富山県内で分離された患者および環境由来レジオネラ・ニューモフィラ血清群１の分子疫学解析

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Molecular epidemiology of *Legionella pneumophila* serogroup 1 isolates from sputum specimens and environmental sources in Toyama Prefecture, Japan

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富山県内で分離された患者および環境由来レジオネラ・ニューモフィラ血清群 1 の分子疫学解析

レジオネラ症は、レジオネラ属菌が原因で引き起こされる感染症で、インフルエンザ様の熱症状を示すポンティアック熱と、重症化しやすいレジオネラ肺炎の 2 つの病型がある。ポンティアック熱は数日で回復する場合がほとんどである。一方、レジオネラ肺炎は 2～10 日の潜伏期を経て、悪寒、39℃以上の高熱、頭痛、筋肉痛などが起こり、呼吸困難、意識障害の症状がしばしば現れ、まれに死亡することもある。現在まで、レジオネラ肺炎の原因となるレジオネラ属菌の病原遺伝子は報告されておらず、重症化の原因は解明されていない。

2013 年には全国で 1,124 人の患者が報告され、そのうち富山県内の患者数は 39 人であった。月別に見ると、レジオネラ症患者報告数は梅雨時の 7 月に最も多かった。富山県の罹患率（人口 10 万人対）は、全国平均 0.88 に比べ 3.57 を記録しており全国で最も高い県であり、この傾向は 2005 年以降続いている。また、富山県内での過去 10 年間の罹患率を地域別に見ると、県西部が 20.8、県東部が 12.0 となり、県西部で高い傾向にある。

レジオネラ属菌は、土壌や河川などの自然環境だけでなく、入浴施設や冷却塔など人工的な環境からも広く検出される。これまでレジオネラ属菌は 58 菌種が報告されているが、レジオネラ症患者から分離されるレジオネラ属菌の 8 割以上が *Legionella pneumophila* 血清群 1 である。

国立感染症研究所の感染症サーベイランスシステムによると、国内におけるレジオネラ症患者の主な感染源は浴用施設である。富山県において、疫学調査によって感染源を推定できた患者は約半数であり、そのほとんどが浴用施設であったが、疫学調査が十分実施できない場合もあり、その他の患者の感染源は不明の場合が多い。国内における患者由来株については、国立感染症研究所で Sequence-Based Typing（SBT）による遺伝子解析が行われているが、中でも患者由来株の 5.3%を占める遺伝子型である *L. pneumophila* 血清群 1 の ST120 はこれまで環境由来株として分離された報告はなく、この株に感染した患者の感染源は不明である。近年の報告では、浴用施設、冷却塔、土壌などの環境中

からレジオネラ属菌が多数分離され、遺伝子解析が行われている。しかしながら、アスファルト道路の水たまりについては、1 年を通して気温や周囲の環境などの条件を加えた調査は実施されておらず、水たまりにおけるレジオネラ属菌の詳細な分布は不明である。また、水たまりが感染源になりうる可能性について、分離菌を用いた詳細な分子疫学解析も行われていない。

そこで本研究では、レジオネラ症患者の浴用施設以外の感染源を解明するため、アスファルト道路の水たまりからのレジオネラ属菌の検出と、分離された *L. pneumophila* 血清群 1 について、当所に保存されている患者および浴用水由来株も含めて分子疫学的解析を行い、以下の結果を得た。

1）2010 年 11 月～2011 年 10 月にかけて、月に 1 度、雨天の当日あるいは翌日に、県内 6 か所のアスファルト道路の水たまりから採水した。これら検体からのレジオネラ属菌の検出率は 47.8%（33/69 検体）であった。地域による検出率に偏りは見られず、レジオネラ属菌は 6 か所のすべての地点から検出された。陽性であった 33 検体の菌数は、10～90 CFU/100 ml が 18 検体、100～990 CFU/100 ml が 14 検体、1,000 CFU/100 ml 以上が 1 検体であった。気温 20℃を基準に見ると、検出率は 20℃以上の時（50.0%、12/24 検体）と 20℃未満の時（46.7%、21/45 検体）で有意差はなかったが（χ^2 検定、$P > 0.05$）、菌数の幾何平均 ± SD（$\log_{10}$ CFU/100 ml）は 2.30 ± 0.68（20℃以上）と 1.63 ± 0.47（20℃未満）となり、気温が高い時の方が有意に高かった（t 検定、$P < 0.05$）。一方で、採水時の平均気温が 0℃以下であった 1 月においても、4/5 検体（80.0%）からレジオネラ属菌が検出された。これらの結果から、1 年を通してレジオネラ属菌は水たまりに分布しており、気温の高い時期には菌数が増加していることが明らかとなった。

2）33 検体から分離されたレジオネラ属菌 325 株について菌種同定を行った結果、75.4%（n = 245）が *L. pneumophila* であり、その他のレジオネラ属菌が 24.6%（n = 80）であった。これらのうち、無作為に選択したの 31 株について 16S rRNA 遺伝子のシークエンスを行ったところ、*L. gresilensis* が 22 株（71.0%）、*L. longbeachae* が 6 株（19.4%）、このほか *L. oakridgensis*、*L. sainthelensi*、*L. waltersii* がそれぞれ 1 株（3.2%）分離された。また血清型別

においては、分離された 245 株の *L. pneumophila* のうち、国内の患者由来株の 8 割以上を占める血清群 1 が 26 検体から 62 株（25.3%）分離され、最も多かった。次いで血清群 5 が 56 株（22.9%）、血清群 8 が 50 株（20.4%）分離された。以上のことから、水たまりにはレジオネラ症の原因となる *L. pneumophila* 血清群 1 をはじめとした様々な菌種が分布しており、市中肺炎の原因として重要であることが明らかとなった。

3）水たまり由来 62 株および当所保存 78 株（浴用水由来 51 株、患者由来 19 株、冷却塔水由来 5 株、シャワー水由来 3 株）の *L. pneumophila* 血清群 1 は、SBT によって 74 種類の遺伝子型（ST）に分類された。このうち、過去に報告されたことのない富山県特有の遺伝子型である ST505 が 9 株（浴用水由来 5 株、患者由来 4 株）分離され、最も多かった。この 9 株は Pulsed-Field Gel Electrophoresis（PFGE）によるバンドパターンの比較でも、3 株に 2 バンドの違いが認められたが、同一クローンであると考えられた。このうち、ST505 が分離された患者 3 名は、いずれも感染が疑われる時期に浴用施設を利用しており、患者由来 2 株は利用した浴用施設から分離された株と PFGE によるバンドパターンがそれぞれ一致した。また、ST505 が検出された患者の住所および浴用施設の所在地は、すべて県西部にある河川に沿って分布していた。これらの結果から、富山県特有の遺伝子型である ST505 が県西部の河川を中心とする地域に広く分布しており、県西部でレジオネラ症患者罹患率が高い原因の 1 つであると考えられた。

SBT 型別で次に多かった遺伝子型は、土壌からも最も多く分離される遺伝子型である ST48（水たまり由来 7 株、冷却塔水由来 1 株）と、環境検体から過去に報告されたことのない ST120（水たまり由来 7 株、患者由来 1 株）であり、それぞれ 8 株が該当した。水たまり由来 ST48 は 4 か所、ST120 は 3 か所の採水地点から分離された。また、ST48 は 2010 年 11 月、2011 年 2 月、5 月、6 月から、ST120 は 2010 年 11 月、2011 年 1 月、5 月からそれぞれ分離された。なお、今回 ST120 が分離された患者 1 名は、疫学調査においても浴用施設を利用していなかった。これらの結果から、水たまりから検出された ST には地域・季節による特徴はなく、環境由来株としてこれまで報告のなかった遺伝子型（ST120）の *L. pneumophila* 血清群 1 が水たまりには広く分布していたことか

ら、患者の感染源となりうることが明らかとなった。
4) 水たまり由来 62 株の *L. pneumophila* 血清群 1 について *lag-1* 遺伝子の検出を行った。本遺伝子は、環境由来株に比べて患者由来株で有意に高い割合で保有されており、レジオネラ症に関連すると考えられている。PCR の結果、59.7%（37/62 株）が *lag-1* 遺伝子を保有していた。ST 別に検出率を見ると、ST120 の 8 株はすべて保有していたのに対し、ST48 の 8 株はすべて保有していなかった。したがって、水たまり由来株の半数以上が *lag-1* 遺伝子を保有していたこと、ST と *lag-1* 遺伝子の保有率に関連があることが明らかとなった。
5）140 株（水たまり由来 62 株および当所保存 78 株）の *L. pneumophila* 血清群 1 を対象とし、菌株間の遺伝学的関係を推定する解析ソフトである eBURST V3（http://eburst.mlst.net/）を用いて SBT による系統解析を行った。SBT で解析する 7 遺伝子のうち、3 遺伝子以内の Variant を Clonal Group（CG）とした。系統解析の結果、全体の 80.0%（112/140 株）が 8 つの CG [CG1（n = 46）、CG2（n = 28）、CG3（n = 19）、CG4（n = 6）、CG5（n = 5）、CG6（n = 4）、CG7（n = 2）、CG8（n = 2）] を形成した。水たまり由来株の 66.1%（41/62 株）は、主に CG1 と CG4 の 2 つの CG を形成し、CG1 には水たまり由来株の 58.1%（36/62 株）、CG4 には 8.1%（5/62 株）が含まれた。一方、浴用水由来株の 74.5%（38/51 株）は CG2 と CG3 の異なる 2 つの CG を形成し、CG2 には浴用水由来株の 49.0%（25/51 株）、CG4 には 25.5%（13/51 株）が含まれた。また、患者由来株は 5 つの CG に分類され、CG1 には患者由来株の 47.4%（9/19 株）、CG2 には 15.8%（3/19 株）、CG3 には 26.3%（5/19 株）、CG4 と CG5 にはそれぞれ 5.3%（1/19 株）が含まれた。これらの結果から、*L. pneumophila* 血清群 1 は生息環境により遺伝子型に特徴があり、患者由来株の解析から感染源が推定できる可能性が示唆された。

上述のように、気温や場所に関わらず 1 年を通してレジオネラ属菌は水たまりから検出され、気温の高い時期には菌数が増加していることが明らかとなった。また、水たまりにはレジオネラ症の原因となる *L. pneumophila* 血清群 1 をはじめとした様々な菌種が分布しており、市中肺炎の原因として重要である。遺伝子型別の結果では、富山県特有の遺伝子型である ST505 が浴用水および患

者から広く検出され、しかも県西部にある河川に沿って分布していたことから、県西部で患者罹患率が高い原因の 1 つであると考えられた。水たまりは、環境由来株としてこれまで報告のなかった遺伝子型の ST120 に該当する *L. pneumophila* 血清群 1 が地域・季節に関連なく広く分布している環境検体であり、レジオネラ症の感染源となりうることが明らかとなった。さらに、水たまり由来株の半数以上が *lag-1* 遺伝子を保有していたことも感染源となりうることを裏付けていた。系統解析による結果では、*L. pneumophila* 血清群 1 は生息環境により遺伝子型に特徴が見られ、患者由来株の解析から感染源が推定できる可能性が示唆された。以上のように、富山県特有の遺伝子型である ST505 の分布状況と、県西部で患者罹患率が高いこととの関連性を明らかにすることができた。また、水たまりは、レジオネラ症の感染源となりうる環境検体であるとの結論に達した。

Molecular epidemiology of *Legionella pneumophila* serogroup 1 isolates from sputum specimens and environmental sources in Toyama Prefecture, Japan

Legionellosis caused by *Legionella* species has 2 distinct forms: Pontiac fever, which is an influenza-like illness, and Legionnaires' disease, which is a more severe form that causes pneumonia. Pontiac fever is self-limited, generally lasting from 2 to 5 days. On the other hand, Legionnaires' disease, which is characterized principally by chill, fever, headache, and dyspnea, is a potentially fatal pneumonia. To date, pathogenic genes of *Legionella* species causing Legionnaires' disease have not been identified, so the mechanism of increasing clinical severity remain unclear. In 2013, 39 patients with legionellosis were reported in Toyama Prefecture among 1,124 patients in Japan. Monthly distribution revealed that the largest number of legionellosis cases was reported in July, the rainy season in Japan. Toyama Prefecture has the largest number of patients with legionellosis per 100,000

population in 2013 (3.57 in Toyama Prefecture and 0.88 in Japan). This trend has been going on for 9 years. Furthermore, the number of patients with legionellosis per 100,000 population in the western part of Toyama Prefecture for the last 10 years (20.8) was larger than those in the eastern part (12.0).

Legionella species is ubiquitous in natural environments. In addition, it has been found in anthropogenic environments, such as cooling towers, baths, and showers. Although 58 species of *Legionella* species have been identified, more than 80% of legionellosis cases are caused by *L. pneumophila* serogroup (SG) 1. In Japan, public baths are the major source of infection, according to the National Epidemiological Surveillance of Infectious Diseases. In Toyama Prefecture, infection sources of about half of legionellosis cases were bath water as determined by epidemiological investigation, although the remaining cases were unclear.

L. pneumophila isolates can be characterized by sequence-based

typing (SBT) using the 7 loci (*flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, and *neuA*) proposed by the European Working Group for *Legionella* Infections. A previous study revealed that sequence type (ST) 120 clinical strains of *L. pneumophila* SG 1 were detected in 5.3% of isolates from patients with legionellosis in Japan, although the sources of the bacteria remain unclear. Recently, isolates of *L. pneumophila* SG 1 from several environments, such as public baths, soil, and cooling towers were characterized, and then compared to clinical isolates to determine relations between isolates from different environments and from patients. However, the prevalence of *Legionella* species isolated from puddles on asphalt roads throughout the year and the genetic relationships between strains from clinical specimens and this environmental source by molecular typing techniques have not been clearly analyzed. In this study, to elucidate the potential new sources of infection, we characterized the genetic relationship between *L. pneumophila* SG 1 isolates from puddles and from stock strains previously isolated from

sputum specimens, public baths, and some other environmental sources. The results were described below.

1) 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. *Legionella* species were detected in 33/69 samples (47.8%) from all sampling locations. 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 100–999 CFU/100 ml, and 1 (3.0%) sample contained 7520 CFU/100 ml. Even when mean temperature was <0°C in January, *Legionella* species were isolated from 4 of 5 samples, and 3 samples contained 100–999 CFU/100 ml. Furthermore, the isolation rates of *Legionella* species at mean temperature ≥20°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 7520 CFU/100 ml at mean temperature ≥20°C and from 10 to 240 CFU/100 ml at mean temperature <20°C. Student's *t* tests revealed that the concentrations of *Legionella* species at mean temperatures ≥20°C and <20°C were significantly different ($P < 0.05$): the geometric means ± SD ($\log_{10}$ CFU/100 ml) in *Legionella*-positive puddles were 2.30 ± 0.68 and 1.63 ± 0.47, respectively. These results indicated that *Legionella* species were frequently detected in puddles throughout the year, regardless of sampling locations. Furthermore, an increase in the number of CFU of *Legionella* species was seen during the warm season.

2) We isolated 325 colonies from puddles from the 6 sampling locations. 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 untypable; *n* = 77, 31.4%). 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%). Thus, puddles on asphalt roads are important in the etiology of community-acquired pneumonia.

3) We analyzed 62 *L. pneumophila* SG 1 isolates obtained from puddles on asphalt roads in comparison with 73 *L. pneumophila* SG 1 stock strains (51 strains from 24 public baths, 4 strains from 2 cooling towers, 1 strain from a shower, and 17 strains from 16 patients with legionellosis) from a previous study and 5 isolates (1 isolate from a cooling tower, 2 isolate from 2 showers, and 2 isolates from 2 patients) from this study. A total of 140 *L. pneumophila*

SG 1 isolates were classified into 74 STs. Among these, ST505 strain, the most-prevalent strain, was identified in 5 isolates from public baths and 4 isolates from patients, and these isolates belonged to 2 PFGE patterns. These, however, were similar because of the difference with only 2 restriction fragments, indicating that ST505 strain was prevalent among *L. pneumophila* SG 1 isolates in Toyama Prefecture. Furthermore, ST505 strain was widely distributed along the river in the western part of Toyama Prefecture, suggesting that it is one of the etiologies of the large number of patients with legionellosis in the western part of Toyama Prefecture.

ST48 (n = 8) and ST120 (n = 8) strains were the second most-prevalent strains. ST48 strain, which was primarily isolated from soil, was identified in 7 isolates from puddles and 1 isolate from a cooling tower. ST120 strain was identified in 7 isolates from puddles and 1 isolate from a patient. Environmental ST120 strain was not mentioned in the previous report, or in the EWGLI SBT database. Puddle isolates of ST48 were

detected in isolates from 4 locations and puddle isolates of ST120 in isolates from 3 locations. Furthermore, ST48 and ST120 strains were isolated during 3 (November 2010; February, May, and June 2011) and 4 (November 2010; January and May 2011) different months, respectively. These results showed that ST120 strain, which was found in an environmental source for the first time in this study, was widely distributed in Toyama Prefecture regardless of the season and sampling locations. Furthermore, puddles on asphalt roads may be considered potential new source of infection.

4) PCR amplification of the *lag-1* gene, a tentative marker for clinical isolates, was carried out. Thirty-seven out of 62 *L. pneumophila* SG 1 isolates from puddles harbored the *lag-1* gene (59.7%). Among the isolates belonging to ST48 and ST120, which were the major STs of puddle isolates, the *lag-1* gene was missing in all ST48 isolates (0/8), and was present in all ST120 isolates (8/8), indicating the correlation between allelic profiles of ST and the

virulence of *L. pneumophila* SG 1.

5) Clonal analysis was performed using *L. pneumophila* SG 1 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) 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). A total of 8 CGs, which included 80.0% of isolates, were generated [CG1 (*n* = 46), CG2 (*n* = 28), CG3 (*n* = 19), CG4 (*n* = 6), CG5 (*n* = 5), CG6 (*n* = 4), CG7 (*n* = 2), CG8 (*n* = 2)]. Puddle isolates formed 2 major CGs, CG1 and CG4, which included 58.1% and 8.1% of puddle isolates, respectively. 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 including 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. Infection sources of legionellosis cases may be elucidated by SBT analysis, because our results suggest that each environment constitutes an independent habitat.

As described above, *Legionella* species were frequently detected in puddles throughout the year and regardless of sampling locations. Furthermore, an increase in the number of CFU of *Legionella* species was seen during the warm season. Six *Legionella* species, including *L. pneumophila*, were detected in puddles. Thus, puddles on asphalt roads are important in the etiology of community-acquired pneumonia. By SBT analysis, ST505 strain

was widely distributed along the river in the western part of Toyama Prefecture, suggesting that it is one of the etiologies of the large number of patients with legionellosis in the western part of Toyama Prefecture. Environmental ST120 strains of *L. pneumophila* SG 1 were isolated from puddles on asphalt roads for the first time in this study throughout the year and regardless of sampling locations. Furthermore, the *lag-1* gene, a tentative marker for clinical isolates, was prevalent in puddle isolates (61.3%). By SBT analysis using eBURST V3, puddle and bath isolates formed 2 (CG1 and CG4) and 2 (CG2 and CG3) clonal groups, respectively, suggesting that each environment constitutes an independent habitat. To identify unrecognized sources of infection in legionellosis cases, we need to isolate *L. pneumolhila* strains from clinical specimens and various environmental sources, including water from puddles on roads, and to analyze these strains by the combination of SBT analysis and epidemiological investigation. In conclusion, distribution of endemic ST505

strain is correlated with the large number of patients with legionellosis in the western part of Toyama Prefecture. Furthermore, puddles on asphalt roads serve as potential reservoirs for *L. pneumophila* in environment.

TITLE

Molecular epidemiology of *Legionella pneumophila* serogroup 1 isolates identify a prevalent sequence type, ST505, and a distinct clonal group of clinical isolates in Toyama prefecture, Japan

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ABSTRACT

We performed comparative analyses of *Legionella pneumophila* serogroup (SG) 1 isolates obtained during 2005–2012 in Toyama prefecture, Japan, by sequence-based typing (SBT) and pulsetd-field gel electrophoresis (PFGE). Seventy-three isolates of *L. pneumophila* SG 1, including 17 isolates from patients, 51 from public baths, 4 from cooling towers, and 1 from a shower, were analyzed. The isolates were classified into 43 sequence types (STs) by SBT and 52 types by PFGE. Fourteen STs were unique to Toyama prefecture, as determined from the SBT database of European Working Group for *Legionella* Infections (EWGLI), as of October 31, 2012. ST505 strain was identified in 4 isolates from patients and 5 isolates from public baths, and these isolates belonged to 2 PFGE types. These, however, were similar because of the difference with only 2 restriction fragments, indicating that ST505 strain was prevalent among *L. pneumophila* SG 1 isolates in this area. ST505 strains isolated from patients and public baths were distributed along the river in a western part of Toyama prefecture. SBT and PFGE profiles of 3 clinical isolates were identical with those of 3 environmental

isolates from the suspected origins of the infection in each case, respectively. This finding suggested that SBT and PFGE were useful for epidemiological study. Furthermore, by SBT analysis, we identified a clonal group formed only by 7 clinical isolates that are not associated with bath water, suggesting that they were derived from unrecognized sources.

KEY WORDS

Legionella pneumophila, Molecular epidemiology, Molecular typing

INTRODUCTION

Legionella are pathogenic gram-negative bacteria that cause legionellosis and are ubiquitously found in the environment. Although 55 species and more than 70 serogroups of *Legionella* spp. have been identified [1], more than 90% of legionellosis cases are caused by *Legionella pneumophila* [2]. Among 15 serogroups of *Legionella pneumophila*, most clinical strains (80%) belonged to serogroup (SG) 1 in Japan [3].

Legionellosis is usually acquired through inhalation of aerosolized water contaminated with *Legionella* spp. [4]. Legionellosis has 2 distinct forms: Pontiac fever, which is an influenza-like illness, and Legionnaires' disease, which is a more severe form that causes pneumonia [5, 6]. *Legionella* spp. have been found in artificial environments such as cooling towers, baths, showers, and decorative fountains [7–10]. Therefore, these facilities are potential sources of sporadic or outbreak cases of infection. In Japan, public baths are a major source of infection according to the National Epidemiological Surveillance of Infectious Diseases [11]. Fatal cases have been reported in homes and spa pools [12, 13].

When a case of legionellosis is reported, it is important to identify the source of infection by molecular typing methods for public health purposes. Pulsed-field gel electrophoresis (PFGE) is commonly used to determine the source of infection [9, 14, 15]. However, this typing method is time consuming. Sequence-based typing (SBT) is a rapid identification method developed 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* [16–18]. Like PFGE, SBT has been considered as a powerful epidemiological tool [19].

Toyama prefecture in Japan has the largest number of patients with legionellosis per 100,000 population from 2008 to 2010 (1.98 [1.80–2.07] in Toyama prefecture and 0.62 [0.56–0.70] in Japan) [20]. However, in many cases, the sources of infection have been unclear. Comparative analysis of *L. pneumophila* SG 1 isolates from clinical specimens and public baths in a local area has been rarely reported. In this study, we performed comparative analyses of *Legionella pneumophila* SG 1 isolates from clinical

specimens and public baths obtained during 2005–2012 in Toyama prefecture by SBT and PFGE, and we found that *L. pneumophila* SG 1 strain ST505 was prevalent in this area. We also found a clonal group formed only by clinical isolates distinct from bath isolates, and we discussed the origin of these clinical isolates.

MATERIALS AND METHODS

Bacterial strains. Seventy-three strains of *L. pneumophila* SG 1 were isolated and collected during 2005–2012 in Toyama prefecture (Table 1). Fifty-one strains from 24 public baths (PB1–PB24) were isolated in our laboratory. Four strains from 2 cooling towers (CT1 and CT2) and 1 strain from a shower (SH1) were collected from each building. Seventeen strains from 16 patients (PA1–PA16) with legionellosis were collected from 4 hospitals in Toyama prefecture. Of the 17 clinical isolates, 15 were obtained from 15 patients. The remaining 2 isolates were obtained from patient PA11 but belonged to different STs and PFGE types. The incubation period was 2–10 days, depending on the diagnosis by the physician.

Isolation of *L. pneumophila* SG 1 from environmental sources. Water samples (500 ml) were filtered with a 0.22-μm pore size membrane (cat. no. GTTP04700, Millipore, MA, USA) and resuspended in 5 ml of distilled water. After the concentrated samples were heated at 50°C for 20 min, they were spread onto glycine-vancomycin-polymyxin B-cycloheximide agar plates (bioMerieux, Lyon, France). These agar plates were incubated at 35°C for 7 days in a moist chamber. Smooth gray colonies were subcultured onto buffered charcoal yeast extract (BCYE) agar plates (bioMerieux) and blood agar plates (Eiken Chemical, Tokyo, Japan). Suspected colonies that grew only on BCYE agar plates were tested by slide agglutination with commercial antisera (Denka Seiken, Tokyo, Japan) to identify *L. pneumophila* SG 1 strains among various *Legionella* spp. and serogroups.

SBT analysis. Isolates were suspended in distilled water. The suspension was boiled at 100°C for 10 min and then centrifuged at 20,000 × *g* for 5 min at room temperature. The supernatant was used as a DNA template. PCR of the SBT scheme was carried out according to the protocol of EWGLI

(http://www.hpa-bioinformatics.org.uk/legionella/legionella_sbt/php/sbt_homepage.php), as described previously [16, 17]. Novel alleles and sequence types (STs) were submitted to the EWGLI SBT database for assigning the newly identified alleles and STs. A phylogenetic tree with concatenated sequences of 7 SBT alleles was constructed by the neighbor-joining method, using the MEGA 4 software [21]. A bootstrapping test was performed 1000 times. Clonal analyses were performed by using eBURST V3 (http://eburst.mlst.net). Groups were generated with single- and double-locus variants and they were defined as clonal groups.

PFGE analysis. PFGE was carried out as previously described [22] with a slight modification. Genomic DNA in the plug was digested overnight with 30 U of *Sfi*I (TaKaRa Bio, Shiga, Japan) at 50°C. Electrophoresis was carried out at 6 V/cm for 19 h with the pulse time ranging from 5 to 50 s, using the CHEF DRIII system (Bio-Rad Laboratories, CA, USA). A dendrogram showing the genetic similarity between PFGE profiles was constructed by the UPGMA method with the Fingerprinting II software (Bio-Rad Laboratories) using a Dice coefficient at 1.2% of tolerance and 1.0% of

optimization. Reproducibility was confirmed by repeat analysis of 17 randomly selected isolates. PFGE types were defined at the 100% similarity breakpoint given by the software. PFGE with *Sfi*I digestion had the ability to type all *L. pneumophila* isolates in this study.

Indices of discrimination. To assess the molecular typing methods, we calculated the indices of discrimination (IODs) of isolates from patients and public baths as described previously [23].

RESULTS

SBT analysis. Seventy-three isolates were divided into 43 STs (Table 1). The IODs of 17 isolates from patients and 38 isolates from public baths were 0.934 (95% confidence interval [CI] 0.859–1.000) and 0.986 (95% CI 0.971–1.000), respectively; strains obtained on the same day from the same public bath and with identical STs were represented as a single strain. Fourteen STs were unique to this area in the EWGLI SBT database, as of October 31, 2012. Among these, 9 ST505 isolates were

obtained from 4 patients and 3 public baths along the Shou River (Fig. 1; LG0003, LG0215, LG0585, LG0613; LG0007, LG0030, LG1116, LG0254, and LG0626 in Table 1). The ST of 3 of 4 isolates (75%) from cooling towers and 1 isolate from a shower was ST1. A phylogenetic tree was constructed and 7 clonal groups were generated by SBT (Fig. 2). Among the 7 clonal groups (CG1–CG7), CG3 was formed by isolates from 7 patients (LG0123, LG0124, LG0232, LG0392, LG0586, LG0716, and LG1060; Table 1). No environmental isolates were present in CG3. Isolates belonging to CG3 found by using eBURST V3 were also clustered using the neighbor-joining method by the MEGA4 software, as shown by the bootstrap support value of 67%.

PFGE analysis. A dendrogram of the PFGE pattern was constructed (Fig. 3). Fig. 4 showed the original gel image of band patterns of isolates belonging to 3 STs (ST1, ST278, and ST505) among 13 STs (ST1, ST59, ST122, ST128, ST278, ST384, ST505, ST644, ST763, ST769, ST1094, ST1098, and ST1101) that were found in more than 1 isolate. Seventy-three isolates were divided into 52 PFGE types. The IODs of 17

isolates from patients and 46 isolates from public baths were 0.978 (95% CI 0.934–1.000) and 0.976 (95% CI 0.949–1.000), respectively; strains obtained on the same day from the same public bath and with the identical type by PFGE were represented as a single strain. Although 9 ST505 isolates belonged to 2 PFGE types (P13 and P14; Fig. 3), band patterns of these types were different by only 2 restriction fragments with similarity of approximately 90% (Fig. 4). The CG3 consisting of 7 clinical isolates was split into 2 PFGE groups with similarity of more than 80% each (Fig. 3). Epidemiologically unrelated ST1 isolates obtained from a cooling tower and a shower had the same PFGE type (LG0593 and LG1948; Fig. 3). However, band patterns of other isolates belonging to ST1 were different by more than 3 restriction fragments (Fig. 4). The other isolates from different environmental sources did not have identical PFGE types.

DISCUSSION

In this study, we found ST505 to be the most prevalent strain in Toyama

prefecture, Japan, and identified a clonal group (CG3, Fig. 2) formed only by 7 clinical isolates that were not associated with bath water. Travel histories of 14 out of the 16 patients during the likely exposure period were available. Although patient PA5 had a history of a visit outside Toyama prefecture, we couldn't identify whether this patient had been infected in Toyama prefecture or not. However, the remaining 13 patients had been in Toyama prefecture, suggesting that most patients had been infected in Toyama prefecture. ST1 strain was isolated from public baths (1 of 51, 2.0%), cooling towers (3 of 4, 75%), and a shower (1 of 1, 100%). ST1 strain was not isolated from clinical specimens in this study, although this strain has been frequently isolated worldwide from clinical specimens and environmental sources [24–26]. Cases of legionellosis from cooling towers and showers have not been reported yet in Toyama prefecture by epidemiological investigation, but these environmental sources, as well as public baths, are still possible infection sources of legionellosis in this area.

The ST505 strain was the most frequently isolated from patients and bath facilities, and 2 PFGE types of the isolates were similar because of the difference with

only 2 restriction fragments (Fig. 4), indicating that this strain was prevalent among *L. pneumophila* SG 1 isolates in this area. A recent study observed high diversity and high abundance of *Legionella* spp. in a river by 16S rRNA gene sequencing and quantitative PCR [27]. Because the ST505 isolates were obtained along the Shou River, this strain was likely to be distributed along this river and may contaminate artificial environments such as public bath facilities. Alternatively, other sources of bacterial contamination may be present upstream of the river, as reported in the previous paper in which the presence of *L. pneumophila* in the river was due to the release of wastewater from industrial aeration ponds [28].

The isolation rates of the ST505 strain from patients and public baths were 23.5% (4 of 17) and 9.8% (5 of 51), respectively. Several studies of endemic clones have been reported. In Ontario, Canada, endemic ST211 (*flaA*3, *pilE*10, *asd*1, *mip*1, *mompS*14, *proA*9, and *neuA*11) and ST222 (*flaA*2, *pilE*19, *asd*5, *mip*10, *mompS*18, *proA*1, and *neuA*10) strains were detected in 7.7% (15 of 194) and 6.7% (13 of 194) of the total clinical isolates, respectively [29]. Thus, the higher isolation rate of clinical

ST505 strain found in this study suggests that this strain may be highly pathogenic. In South Korea, ST-K1 (*flaA*7, *pilE*12, *asd*17, *mip*3, *mompS*35, *proA*11, and *neuA*11) strains accounted for 36.1% of the total isolates in hot-water samples [26]. It is notable that ST505 is a triple-locus variant of ST-K1. These endemic clones were not detected in this study. Further investigation of endemic clones is required, as our study, in addition to previous findings, suggested that it was important to determine the infection source of legionellosis by the combination of molecular typing methods such as PFGE and SBT analyses, monoclonal antibody subgrouping [3], and epidemiological investigation in certain areas.

By SBT and PFGE analyses, LG0003 strain from PA2 and LG0007 strain from PB1 as the suspected origin of the infection in this case had the same profile (ST505 and P14; Table 1). In another case, LG0604 and LG0613 strains that were obtained on the same day from PA11 had different profiles (ST644 and P27; ST505 and P13 in Table 1). These profiles were identical with those of LG0643 strain from PB12 and LG0626 strain from PB13, respectively, that were obtained from the suspected origins

of the infection. Therefore, this patient might be serially infected with 2 different strains by using several public baths. These findings indicated that SBT and PFGE were useful for epidemiological study and that several colonies should be isolated from a patient for epidemiological study.

By SBT analysis, the 7 clinical isolates belonged to CG3 (Fig. 2), in which no environmental isolates were present. Among the 7 clinical isolates, 6 were not associated with bath water by epidemiological investigation. The STs of clinical strains in this clonal group were ST120, ST132, ST384, ST506, and ST507. All registered strains belonging to these STs in the EWGLI SBT database were isolated only from patients and not from the environment. Amemura-Maekawa et al. suggested the possibility of habitat segregation of *L. pneumophila* [30]. Thus, these clinical isolates belonging to the same clonal group were originally derived from unrecognized environmental sources. These STs have single-, double-, and triple-locus variants of STs belonging to group S1, which mainly consisted of isolates from soil as well as from bath water in rare cases, but not isolates from cooling towers [30], suggesting that

the clinical strains belonging to the 5 STs in this study may originate from soil. Although the LG0123 strain in CG3 (Fig. 2) was suspected to be derived from bath water by epidemiological investigation, *L. pneumophila* SG 1 strains were not isolated from the suspected origin of the infection in this case. Our findings, in addition to those of previous reports, may reveal potential major routes of infection from soil. Alternatively, it is important to type more than 1 isolate from an environmental source because otherwise the causative strain might be not detected. Further investigation by SBT analysis of isolates from various environmental sources, including soil, and those from patients is required to reveal potential major routes of *Legionella* infection.

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Conflict of interest

None.

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Table 1 SBT and PFGE profiles of *L. pneumophila* SG 1 isolates used in this study

No.	Strain	Origin[a]	Year	Month	SBT profile							ST	PFGE type	Sources of infection
					flaA	*pilE*	*asd*	*mip*	*mompS*	*proA*	*neuA*			
1	LG0002	PA1	2005	May	6	10	19	3	19	4	6	502	P39	unknown
2	LG0003	PA2	2005	Aug	7	6	17	3	11	11	9	505[b]	P14	bath water[c]
3	LG0122	PA3	2006	Sep	8	10	6	15	51	1	6	353	P6	unknown
4	LG0123	PA4	2006	Sep	2	3	6	13	2	1	6	506[b]	P3	bath water
5	LG0124	PA5	2006	Sep	2	3	5	10	2	1	6	507	P11	unknown
6	LG0215	PA6	2006	Oct	7	6	17	3	11	11	9	505[b]	P13	bath water
7	LG0232	PA7	2006	Nov	2	3	5	11	2	1	6	120	P12	unknown
8	LG0392	PA8	2007	Feb	2	3	9	10	2	1	10	384	P2	unknown
9	LG0585	PA9	2008	May	7	6	17	3	11	11	9	505[b]	P13	unknown
10	LG0586	PA10	2008	Jun	2	3	9	10	2	1	10	384	P4	unknown
11	LG0604	PA11	2008	Sep	6	10	20	10	9	14	11	644	P27	bath water[c]
12	LG0613	PA11	2008	Sep	7	6	17	3	11	11	9	505[b]	P13	bath water[c]
13	LG0716	PA12	2008	Sep	2	1	6	15	2	1	6	132	P5	unknown
14	LG0977	PA13	2008	Dec	6	10	19	3	19	4	9	2	P38	bath water
15	LG1008	PA14	2009	Feb	7	6	17	10	13	9	11	682	P17	bath water
16	LG1060	PA15	2009	Jun	2	3	9	10	2	1	10	384	P1	unknown
17	LG1171	PA16	2009	Dec	4	7	11	3	11	12	9	42	P24	bath water
18	LG0017	PB1	2005	Aug	6	10	19	28	19	4	11	763	P37	
19	LG0006	PB1	2005	Aug	6	10	19	28	19	4	11	763	P40	
20	LG0007	PB1	2005	Aug	7	6	17	3	11	11	9	505[b]	P14	
21	LG0029	PB1	2005	Nov	6	10	19	28	19	4	11	763	P37	
22	LG0030	PB1	2005	Nov	7	6	17	3	11	11	9	505[b]	P13	
23	LG1116	PB1	2009	Nov	7	6	17	3	11	11	9	505[b]	P14	
24	LG1119	PB1	2009	Nov	2	10	14	10	19	4	3	285	P32	
25	LG0128	PB2	2006	Sep	3	13	1	28	14	9	11	493	P22	
26	LG0129	PB2	2006	Sep	7	10	17	13	14	11	11	1091[b]	P16	
27	LG0156	PB3	2006	Oct	6	10	15	28	4	14	11	278	P43	
28	LG0326	PB3	2006	Dec	6	10	15	28	4	14	11	278	P43	
29	LG0347	PB3	2006	Dec	7	4	31	10	48	15	11	1092[b]	P19	
30	LG0218	PB4	2006	Oct	3	13	1	3	14	9	9	664	P22	
31	LG0219	PB4	2006	Oct	6	10	17	6	9	4	9	136	P50	
32	LG0254	PB5	2006	Nov	7	6	17	3	11	11	9	505[b]	P13	
33	LG0258	PB6	2006	Dec	6	10	15	13	17	14	11	122	P33	
34	LG0478	PB6	2007	Oct	6	10	15	13	17	14	11	122	P34	
35	LG0490	PB6	2007	Oct	10	12	7	3	16	18	6	138	P48	
36	LG0301	PB7	2006	Dec	10	12	7	21	16	18	9	769	P49	
37	LG0534	PB7	2007	Nov	10	12	7	21	16	18	9	769	P49	
38	LG0449	PB8	2007	Sep	7	43	31	3	48	15	40	1151	P20	
39	LG0453	PB9	2007	Oct	6	10	19	28	19	4	11	763	P37	
40	LG0454	PB9	2007	Oct	7	6	17	3	13	11	11	59	P15	
41	LG0469	PB10	2007	Oct	6	10	15	14	21	7	6	1093[b]	P36	

42	LG0516	PB11	2007	Oct	7	6	17	3	13	11	40	1152[b]	P13
43	LG0622	PB12	2008	Sep	6	10	20	10	9	14	11	644	P29
44	LG0643	PB12	2008	Sep	6	10	20	10	9	14	11	644	P27
45	LG0646	PB12	2008	Sep	6	10	20	10	9	14	11	644	P28
46	LG0638	PB12	2008	Sep	6	10	20	10	9	4	9	1094[b]	P30
47	LG0641	PB12	2008	Sep	6	10	20	10	9	4	9	1094[b]	P31
48	LG0626	PB13	2008	Sep	7	6	17	3	11	11	9	505[b]	P13
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51	LG0709	PB14	2008	Sep	7	6	17	3	14	11	11	128	P16
52	LG0710	PB14	2008	Sep	7	6	17	3	14	11	11	128	P13
53	LG0864	PB15	2008	Nov	7	6	17	3	13	11	11	59	P13
54	LG0903	PB16	2008	Nov	6	10	20	28	9	4	9	1097[b]	P26
55	LG0909	PB17	2008	Nov	2	12	3	6	8	14	9	141	P51
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58	LG0964	PB18	2008	Nov	7	6	17	6	13	11	9	1099[b]	P18
59	LG1132	PB18	2009	Nov	6	6	15	3	9	14	11	1101[b]	P44
60	LG1134	PB18	2009	Nov	10	22	7	3	16	9	6	162	P47
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69	LG0808	CT1	2008	Oct	1	4	3	1	1	1	1	1	P9
70	LG1948	CT2	2012	Apr	1	4	3	1	1	1	1	1	P7
71	LG1949	CT2	2012	Apr	1	4	3	1	1	1	1	1	P8
72	LG1950	CT2	2012	Apr	5	2	22	27	6	10	12	48	P21
73	LG0593	SH1	2008	Aug	1	4	3	1	1	1	1	1	P7

[a] PA = patient, PB = public bath, CT = cooling tower, SH = shower

[b] Fourteen of 43 STs were unique to this area, as of October 31, 2012

[c] confirmed by PFGE with environmental isolates

LEGENDS TO FIGURES

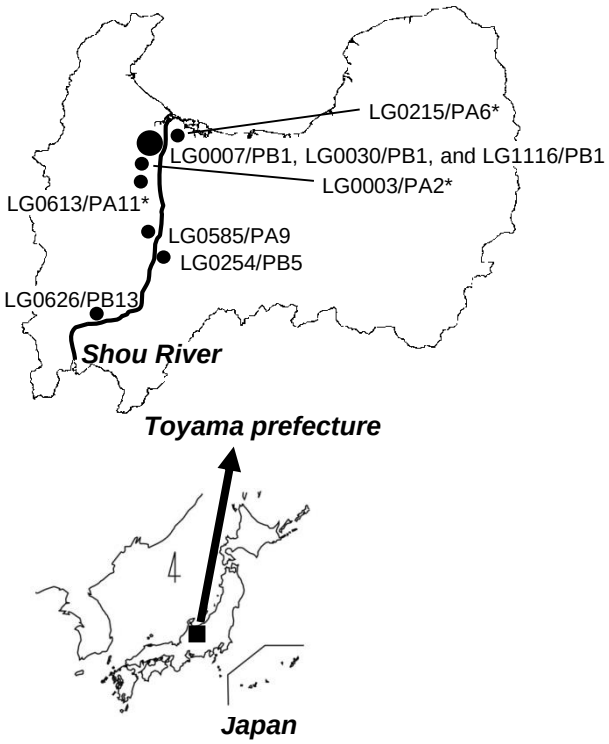
Fig. 1 Geographic distribution of ST505 strain. Isolate name indicates the strain/origin as described in Table 1. The size of the circle indicates the number of isolates. Asterisk indicates the clinical isolates associated with bath water by epidemiological investigation

Fig. 2 Phylogenetic analysis of the concatenated sequences (*flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, and *neuA*) of *L. pneumophila* SG 1 isolates in this study. Isolate name indicates the strain/origin/month/year as described in Table 1. Isolates in boldface are from patients. Asterisk indicates the clinical isolates associated with bath water by epidemiological investigation. More than 60% of bootstrap values are shown on the branches. Clonal groups (CG1–CG7) were generated with single- and double-locus variants by using eBURST V3 (http://eburst.mlst.net)

Fig. 3 A dendrogram of the PFGE pattern constructed from *L. pneumophila* SG 1

isolates in this study. Isolate name indicates the strain/origin/month/year as described in Table 1. Isolates in boldface are from patients. Asterisk indicates the clinical isolates associated with bath water by epidemiological investigation. Two PFGE types (P13 and P14) and ST505 are denoted by boldface. Italic letters indicate STs belonging to CG3

Fig. 4 PFGE patterns with *Sfi*I digestion of *L. pneumophila* SG 1 isolates. Lanes: M, *S. enterica* serovar Braenderup H9812 strain digested with *Xba*I as a size marker; 1, LG0215; 2, LG0254; 3, LG0030; 4, LG0613; 5, LG0585; 6, LG0626; 7, LG0003; 8, LG0007; 9, LG1116; 10, LG0593; 11, LG0808; 12, LG1167; 13, LG1948; 14, LG1949; 15, LG0156; 16, LG0326; 17, LG0976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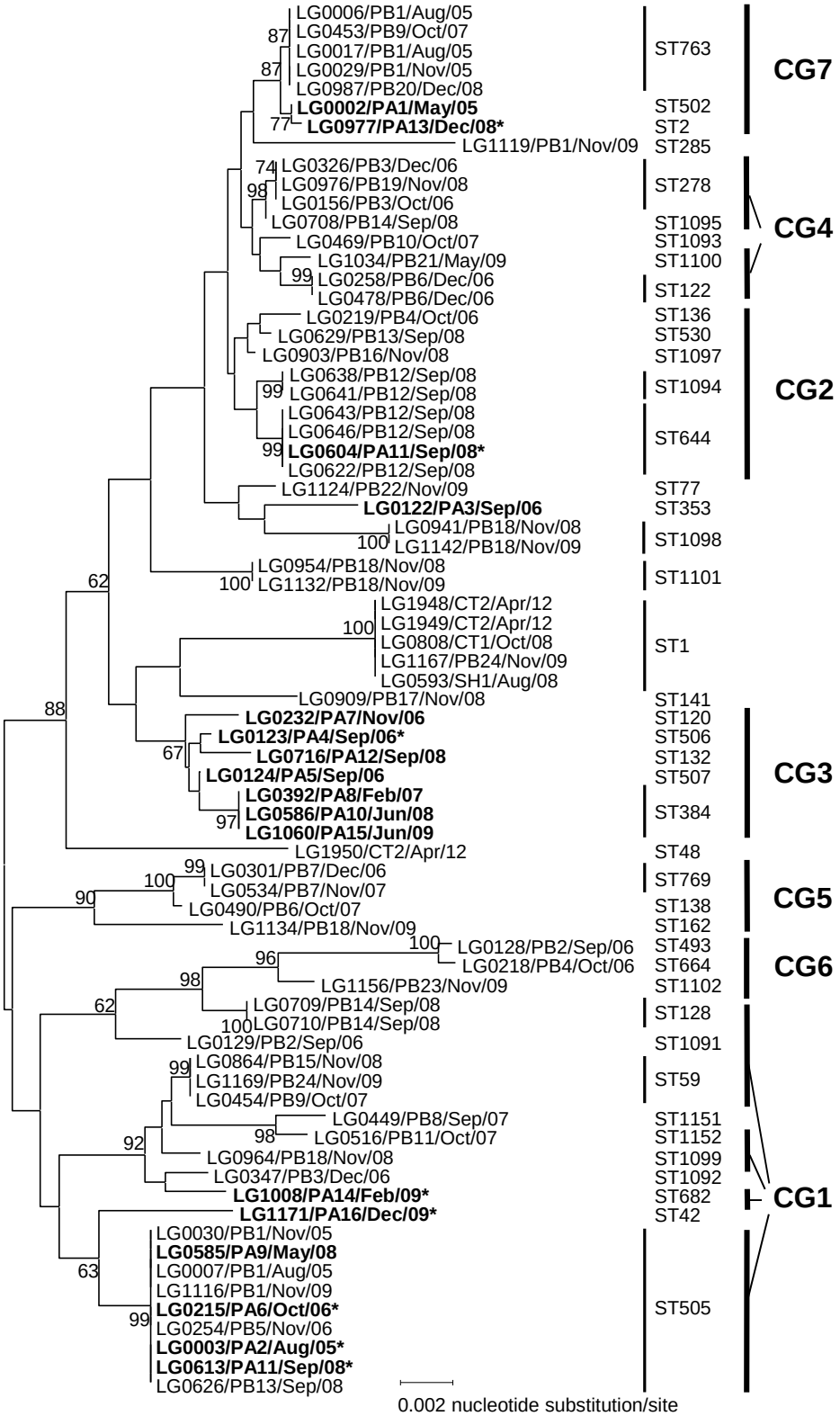
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ST1101
ST1
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ST506
ST132
ST507
ST384
ST48
ST769
ST138
ST162
ST493
ST664
ST1102
ST128
ST1091
ST59
ST1151
ST1152
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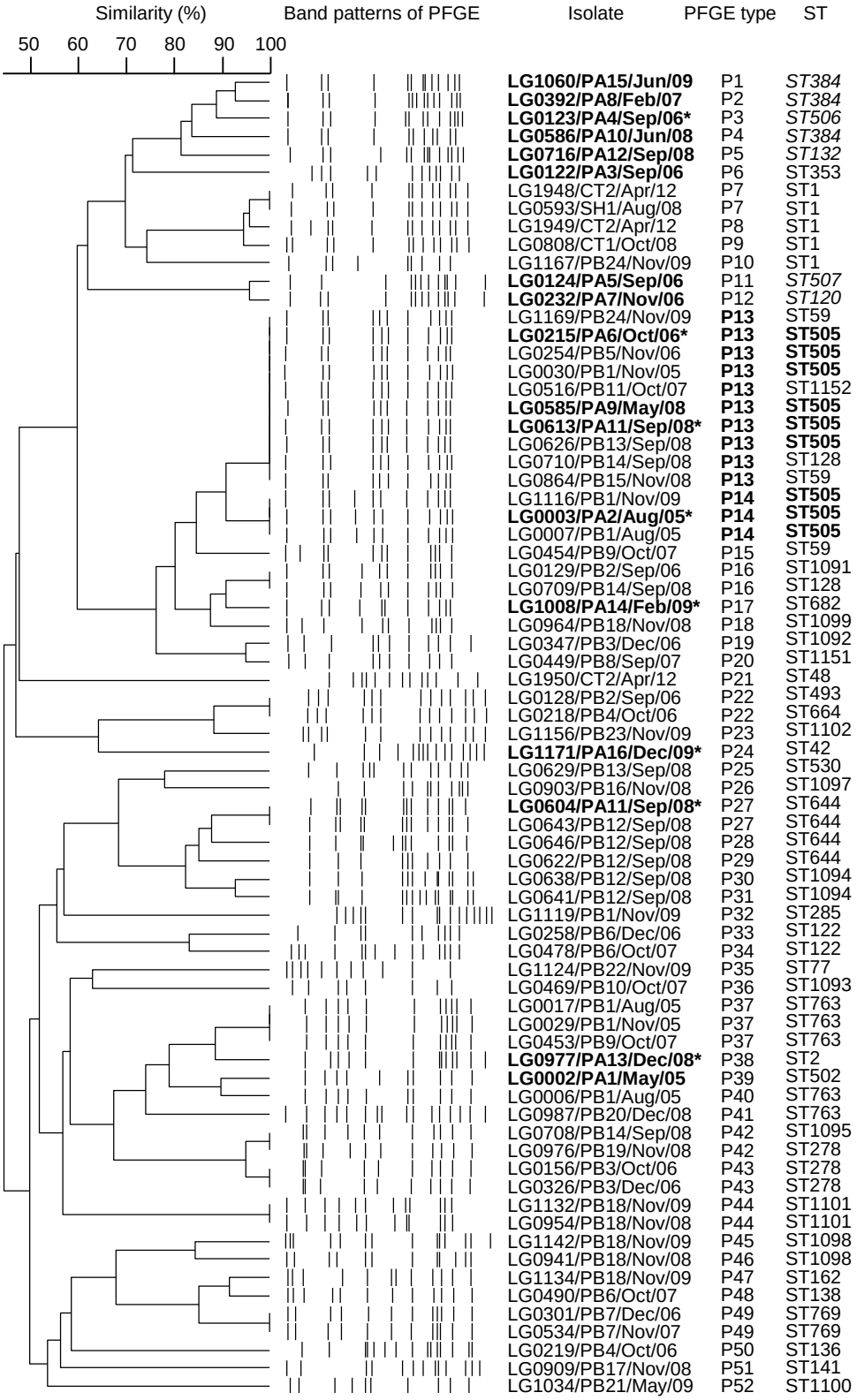
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70
80
90
100
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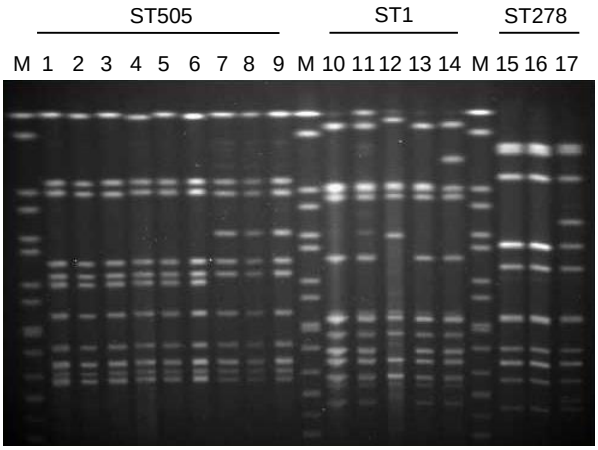
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Close Genetic Relationship between *Legionella pneumophila* Serogroup 1 Isolates from Sputum Specimens and Puddles on Roads, as Determined by Sequence-Based Typing

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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 potential 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-μm-pore-size polycarbonate membrane (catalog no. GTTP04700; 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 3130*xl* genetic analyzer (Applied Biosystems, Foster City, CA, USA) and primers 27f (AGAGTTTGATCCTGGCTCAG) 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 μl 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 × *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 ≥20°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 ≥20°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 ≥20°C and <20°C were significantly different ($P < 0.05$): the geometric means ± 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.

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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 *lag-1*
		Yr	Mo	*flaA*	*pilE*	*asd*	*mip*	*mompS*	*proA*	*neuA*		
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

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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.

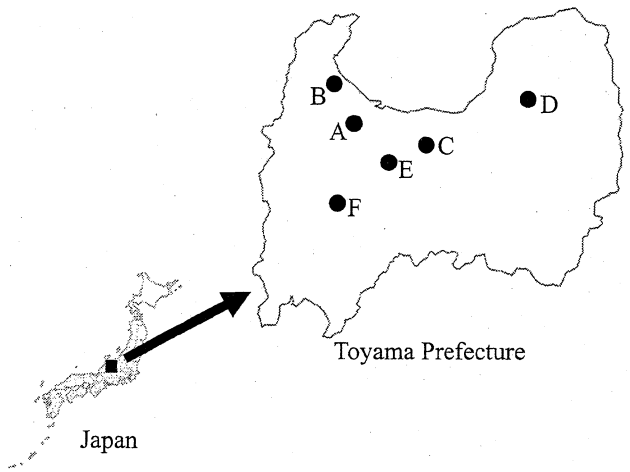

FIG 1 Locations of the 6 fixed points for sampling from puddles on roads. Sixty-nine samples were collected from the 6 locations as follows: A (n = 12), B (n = 12), C (n = 12), D (n = 12), E (n = 12), and F (n = 9).

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

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

Yr	Mo	Mean temp (°C)	No. of samples (no. with *L. pneumophila*): Analyzed	*Legionella* positive	With CFU/100 ml of: 10–99	100–999	>1,000	Geometric mean ± SD (log_{10} CFU/100 ml) in *Legionella*-positive puddles
2010	Nov	11.4	5	4 (4)	4 (4)	0	0	1.3 ± 0.3
	Dec	10.4	5	3 (2)	3 (2)	0	0	1.4 ± 0.3
2011	Jan	−0.6	5	4 (4)	1 (1)	3 (3)	0	2.0 ± 0.1
	Feb	9.0	6	5 (5)	4 (4)	1 (1)	0	1.6 ± 0.5
	Mar	11.5	6	4 (1)	3 (1)	1 (0)	0	1.7 ± 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 ± 0.4
	Jun	32.2	6	2 (2)	1 (1)	1 (1)	0	1.8 ± 0.2
	Jul	31.0	6	3 (1)	0	2 (1)	1 (0)	3.2 ± 0.5
	Aug	26.2	6	3 (3)	1 (1)	2 (2)	0	2.2 ± 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

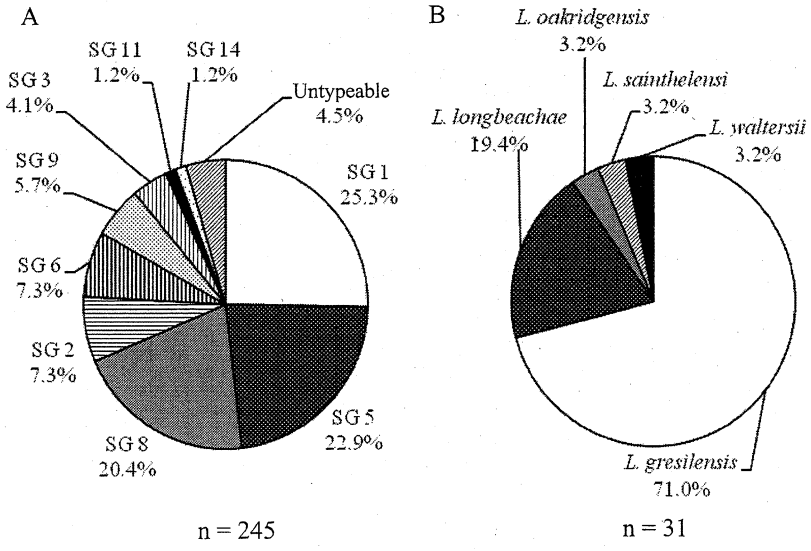

FIG 2 Distribution of *Legionella* species from puddles. (A) Frequencies of serogroups among *L. pneumophila* isolates. (B) Frequencies of *Legionella* species among non-*L. pneumophila* isolates identified by 16S rRNA gene sequencing.

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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 *lag-1*	
		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. Furuhata, 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. sainthelensi* 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.

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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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出展

なお、本研究の成果の一部は、以下の学術雑誌において掲載されている。

・Jun-ichi Kanatani, Junko Isobe, Keiko Kimata, Tomoko Shima, Miwako Shimizu, Fumiaki Kura, Tetsutaro Sata, Masanori Watahiki: Molecular epidemiology of *Legionella pneumophila* serogroup 1 isolates identify a prevalent sequence type, ST505, and a distinct clonal group of clinical isolates in Toyama Prefecture, Japan. *Journal of Infection and Chemotherapy*, 19:644-652, 2013.
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