Japan, unpublished data). Furthermore, 31 of the 36 puddle isolates belonging to CG1 revealed the same or single-

double-, and triple-locus variants of STs derived from soil (group S1 [27]) and 3 of the 5 puddle isolates belonging to CG4 revealed triple-locus variants of STs derived from soil (group S3 [27]). These results showed that the isolates from puddles and soil were genetically and serologically close to each other. However, the only common STs detected in samples from both puddles and soil were ST22 and ST48. To elucidate the habitat segregation of *L. pneumophila* strains in these environments, further investigation of isolates from puddles and soil is required.

In this study, *Legionella* species were isolated even at low temperatures. In this regard, Söderberg et al. reported that *L. pneumophila* strains could survive in tap water at 4°C for about a year, while a gradual decline in the number of CFU was seen (34). Although *Legionella* species were not detected in puddles during the last 2 months of collection for this study (September and October 2011), 4 of 6 puddles regained positivity for *L. pneumophila* and/or other *Legionella* species 2 months later (data not shown). Thus, *Legionella* species, including *L. pneumophila*, were distributed in puddles regardless of the season. A previous study in metropolitan Tokyo reported that the isolation rates of *L. pneumophila* strains from puddles increased with increasing mean temperatures and that *L. pneumophila* SG 1 strains were the most frequently isolated (20). Because *L. pneumophila* was frequently detected in samples from puddles, the possibility of contracting legionellosis in daily life should be considered. It may be important to recommend that individuals who are immunocompromised or elderly wear masks to avoid acquiring the disease, especially during the warm rainy season. Among the 5 other *Legionella* species detected in samples from puddles in this study, to the best of our knowledge, *L. longbeachae*, *L. oakridgensis*, and *L. saintel-helmsi* have been isolated from patients with pneumonia (35–37) and *L. waltersii* DNA was identified in a patient with pneumonia (38). Thus, these species may also be important in the etiology of community-acquired pneumonia.

In conclusion, ST120 environmental strains were isolated from puddles on an asphalt road for the first time in this study. Furthermore, 33.3% of patients with legionellosis in Toyama Prefecture, Japan, may be infected directly or indirectly through a puddle route. Our findings by SBT analysis suggest that puddles on as-

TABLE 4 Distributions of clonal groups from 74 sequence-based typing profiles of *L. pneumophila* serogroup 1 isolates in Toyama Prefecture, Japan^a

CG (total no. of STs; no. of isolates)	No. (%) of isolates ($n = 140$) from:					ST(s) detected in both clinical and environmental isolate(s) ^b (no. and sources of isolates with ST)
	PU	PB	CT	SH	PA	
CG1 (25; 46)	36 (58.1)	1 (2.0)	0	0	9 (47.4)	120 (7 from PU, 1 from PA), 384 (2 from PU, 3 from PA), 507 (3 from PU, 1 from PA), 132 (2 from PU, 1 from PA)
CG2 (15; 28)	0	25 (49.0)	0	0	3 (15.8)	644 (4 from PB, 1 from PA)
CG3 (7; 19)	0	13 (25.5)	0	1	5 (26.3)	505 (5 from PB, 4 from PA)
CG4 (6; 6)	5 (8.1)	0	0	0	1 (5.3)	
CG5 (3; 5)	0	4 (7.8)	0	0	1 (5.3)	138 (1 from PB, 1 from PA)
CG6 (4; 4)	1 (1.6)	3 (5.9)	0	0	0	
CG7 (2; 2)	1 (1.6)	1 (2.0)	0	0	0	
CG8 (2; 2)	0	2 (3.9)	0	0	0	
Singletons (10; 28) ^b	19 (30.6)	2 (3.9)	5	2	0	
Total	62 (100)	51 (100)	5	3	19 (100)	

^a CG, clonal group; PU, puddle; PB, public bath; CT, cooling tower; SH, shower; PA, patient; ST, sequence type.^b Singletons include 10 STs that are not grouped into any CGs.

phalt roads serve as potential reservoirs for *L. pneumophila* in the environment, which could increase potential opportunities for exposure. To identify unrecognized sources of infection in legionellosis cases, we need to isolate *L. pneumophila* strains from clinical specimens and various environmental sources, including water from puddles on roads, and to analyze these strains by a combination of molecular typing techniques and epidemiological investigation.

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