表５ 浴槽水、ろ過材、患者由来の*M. avium* のアリルプロファイル例

Primer No.	浴槽水由来菌 K-147			ろ過材 K-152			患者喀痰 Ka316		
	実測値	換算値	TR数	実測値	換算値	TR数	実測値	換算値	TR数
1	281.8	281	1	328.0	334	2	285.4	281	1
2	247.6	247	1	245.7	247	1	248.3	247	1
3	248.0	248	1	248.2	248	1	248.7	248	1
4	270.5	274	2	271.8	274	2	271.8	274	2
5	248.3	249	2	251.1	249	2	249.0	249	2
6	268.9	268	1	271.2	268	1	269.4	268	1
7	276.4	277	1	276.9	277	1	278.0	277	1
8	220.2	220	2	161.4	163	1	171.1	163	1
9	483.2	490	3	480.5	490	3	486.3	490	3
11	333.4	339	1	329.6	339	1	333.7	339	1
12	556.3	542	3	552.0	542	3	565.9	542	3
13	235.2	235	0	231.3	235	0	239.1	235	0
14	392.9	389	3	390.7	389	3	397.2	389	3
15	304.2	308	2	305.7	308	2	305.0	308	2
16	358.5	359	2	422.0	418	3	360.9	359	2

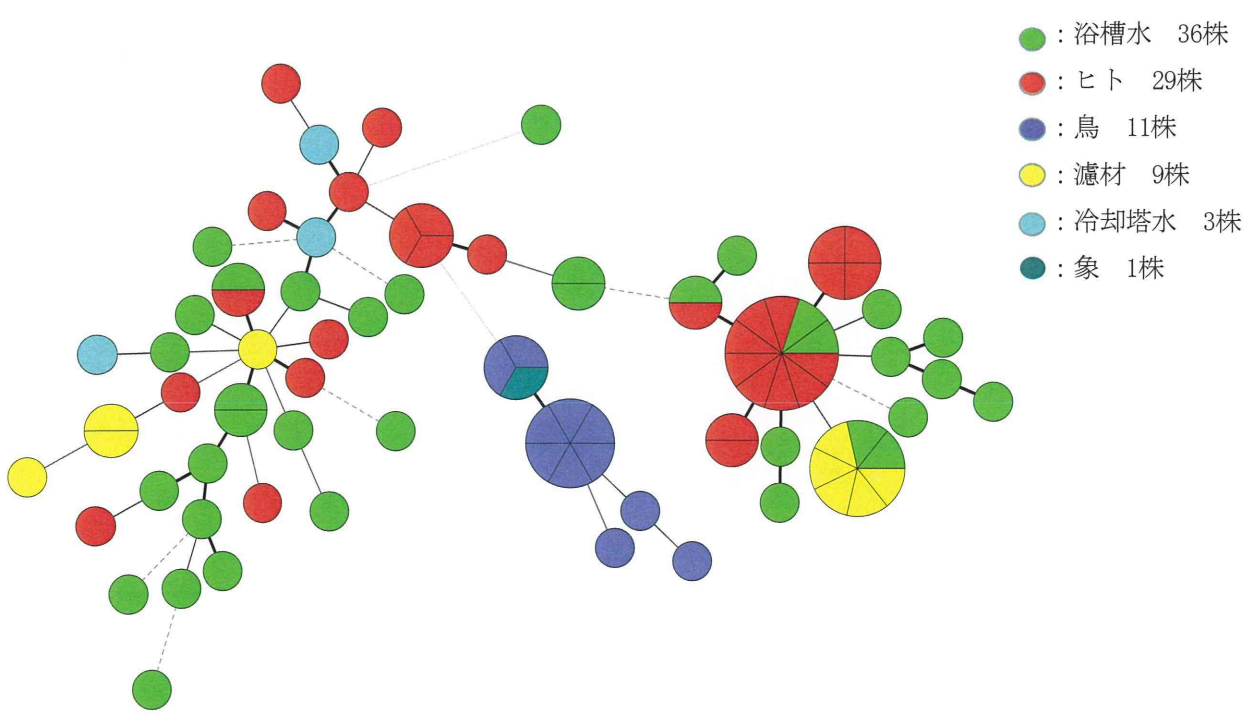


図１　分離源が異なる*M. avium*のminimum spanning tree

平成22年度厚労科研（健康安全・危機管理対策総合研究事業）
「公衆浴場等におけるレジオネラ属菌対策を含めた総合的衛生管理手法に関する研究」
平成24年度　分担研究報告書

研究分担者　　国立感染症研究所　寄生動物部　八木田健司
研究協力者　　国立感染症研究所　寄生動物部　泉山　信司

レジオネラ属菌遺伝子型とアメーバ感受性

研究要旨：様々な環境から分離した *L. pneumophila* SG1 株とアメーバ類を用いて実験感染を行い、菌の遺伝子型とアメーバのとの関係を調べた。*L. pneumophila* SG1 株は土壌および冷却塔由来の計 5 グループについて、土壌、温泉浴槽あるいは冷却塔からの *Acanthamoeba* sp および *Vannella* sp を用いて試験した。*Acanthamoeba* においては菌の特定のグループが特定の環境の *Acanthamoeba* と関連している傾向は認められず、菌の遺伝子型分布に *Acanthamoeba* が関連する証左は得られなかった。また *Vannella* においては全般的にアメーバ株による特異性が見られる中で、浴槽分離の株に菌の特定遺伝子型と関連していることを示唆する結果が得られた。レジオネラ属菌感受性の *Vannella* に関しては、同アメーバがシスト化しない性質から実際の環境における宿主としての重要性が高いものと考えられた。

A. 研究目的

環境中に生息する *Acanthamoeba* 等の自由生活性アメーバは、レジオネラ属菌の自然宿主として知られている。公衆衛生学的には宿主アメーバ類の生態がレジオネラ感染症の成立に大きく影響を及ぼしており、生活環境中における宿主アメーバ調査とその管理は重要な問題である。最近の研究から、レジオネラ属菌の遺伝子型が分離される環境によりその分布に違いが見られることが明らかになってきている。その遺伝子型の分布に宿主アメーバが関連している可能性を知るため、本研究では様々な環境から分離した *L. pneumophila* SG1 株とアメーバ類を用いて、菌の特定の遺伝子型の増殖をサポートするアメーバが存在するのかどうか、菌の遺伝子型とアメーバの感受性の関係を調べた。

B. 研究方法

1.　レジオネラ属菌株

土壌および冷却塔より分離した *L. pneumophila*（以下 *L.p*）SG1 株を用いた。遺伝子型（ST）に基づき冷却塔分離は 2 グループ（C1、C2）、土壌分離は3グループ（S1、S2、S3）の計 5 グループに分類し、各グループにつき 2 株を調べた。菌株は BCYE α 培地にて 30℃、3 日間培養したものを用いた

2. アメーバ株

Acanthamoeba sp.株は土壌分離 9 株（S1－S9）、温泉浴槽水分離 11 株（HS1-HS11）、冷却塔水 4 株（CT1-CT4）を用いた。また *Vannella* sp 株として土壌分離の1株（VNS1）ならびに浴槽水分離の3株（VNHS1-VNHS3）を用いた。アメーバ類は大腸菌塗布培地を用いて30℃で培養した。

3. 感染実験

培養した *L. p* 菌株をアメーバ用生理食塩水（AS）で希釈し濃度約0．1OD の菌液を調整した。加熱により不活化処理した大腸菌を用いて同様に菌液を調整し、2 つの菌液を等量混合した。無栄養寒天培地に約 0.5ml の混合菌液を塗布し風乾させ試験培地とした。供試するアメーバについては増殖中の栄養体を培地上より回収し、AS で浮遊液を調整した。レジオネラ属菌を塗布した試験培地上に供試アメーバ浮遊液を滴下し風乾させ、アメーバと菌を

接触させた(図1)。大腸菌のみを塗布した培地を用い同様にアメーバ浮遊液を滴下し、これを対照実験とした。培養は 20℃および 30℃で行った。菌の感染を生きた栄養体の有無で評価した。評価に際してのスコアーは以下のようにした。(+)は栄養体の増殖が認められ、また菌の細胞内増殖による破壊が認められない、菌の感染は成立しなかった状態。(±)は栄養体の増殖が認められるも、一部で菌の感染があり細胞内増殖による破壊が認められる。(−)は菌の感染後細胞内増殖により破壊され、栄養体の増殖が認められない。死滅状態。

C. 研究結果

L. p SG1 の 5 つのグループ(各 2 菌株)を *Acanthamoeba* sp.株に感染、30℃で培養したときの結果を表-1 に示した。ほとんどすべての *Acanthamoeba* sp.株は試験培地上に滴下した範囲の中で死滅したが、対象実験である大腸菌のみの培地上では活発な増殖を示した。土壌株の一つ S9 が、ほとんどすべての菌株に対し、菌が存在する条件であっても増殖を示したが、増殖するアメーバの一部では明らかに菌の細胞内増殖による破裂があったことが認められる場合があった(図-2)。表-2 には、一部のアメーバ株を選択して、5 グループの *L. p* SG1 の存在下、20℃で培養したときの結果を示した。30℃における感染結果と同様、土壌の S9 株が菌の存在下で増殖を示した。他に 30℃の試験では死滅したアメーバ株S1およびHS1が20℃培養においては一部の菌株に対し増殖性を示した。

表-3 に *Vannella* sp.株に関する試験結果を示した。図 3 に用いた *Vannella* sp.株の形態画像を示す。土壌分離の VNS1がすべての菌株に対して死滅する結果を示したが、対照的に浴槽分離の VNHS1 および HS2 はすべての菌株存在下で活発な増殖を示した。一方浴槽分離の VNHS3 は限定的な増殖を示した。即ち土壌分離の L.pSG1 の 3 グループすべてで死滅し、また冷却塔分離の C1 グループに対しては 2 株とも死滅の結果を示したが、冷却塔 C2 グループの 2 株にはいずれに対しても対照実験と変わらない増殖を示した。

D. 考　察

レジオネラ属菌の遺伝子型が環境により検出頻度が異なることが明らかになっている。遺伝子型が菌の病原性を含めた細菌学的性質に影響を及ぼす因子となることを考えれば、特定の環境が感染源となる可能性についてそこに生息するアメーバが菌の宿主となりうるかどうかが極めて重要な要因となる。本研究では、異なる環境から分離した *L. p* SG1 とアメーバ類を相互感染実験し、その感染適合性を調べた。限定された培養温度条件の結果ではあるが、*L. p* SG1 の特定のグループが特定の環境の *Acanthamoeba* と関連している傾向は認められず、レジオネラ属菌の遺伝子型分布に *Acanthamoeba* が関連する証左は得られなかった。この結果は *L. p* SG1 は概ねその環境にいる *Acanthamoeba* に対して、非選択的に宿主として利用していること、一方 *Acanthamoeba* の中には菌のグループの違いに関わらず一定の抵抗性を示す場合もあり、菌の宿主となるかどうかは宿主アメーバ株の細胞学的・遺伝学的性質の違いにより決まることではないか、ということを示唆している。遺伝子型ではなくレジオネラ属菌の種類によっては *Acanthamoeba* が宿主であっても、その細胞内増殖性が大きく異なることが分かっている。例えば *L.londinensis* はほとんど *Acanthamoeba* では増殖せず、その好適宿主は *Naegleria* sp である。レジオネラ属菌の種における分布様式に関しては、そこに生息するアメーバ種のポピュレーションが影響していることが推測される。本研究では *Acanthamoeba* 以外のアメーバ種として *Vannella* の菌宿主特異性を調べた。少ない株数を調べた結果ではあるが、*L. p* SG1 全体として *Vannella* 株による宿主特異性が見られる一方、浴槽分離の VNHS3 が冷却塔の C2 グループの *L. p* SG1 に対しては増殖を示した点は、*L. p* SG1 の遺伝子型が宿主特異性に関連していることを示唆する結果であり興味深い。菌の遺伝子型とアメーバ類の感染適合性に関しては、土壌環境を考えると低温(10−15℃)での試験の重要性も考えられ、温度を厳密にコントロールした上での感染適合性の試験を行うことが必要と考えられる。

本研究では *Vannella* が *L. p* SG1 の宿主となり得る結果が示された。*Vannella* がシスト化しないタイプのアメーバで、旺盛な増殖力を示し、特に水系環境中では一般的な存在であることを考えると、シスト化する *Acanthamoeba* などと違って常にレジオネラ属菌の宿主となる可能性があり、レジオネラ属菌による汚染実態を考える上では見逃せない存在であることが指摘される。これまでは実験モデル化しやすい *Acanthamoeba* などのシスト形成アメーバが研究の対象となっていたが、今後はシスト化しないアメーバに関しても宿主としての研究の重要性があると考えられる。

E. 結　論

レジオネラ属菌の宿主アメーバが環境中でのレジオネラ属菌の生物学的因子として働いているが、とりわけ *Acanthamoeba* はいかなる環境でもそこにいる菌にとって格好の増殖装置として存在すると考えられる。またレジオネラ属菌による汚染実態には、シストを形成しない *Vannella* などのアメーバ類も関連することが推測され、その宿主としての重要性を明らかにする必要がある。

F. 参考文献

厚生労働省科学研究費補助金　健康安全・危機管理対策総合研究事業「公衆浴場等におけるレジオネラ属菌対策を含めた総合的衛生管理手法に関する研究」平成 22 年度総括・分担研究報告書　研究代表者　倉　文明

G. 健康危惧情報

なし

H. 研究発表

なし

I.知的所有権の出願・登録状況

なし

表-1、異なる環境より分離された *L. pneumophila* SG1 株と *Acanthamoeba* sp.株の相互感染実験結果（30℃試験）

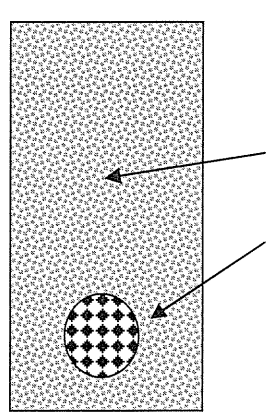
		冷却塔分離 L.p				土壌分離 L.p						対照
		C1		C2		S1		S2		S3		
		1052	1592	590	694	2375	2363	2332	2398	2327	2342	大腸菌
土壌分離 *Acanthamoeba*	S1	—	—	—	—	—	—	—	—	—	—	+
	S2	—	—	—	—	—	—	—	—	—	—	+
	S3	—	±	—	—	—	—	—	—	—	±	+
	S4	—	—	—	—	—	—	—	—	—	—	+
	S5	—	—	—	—	—	—	—	—	—	—	+
	S6	—	—	—	—	—	—	—	—	—	—	+
	S7	—	—	—	—	—	—	—	—	—	—	+
	S8	—	—	—	—	—	—	—	—	—	—	+
	S9	—	+	+	+	±	+	±	+	±	+	+
温泉浴槽分離 *Acanthamoeba*	HS1	—	—	—	—	—	—	—	—	—	—	+
	HS2	—	—	—	—	—	—	—	—	—	—	+
	HS3	—	—	—	—	—	—	—	—	—	—	+
	HS4	—	—	—	—	—	—	—	—	—	—	+
	HS5	—	—	—	—	—	—	—	—	—	—	+
	HS6	—	—	—	—	—	—	—	—	—	—	+
	HS7	—	—	—	—	—	—	—	—	—	—	+
	HS8	—	—	—	—	—	—	—	—	—	—	+
	HS9	—	—	—	—	—	—	—	—	—	—	+
	HS10	—	—	—	—	—	—	—	—	—	—	+
	HS11	—	—	—	—	—	—	—	—	—	—	+
冷却塔分離 *Acanthamoeba*	CT1	—	—	—	—	—	—	—	—	—	—	+
	CT2	—	—	—	—	—	—	—	—	—	—	+
	CT3	—	—	—	—	—	—	—	—	—	—	+
	CT4	—	—	—	—	—	—	—	—	—	—	+

表-2、異なる環境より分離された *L. pneumophila* SG1 株と *Acanthamoeba* sp.株の
相互感染実験結果 (20℃試験)

		冷却塔分離 L.p				土壌分離 L.p						対照
		C1		C2		S1		S2		S3		
		1052	1592	590	694	2375	2363	2332	2398	2327	2342	大腸菌
土壌分離 *Acanthamoeba*	S1	—	±	—	—	—	—	—	—	—	—	+
	S4	—	—	—	—	—	—	—	—	—	—	+
	S6	—	—	—	—	—	—	—	—	—	—	+
	S7	—	—	—	—	—	—	—	—	—	—	+
	S9	—	±	±	±	±	±	±	+	±	±	+
温泉浴槽分離 *Acanthamoeba*	HS1	—	—	—	—	—	—	—	—	±	±	+
	HS2	—	—	—	—	—	—	—	—	—	—	+
	HS6	—	—	—	—	—	—	—	—	—	—	+
	HS9	—	—	—	—	—	—	—	—	—	—	+

表-3、異なる環境より分離された *L. pneumophila* SG1 株と *Vannella* sp.株の
相互感染実験結果 (30℃試験)

		冷却塔分離 L.p				土壌分離 L.p						対照
		C1		C2		S1		S2		S3		
		1052	1592	590	694	2375	2363	2332	2398	2327	2342	大腸菌
土壌分離 *Vannella*	VNS1	—	—	—	—	—	—	—	—	—	—	+
温泉浴槽分離 *Vannella*	VNHS1	+	+	+	+	+	+	+	+	+	+	+
	VNHS2	+	+	+	+	+	+	+	+	+	+	+
	VNHS3	—	—	+	+	—	—	—	—	—	—	+



1) 寒天表面上に大腸菌とレジオネラ属菌を均等混合した液を塗布し乾燥させる。対照実験では大腸菌のみを塗布する。
2) 寒天培地の一端に試験するアメーバ浮遊液を滴下し乾燥させる。
3) 過度の乾燥を防いで培養する

図-1、感染および培養試験法の模式図

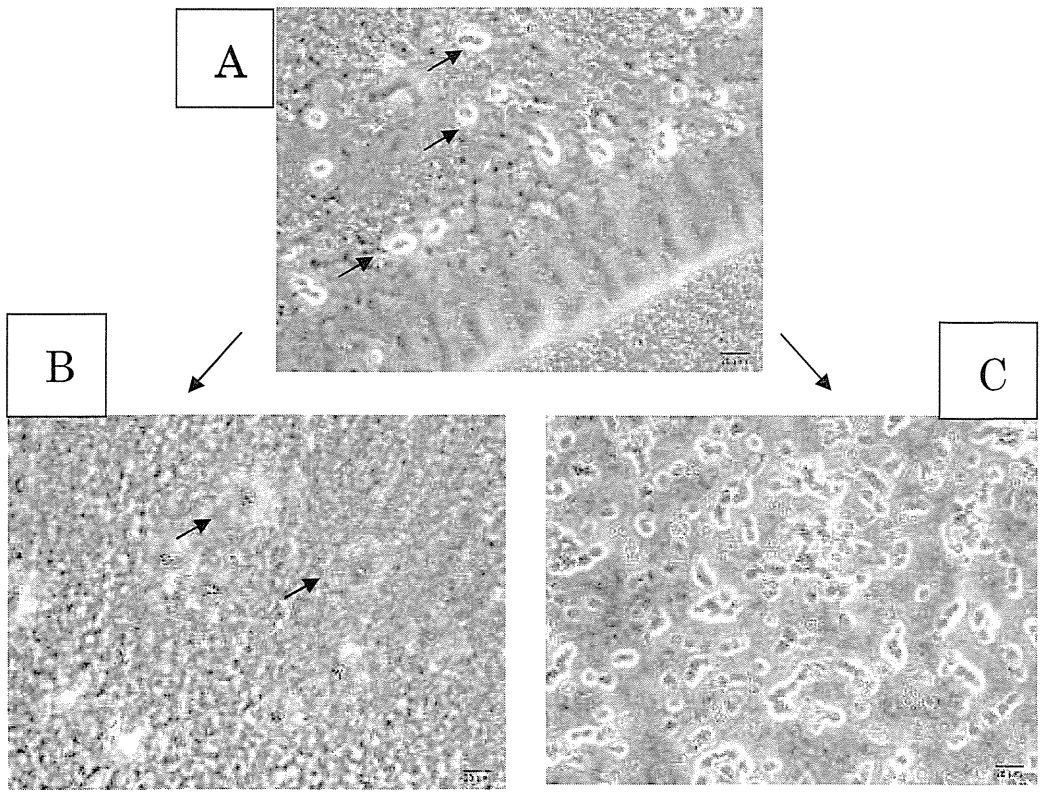


図-2、試験培地での *Acanthamoeba* の培養

A: 培地に接種直後の観察像。アメーバ栄養体が観察される（→）

B: 培養 3 日後の接種部における観察像（判定結果は－）。菌の感染による宿主アメーバでの細胞内増殖の結果、アメーバが破裂崩壊し死滅している（→）。

C: 培養 3 日後の接種部における観察像（班定結果は＋）。菌が存在していてもアメーバの死滅は見られず、増殖が認められる。

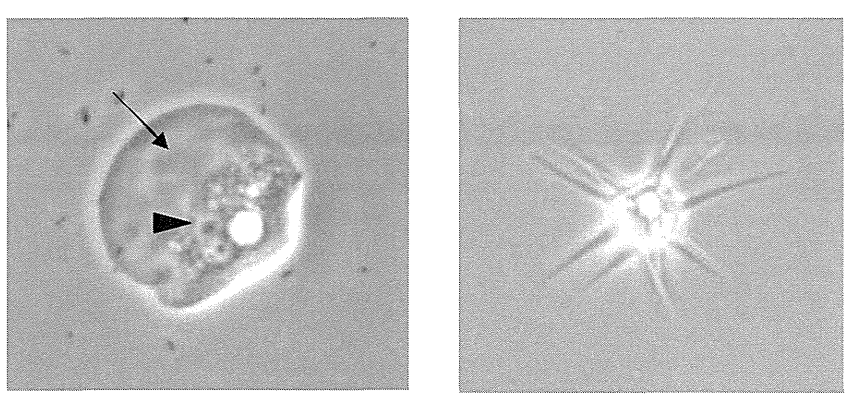


図-3、 *Vannella* sp.VNHS1 株の形態

A: 付着して運動する栄養体。扇状に幅広く伸ばす仮足（→）が特徴。▶ は細胞核を示す。

B: 浮遊する栄養体。付着できない場合は細胞周囲に棘状の構造を多数突出させ浮遊体となる。

III. 研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Kanatani JI, Isobe J, Kimata K, Shima T, Shimizu M, Kura F, Sata T, and Watahiki M	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		in press	
Amemura-Maekawa J, Kikukawa K, Helbig J, Kaneko S, Suzuki-Hashimoto A, Furuhata K, Chang B, Murai M, Ichinose M, Ohnishi M, Kura F and the Working Group for *Legionella* in Japan	Distribution of monoclonal antibody subgroups and sequence-based types among *Legionella pneumophila* serogroup 1 isolates derived from cooling tower water, bath water and soil in Japan.	Applied and Environmental Microbiology		4263-4270	

IV. 研究成果の刊行物・別刷

J Infect Chemother
DOI 10.1007/s10156-012-0537-x

ORIGINAL ARTICLE

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 pulsed-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 two 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 bathwater, suggesting that they were derived from unrecognized sources.

Keywords *Legionella pneumophila* · Molecular epidemiology · Molecular typing

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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 *L. 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 two 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

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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 seven genes (*flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, and *neuA*) for *L. pneumophila* [16–18]. Similar to PFGE, SBT has been considered to be 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 *L. 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 two 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 four 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, Billerica, MA, USA) and resuspended in 5 ml 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. Polymerase chain reaction (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 seven SBT alleles was constructed by the neighbor-joining method, using the MEGA4 software [21]. A bootstrapping test was performed 1,000 times. Clonal analyses were performed by using eBURST V3 (http://eburst.mlst.net). Groups were generated with single- and double-locus variants and 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 *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, Hercules, 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 (IOD)

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

Table 1 Sequence-based typing (SBT) and pulsed-field gel electrophoresis (PFGE) profiles of *Legionella 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	Bathwater[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	Bathwater
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	Bathwater
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	Bathwater[c]
12	LG0613	PA11	2008	Sep	7	6	17	3	11	11	9	505[b]	P13	Bathwater[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	Bathwater
15	LG1008	PA14	2009	Feb	7	6	17	10	13	9	11	682	P17	Bathwater
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	Bathwater
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	

Table 1 continued

No.	Strain	Origin[a]	Year	Month	SBT profile							ST	PFGE type	Sources of infection
					flaA	*pilE*	*asd*	*mip*	*mompS*	*proA*	*neuA*			
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	
49	LG0629	PB13	2008	Sep	6	10	20	6	9	4	9	530	P25	
50	LG0708	PB14	2008	Sep	6	10	15	28	21	14	11	1095[b]	P42	
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	
56	LG0941	PB18	2008	Nov	23	10	3	3	8	4	9	1098[b]	P46	
57	LG0954	PB18	2008	Nov	6	6	15	3	9	14	11	1101[b]	P44	
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	
61	LG1142	PB18	2009	Nov	23	10	3	3	8	4	9	1098[b]	P45	
62	LG0976	PB19	2008	Nov	6	10	15	28	4	14	11	278	P42	
63	LG0987	PB20	2008	Dec	6	10	19	28	19	4	11	763	P41	
64	LG1034	PB21	2009	May	6	10	15	3	17	14	9	1100[b]	P52	
65	LG1124	PB22	2009	Nov	6	10	14	10	2	3	6	77	P35	
66	LG1156	PB23	2009	Nov	3	6	1	28	14	9	11	1102[b]	P23	
67	LG1167	PB24	2009	Nov	1	4	3	1	1	1	1	1	P10	
68	LG1169	PB24	2009	Nov	7	6	17	3	13	11	11	59	P13	
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] *ST* sequence type, *PA* patient, *PB* public bath, *CT* cooling tower, *SH* shower

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

[c] Confirmed by PFGE with environmental isolates

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 31 October 2012. Among these, 9 ST505 isolates were obtained from four patients and three 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 seven clonal groups were generated by SBT (Fig. 2). Among the seven clonal groups (CG1–CG7), CG3 was formed by isolates from seven 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 %.

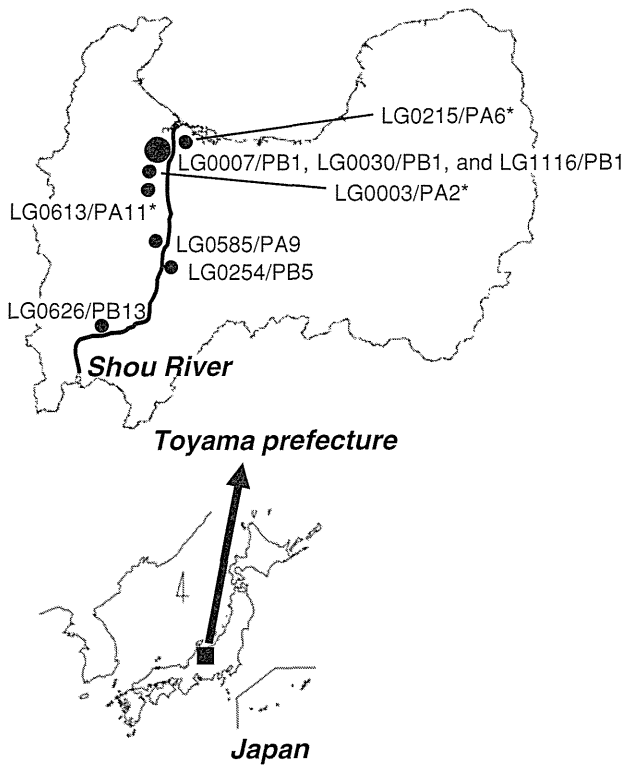

Fig. 1 Geographic distribution of ST505 strain. *Isolate name* indicates the strain/origin as described in Table 1. The *size of the circle* indicates the number of isolates. *Asterisk* indicates the clinical isolates associated with bathwater by epidemiological investigation

PFGE analysis

A dendrogram of the PFGE pattern was constructed (Fig. 3). Figure 4 shows the original gel image of band patterns of isolates belonging to 3 STs (ST1, ST278, ST505) among 13 STs (ST1, ST59, ST122, ST128, ST278, ST384, ST505, ST644, ST763, ST769, ST1094, ST1098, 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 two restriction fragments with similarity of approximately 90 % (Fig. 4). The CG3 consisting of 7 clinical isolates was split into two 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 three 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 seven clinical isolates that were not associated with bathwater. Travel histories of 14 of the 16 patients during the likely exposure period were available. Although patient PA5 had a history of a visit outside Toyama Prefecture, we could not identify whether this patient had been infected in Toyama Prefecture. 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 two PFGE types of the isolates were similar because of the difference with only two 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 caused by 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

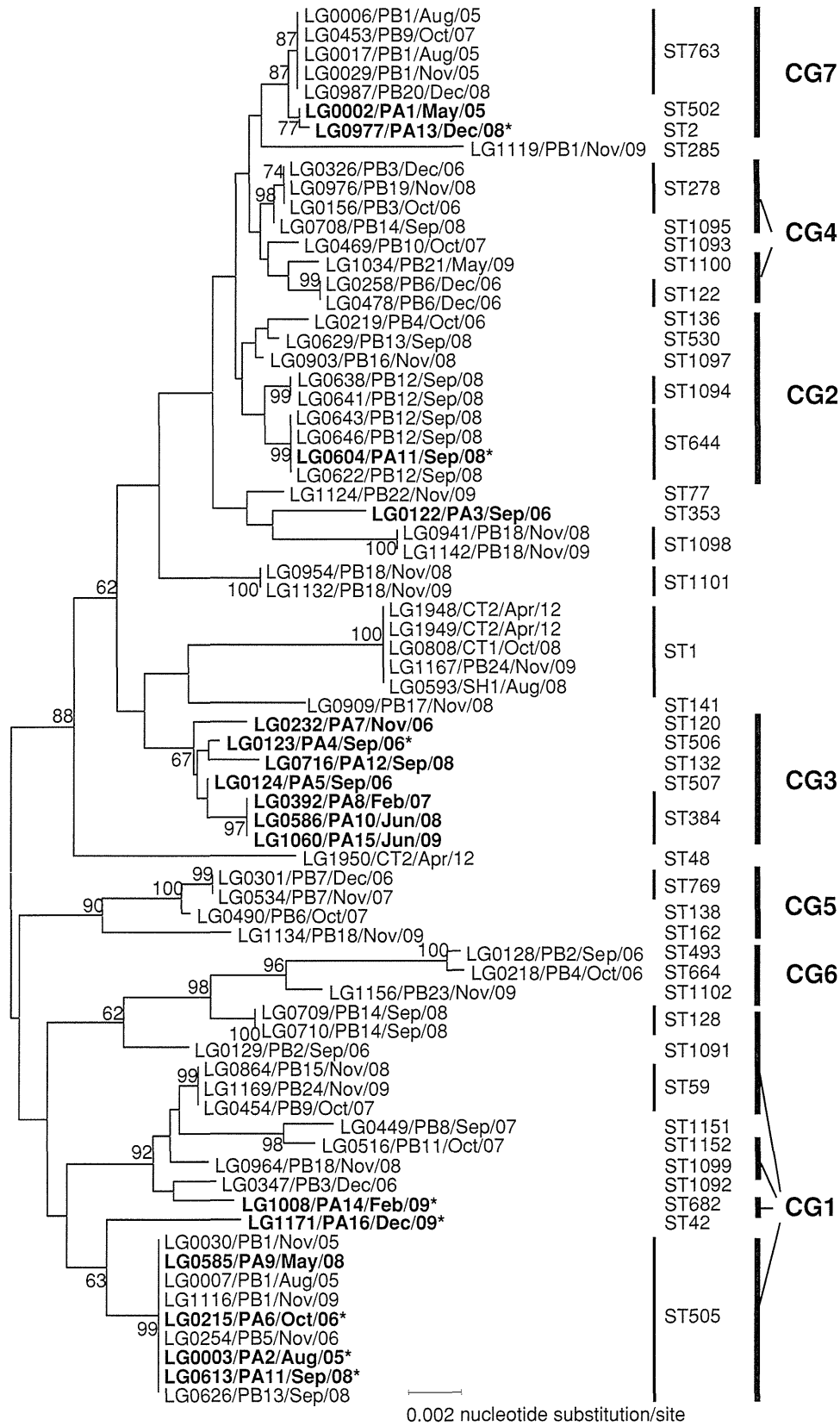

Fig. 2 Phylogenetic analysis of the concatenated sequences (*flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, *neuA*) of *Legionella pneumophila* SG 1 isolates in this study. *Isolate name* indicates the strain/origin/month/year as described in Table 1. *Isolates in boldface* are from patients. *Asterisk* 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)

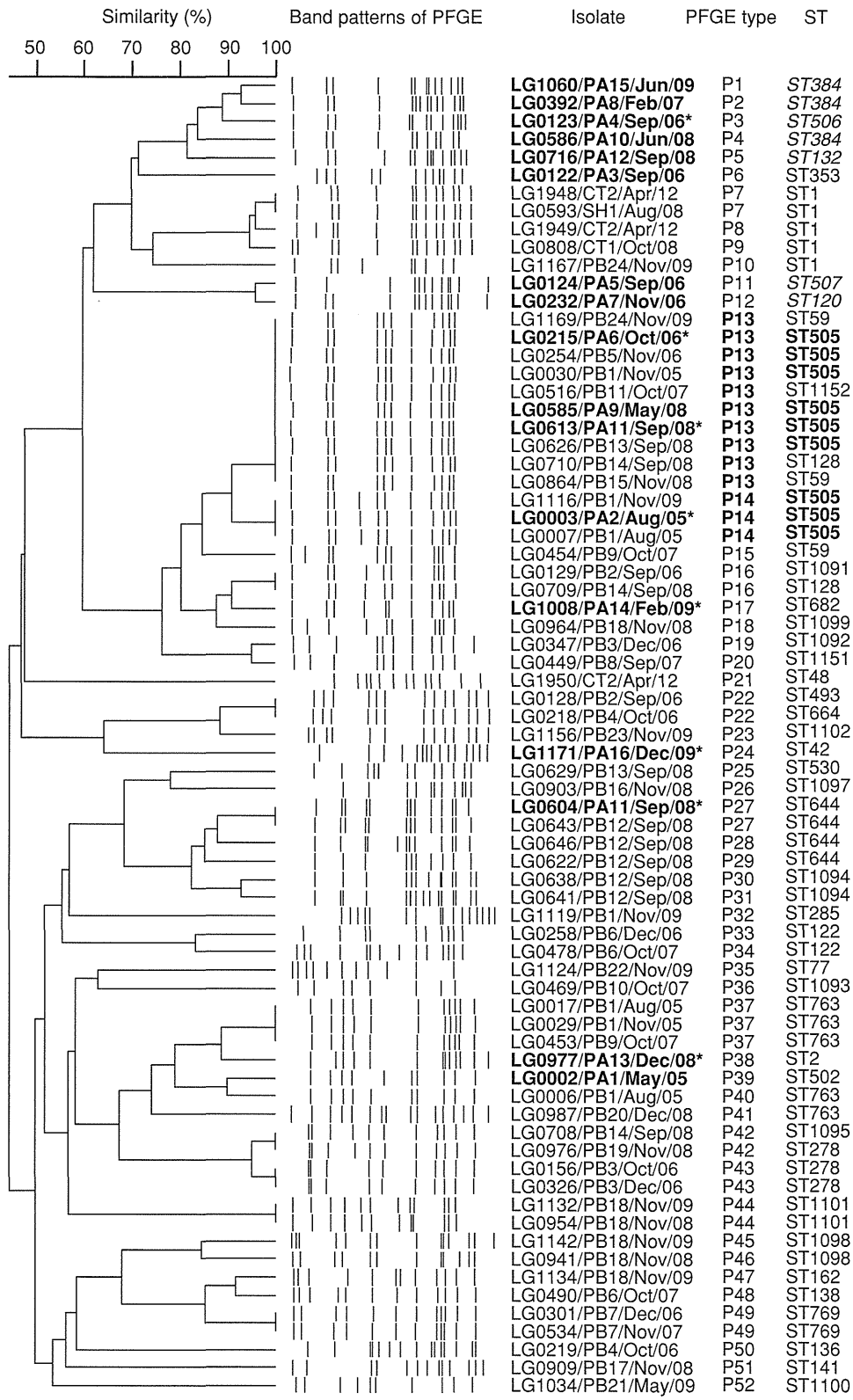

Fig. 3 *Dendrogram* of the pulsed-field gel electrophoresis (PFGE) pattern constructed from *L. pneumophila* SG 1 isolates in this study. *Isolate name* indicates the strain/origin/month/year as described in Table 1. *Isolates in boldface* are from patients. *Asterisk* indicates the clinical isolates associated with bathwater by epidemiological investigation. Two PFGE types (P13 and P14) and ST505 are denoted by *boldface*. *Italic letters* indicate STs belonging to CG3

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

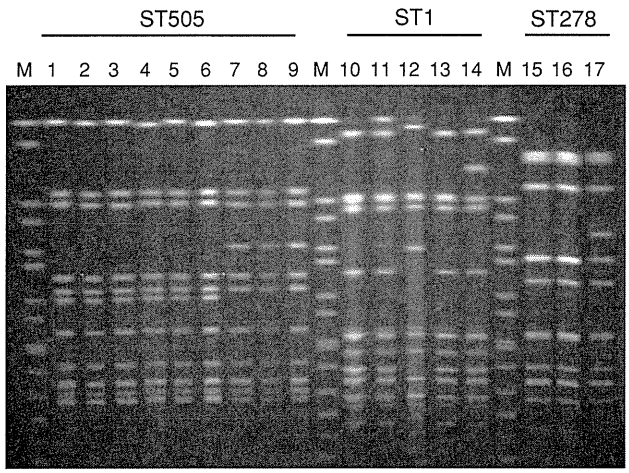

Fig. 4 PFGE patterns with *Sfi*I digestion of *L. pneumophila* SG 1 isolates. *Lanes*: *M Salmonella enterica* serovar Braenderup H9812 strain digested with *Xba*I as a size marker, *1* LG0215, *2* LG0254, *3* LG0030, *4* LG0613, *5* LG0585, *6* LG0626, *7* LG0003, *8* LG0007, *9* LG1116, *10* LG0593, *11* LG0808, 12 LG1167, *13* LG1948, *14* LG1949, *15* LG0156, *16* LG0326, *17* LG0976

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 two 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 seven clinical isolates belonged to CG3 (Fig. 2), in which no environmental isolates were present. Among the seven clinical isolates, six were not associated with bathwater 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. [30] suggested the possibility of habitat segregation of *L. pneumophila*. 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 bathwater 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 bathwater 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 one 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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Distribution of Monoclonal Antibody Subgroups and Sequence-Based Types among *Legionella pneumophila* Serogroup 1 Isolates Derived from Cooling Tower Water, Bathwater, and Soil in Japan

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***Legionella pneumophila* serogroup (SG) 1 is the most frequent cause of legionellosis. This study analyzed environmental isolates of *L. pneumophila* SG 1 in Japan using monoclonal antibody (MAb) typing and sequence-based typing (SBT). Samples were analyzed from bathwater (BW; $n = 50$), cooling tower water (CT; $n = 50$), and soil (SO; $n = 35$). The distribution of MAb types varied by source, with the most prevalent types being Bellingham (42%), Oxford (72%), and OLDA (51%) in BW, CT, and SO, respectively. The ratios of MAb 3/1 positive isolates were 26, 2, and 14% from BW, CT, and SO, respectively. The environmental isolates from BW, CT, and SO were divided into 34 sequence types (STs; index of discrimination [IOD] = 0.973), 8 STs (IOD = 0.448), and 11 STs (IOD = 0.879), respectively. Genetic variation among CT isolates was smaller than seen in BW and SO. ST1 accounted for 74% of the CT isolates. The only common STs between (i) BW and CT, (ii) BW and SO, and (iii) CT and SO were ST1, ST129, and ST48, respectively, suggesting that each environment constitutes an independent habitat.**

Legionella pneumophila serogroup (SG) 1 is the most common agent causing legionellosis found in patients; however, differences in SGs have been found both in isolates from patients and from soil and various freshwater environments (9), such as cooling towers and bathing facilities. In patients, most strains (80%) belonged to SG 1 in our previous study (2). In cooling tower water isolates of Japan, *L. pneumophila* SGs 1 and 7 accounted for 67 and 23%, respectively, with other SGs being rarely isolated. On the other hand, the isolates from bathwater and from soil were more serotypically diverse, but SG 1 was still dominant in both environments, at 31% (1) and 26% (10), respectively. *L. pneumophila* SG 1 can be divided based on having or not having the virulence-associated epitope recognized by monoclonal antibody (MAb) 3/1 (13). In England and Wales, of the clinical isolates, 91.6% were MAb 3/1 positive compared to only 8.3% of the environmental isolates (12).

L. pneumophila isolates can be characterized by sequence-based typing (SBT) using the seven loci (*flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, and *neuA*) proposed by the European Working Group on *Legionella* Infections (EWGLI; http://www.ewgli.org/ [11, 21]). This is a separate classifier from serogroup or MAb subtyping and is generally more precise due to the mutability of the latter factors. It allows for phylogenetic studies and identification of isolates that are closely related. The variation in STs of clinical and environmental isolates of *L. pneumophila* worldwide is very diverse. The indices of discrimination (IODs) (14) of environmental isolates and clinical isolates were determined to be 0.888 and 0.964, respectively, in Canada and 0.822 and 0.946, respectively, in the United States (15, 22). In England and Wales, however, environmental isolates are more variable than clinical ones (IODs of 0.933 and 0.901, respectively [12]), but the diversity is comparably great.

When 69 SG1 clinical isolates from Japan were subjected to SBT, they could be divided into 41 sequence types (STs). The IOD was 0.979. The ST with the most isolates ($n = 7$) was ST1. This is the most common ST occurring in the environment and among patients worldwide. Other major STs were ST306 ($n = 6$), ST120 ($n = 5$), and ST138 ($n = 5$). All ST306 and ST138 isolates, with one exception (ST306), were derived from bathwater (or suspected to be), suggesting that these strains readily adapt to bathwater habitats. The source of all ST1 and ST120 isolates remains unclear (2). In Japan, data from the National Epidemiological Surveillance of Infectious Diseases indicate that hot springs and public baths are primary sources of *L. pneumophila*, rather than cooling towers; however, in most cases the source of the bacteria is unknown (19).

We analyzed here environmental isolates of *L. pneumophila* SG 1, which is the principal cause of legionellosis in bathwater (the main source of infection in Japan), soil (a potential source of contamination for various water systems), and cooling tower water (another major source of legionellosis). Isolates were identified using MAb typing and SBT and then compared to previous clinical isolates (2) to determine relations between isolates from different environments and from patients.

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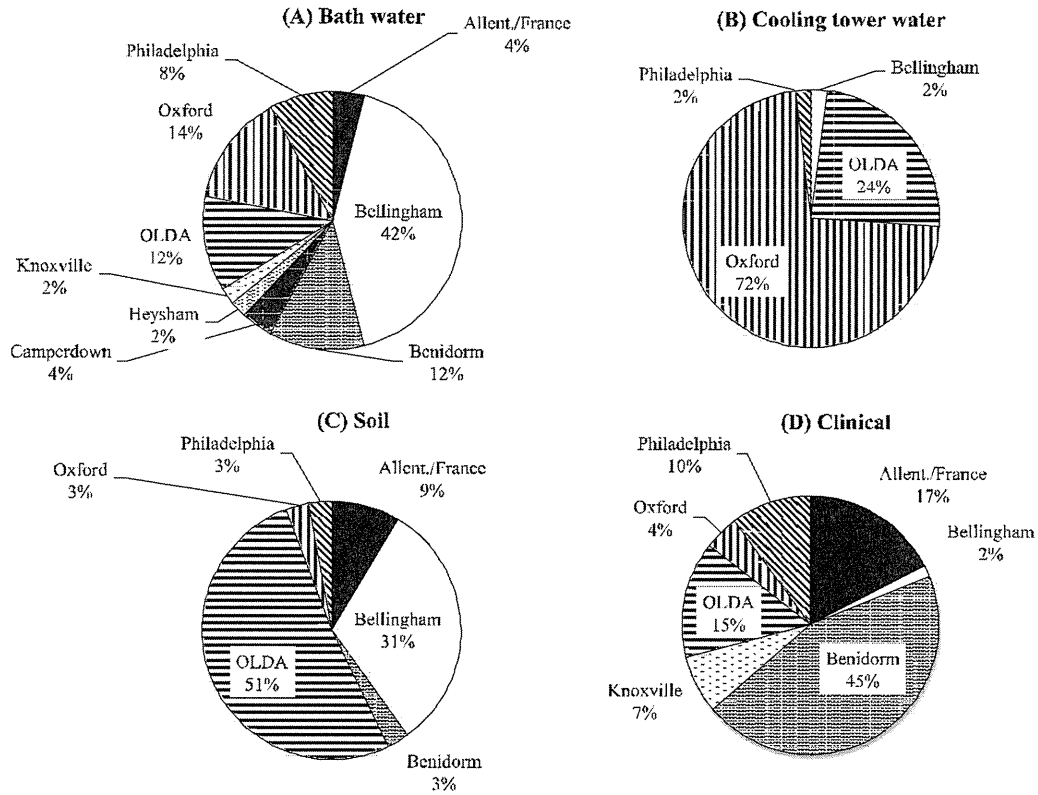

FIG 1 Distributions of MAb types. (A) Isolates from bathwater (n = 50); (B) isolates from cooling tower water (n = 50); (C) isolates from soil (n = 35); (D) isolates from patients of legionellosis (n = 69 [2]). Allentown/France, Benidorm, Knoxville, and Philadelphia are MAb 3/1-positive subgroups. Bellingham, Camperdown, Heysham, OLDA, and Oxford are MAb 3/1-negative subgroups. MAb 3/1 indicates the virulence-associated epitope.

MATERIALS AND METHODS

***L. pneumophila* strains.** A total of 135 environmental strains of *L. pneumophila* SG 1, which were independently isolated and unrelated to cases of infection, were analyzed, including isolates from bathwater (BW; n = 50), cooling tower water (CT; n = 50), and soil (SO; n = 35). All of the CT and BW isolates were obtained from different facilities: 66% of the CT isolates and 42% of the BW isolates originated from the Kanto region in central Japan. The SO isolates, which were independently collected from across Japan, were obtained from topsoil samples from roadsides, farmlands, gardens, etc. (10).

MAb subgrouping. A total of 135 environmental strains of *L. pneumophila* SG 1 were subtyped serologically, using MAbs as described previously, into nine subgroups named Allentown/France, Bellingham, Benidorm, Camperdown, Heysham, Knoxville, OLDA, Oxford, and Philadelphia (13).

SBT. SBT was performed according to the EWGLI SBT protocol (http://www.ewgli.org/) as described previously (11, 21). The isolates that failed amplification of *neuA* (whose indicated allele number was "0") were not given ST numbers but were allocated arbitrary numbers prefixed by J (2). A minimum-spanning tree that had categorical coefficients of similarity and the priority rule of the highest number of single-locus variants as parameters was used to indicate differences in the number of loci among operational taxonomic units (OTU). The neighbor-joining method was then used to find pairs of OTU that minimized the total branch lengths by number of base substitutions on *flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, and *neuA* concatenated sequences (2,501 bp) at each stage of OTU clustering. Both trees were constructed using BioNumerics software (version 6.5; Applied Maths, Sint-Martens-Latem, Belgium).

RESULTS

MAb subgrouping. The isolates examined here were comprised of nine MAb types in all. The BW isolates were comprised of all nine MAb types, the CT isolates were comprised only of four, and the SO isolates were comprised of six. The distributions of MAb subgroups in the environmental isolates differed from one another, and from that found in clinical isolates (Fig. 1). The most common MAb subgroup in BW isolates was the Bellingham subgroup (42%), whereas the Oxford subgroup was the most common in CT (72%) and the OLDA subgroup was the most common in SO (51%). Bellingham, Oxford, and OLDA are MAb 3/1-negative subgroups. On the other hand, the most common subgroup observed in Japanese clinical isolates was Benidorm (45%), which is MAb 3/1 positive (2). Benidorm was detected in 12% of isolates from bathwater and 3% of isolates from soil. Of the 135 environmental isolates, only 14% had the virulence-associated epitope recognized by MAb 3/1: Benidorm, Allentown/France, Philadelphia, and Knoxville (13). In BW, 26% of the isolates were MAb 3/1 positive, compared to 14% in SO and a mere 2% in CT.

SBT. The 135 environmental isolates (with the exception of one SO isolate in which amplification of the *neuA* target failed) could be divided into 50 STs, including 33 singletons (IOD = 0.886; Tables 1 and 2). The ST with the largest number of isolates was ST1 (n = 43, 29%), followed by ST48 (n = 10, 6.7%), ST129 (n = 7, 4.7%), ST739 (n = 6, 4.0%), and ST22 (n = 5, 3.3%). Strains with indigenous STs were isolated from each environment. The only common STs across environments were ST1 (37 from