表１　検出抗酸菌の地研由来別数

	調査地方別数				
	関東	中部	北陸	九州	合計
調査施設数	100	32	96	31	259
浴槽・源泉等数	100	45	263	58	466
検体数	100	61	263	58	482
地研検出菌株数	(16)	26	8	(10)	60
純化後同定菌株数	22	31	9	13	75

()：レジオネラ検出用100倍濃縮液を感染研に輸送、感染研にて検出した数

表２　浴槽水等から検出された抗酸菌の同定結果

ラニオン			由来内訳（株）			
分類	菌種名	菌株数	関東	中部	北陸	九州
I群菌	*M. asiaticum*	1	1			
	M. kansassi	6	5		1	
	M. simiae	2	1		1	
II群菌	*M. gordonae*	15	4	1	9	1
	M. intermedium	1			1	
	M. scrofulaceum	1	1			
	M. paraffinicum	1				1
III群菌	*M. avium*	27	13	7	3	4
	M. celatum	1				1
	M. intracellulare	3	1		2	
	M. terrae	6	2		3	1
IV群菌	*M. fortuitum*	3	1		1	1
	M. mucogenicum	1			1	
	M. peregrinum	2	1			1
	M. phlei	5	1	1		3
	合計	75	31	9	22	13

表３ 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

表４ *L. pneumophila*の血清群別亜種の同定結果

血清群	基準株と参照株 亜種同定結果	株数	国内分離株の亜種同定菌株数 *pneumophila*	*fraseri*	*pascullei*	合計 菌株数
1	*pneumophila*	1	10			11
2	*pneumophila*	1	7			8
3	*pneumophila*	1	9	1		11
4	*fraseri*	1	5	2		8
5	*fraseri* *pascullei*	1 3	16			20
6	*pneumophila*	1	8			9
7	*pneumophila*	1	7			8
8	*pneumophila*	1	8			9
9	*pneumophila*	1	7			8
10	*pneumophila*	1	6			7
11	*pneumophila*	1	2	2		5
12	*pneumophila*	1	7			8
13	*pneumophila*	1	6			7
14	*pneumophila*	1	2			3
15	*fraseri*	1	4			5
	合計	18	104	5	0	127

表５ *L. pneumophila* の国内分離109株における*pilE*遺伝子のタイプ

	*pilE*遺伝子タイプ															
亜種名	1	3	4	5	6	8	10	12	13	14	17	20	21	22	27	計
pneumophila	4	6	11	6	10	2	31	16	11	0	1	1	3	2	0	104
fraseri	0	0	0	0	0	0	0	0	0	4	0	0	0	0	1	5
計	4	6	11	6	10	2	31	16	11	4	1	1	3	2	1	109

表６　血清群１で*pilE*遺伝子のタイプ14と27の国内分離
計19株のＤＤＨ法による亜種の同定

菌株番号	*pilE* type	同定結果
NIIB0565	14	L. *pneumophila* subsp. *fraseri*
NIIB0583	14	L. *pneumophila* subsp. *fraseri*
NIIB0590	14	L. *pneumophila* subsp. *fraseri*
NIIB0591	14	L. *pneumophila* subsp. *fraseri*
NIIB0595	14	L. *pneumophila* subsp. *fraseri*
NIIB0694	14	L. *pneumophila* subsp. *fraseri*
NIIB0718	14	L. *pneumophila* subsp. *fraseri*
NIIB0741	14	L. *pneumophila* subsp. *fraseri*
NIIB0746	14	L. *pneumophila* subsp. *fraseri*
NIIB0778	14	L. *pneumophila* subsp. *fraseri*
NIIB1075	14	L. *pneumophila* subsp. *fraseri*
NIIB1077	14	L. *pneumophila* subsp. *fraseri*
NIIB1098	14	L. *pneumophila* subsp. *fraseri*
NIIB1207	14	L. *pneumophila* subsp. *fraseri*
NIIB1269	14	L. *pneumophila* subsp. *fraseri*
NIIB0847	14	L. *pneumophila* subsp. *fraseri*
NIIB0975	14	L. *pneumophila* subsp. *fraseri*
NIIB1147	14	L. *pneumophila* subsp. *fraseri*
NIIB2752	27	L. *pneumophila* subsp. *fraseri*

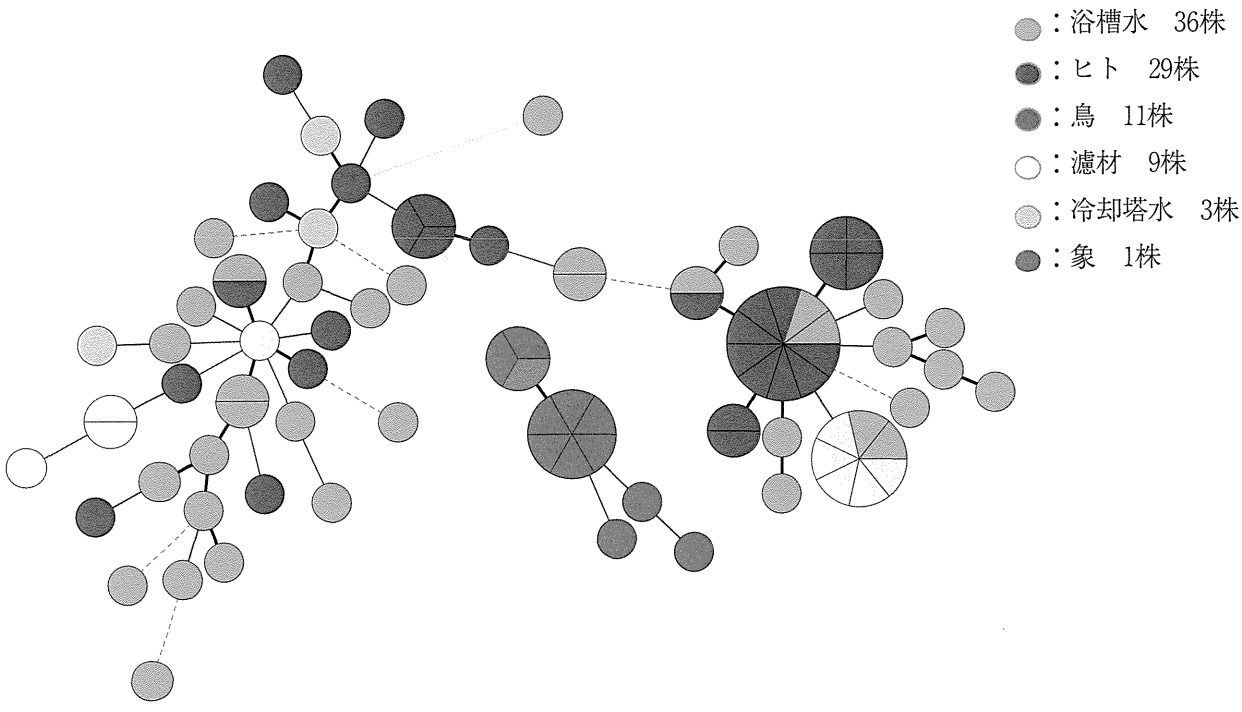

図１　分離源が異なる*M. avium*のminimum spanning tree (MST)

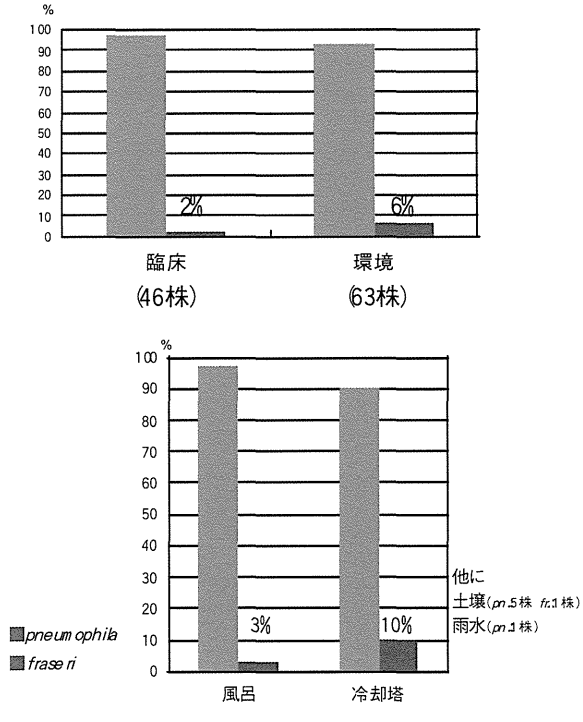

図２　国内分離株の由来別にみた亜種の分布

上）臨床分離株と環境分離株の亜種の内訳
下）環境分離株の由来別による亜種の内訳

レジオネラ属菌対策における宿主アメーバの管理

研究分担者　八木田健司
研究協力者　泉山　信司

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、病原細菌であるレジオネラ属菌等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。本研究では、レジオネラ属菌等共生微生物の宿主アメーバ感染あるいは共生における宿主特異性と、レジオネラ感染症の主要な感染源となる浴槽において宿主アメーバ自体を消毒する方法としてモノクロラミンの有用性を明らかにした。

研究方法

3年間の年次毎にまとめる。

1.　H22年度:レジオネラ属菌以外の微生物とアメーバの共生実態について

浴槽水、ハウスダストから分離した *Acanthamoeba* に関して、ギムザ染色で細胞内共生体を確認した株より共生体を含めた全DNA抽出を行った。共生体として報告のあるChlamydiales等に対するPCRを行い、シークエンス解析および系統解析により共生体を遺伝学的に特定した。

2.　H23年度:モノクロラミンの *Acanthamoeba* に対する不活化効果

Acanthamoeba sp.温泉浴槽水分離株を用いて、栄養体およびシストに対するモノクロラミンの増殖抑制作用を調べた。モノクロラミンは次亜塩素酸ナトリウム溶液と塩化アンモニウム溶液を用いて実験直前に調整した。3mg/Lおよび30mg/L調整したモノクロラミン溶液で所定の時間シストあるいは栄養体を暴露し、チオ硫酸ナトリウム溶液で中和後、大腸菌を用いて培養を行った。モノクロラミン暴露の強さはCT値(濃度・mg/Lx時間・分)で表した。培養は30℃で行い生きた栄養体の有無でアメーバの増殖性を評価した。

3.　H24年度:レジオネラ属菌遺伝子型とアメーバ感受性

土壌、浴槽水および冷却塔水より分離した *Acanthamoeba* sp.株と土壌および浴槽水より分離した *Vannella* sp.株に対し、土壌および冷却塔より分離した遺伝子型(ST)に基づく5つのグループ(C1、C2、S1、S2、およびS3)の *L. pneumophila* SG1株の感染性を調べた。アメーバ類は大腸菌を用いて30℃培養したものを、*L. pneumophila* はBCYEα培地にて30℃、3日間培養したものを用いた。*L. pneumophila* 株ごとに大腸菌と混合して無栄養寒天培地に塗布したものを試験培地とし、そこに供試アメーバ浮遊液を滴下した。大腸菌のみを塗布した培地を用いた条件を対照実験とした。20℃および30℃で培養を行い、アメーバの増殖性を生きた栄養体の有無で評価した。

研究結果

3年間の年次毎にまとめる。

1.　H22年度:レジオネラ属菌以外の微生物とアメーバの共生実態について

浴槽水分離アメーバからはParachlamydiaceae bacteriumCRIB38ならびに *Criblamydia sequanensis* に高い相同性を示す共生体が検出された(図1)。前者の共生体の宿主アメーバ株には、2002年の宮崎におけるレジオネラ集団感時に環境調査で対象施設より分離されたものも含まれた。ハウスダスト分離のアメーバからは *Neochlamydia* sp. CRIB37、Endosymbiont of *Acanthamoeba* sp. R18、Endosymbiont of *Acanthamoeba* sp. UWC22、*Propionibacterium acne*、*Methylophilus* sp.また *Sinorickettsia chlamys* と高い相同性を示す共生体が検出された。共生体のほとんどはアメーバ内に充満するような増殖形態は認められず、またアメーバ自体の脆弱性もみられなかった。

2. H23 年度:モノクロラミンの *Acanthamoeba* に対する不活化効果

短時間作用の結果としては、モノクロラミン 3mg/L、15 分間(平均 Ct 値:55)の作用でシストの生残性は対照と変わらなかったが、30mg/L で 15 分間(平均 Ct 値:444)また 60 分間(平均 Ct 値:1728)の作用では 1000 シスト培養条件でも増殖抑制が認められ、Ct 値 1700 で 3-log 以上のシスト不活化が生じることが示唆された。一方栄養体の場合は、3mg/L、15 分間の作用の結果から、Ct 値 45 程度で 3-log 以上の不活化が生じることが示唆された。長時間作用の結果としては、3mg/L、5 時間(Ct 値:810)作用ではシストの生残性の低下は認められなかったが、24 時間(Ct 値:3700)の長時間作用では 3-log 以上のシスト不活化が生じることが示唆された(表1)。

3. H24 年度:レジオネラ属菌遺伝子型とアメーバ感受性

Acanthamoeba sp.株は土壌分離 9 株、温泉浴槽水分離 11 株、冷却塔水 4 株について *L. pneumophila* 5 つのグループ(各 2 菌株)を試験した。20℃および 30℃培養条件において、ほとんどすべての *Acanthamoeba* sp.株は死滅したが、土壌株の一つはほとんどすべての菌株に抵抗性(アメーバは増殖)を示し、また温泉浴槽水の中の 3 株が土壌由来の 1 菌株(S1)に抵抗性を示した(表-2)。全体としてレジオネラ属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なかった。一方、浴槽水分離の *Vannella* sp.株に関しては、アメーバ株特異性が見られた。30℃培養条件において土壌分離の株はすべての菌株に感受性を示し、浴槽水分離の中の 2 株はすべての菌株・菌グループに抵抗性を示した。浴槽水分離の他の 1 株は冷却塔の C2 グループ 2 菌株に対して抵抗性を示したものの他のグループでは死滅した。

考 察

環境中のアメーバを宿主とする病原細菌としては現在レジオネラ属菌が知られる。これに加え近年アメーバを宿主とする *Parachlamydia* がヒトの呼吸器疾患に関連することが示唆されている。今回検出された Parachlamydiaceae bacteriumCRIB38 はその近縁関係にあり(図-2)、浴槽環境より検出されたことを考えると、国内においても本菌種を始めレジオネラ属菌と同様な呼吸器疾患間連の微生物に関する汚染への留意が必要と考えられる。今後は *Parachlamydia* 科の共生体を中心に、アメーバ共生体のヒトの健康に及ぼす影響に関するエビデンスの蓄積が重要と考えられる。

一方、レジオネラ属菌に関して遺伝子型による環境中の分布に宿主アメーバが関連している可能性があり、実験的相互感染させることで菌の遺伝子型とアメーバの分離環境との関係を調べた。限定された培養温度条件の結果としては、レジオネラ属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、レジオネラ属菌の遺伝子型分布に宿主アメーバが関連する証左は得られなかった。この点に関しては、土壌環境を考えると低温(10−15℃)での試験も重要と考えられる。さらに低温でのアメーバ細胞内増殖性が菌グループにより異なる可能性を検証する。またこれまで研究の進んでいなかった水系環境では一般的な *Vannella* についても調べた結果、株により菌の遺伝子型との関連があり得るということが示唆され今後の検証となった。なお、*Vannella* はシスト化しない性質から環境中で常に菌を増殖させる宿主の可能性があることが指摘された。

宿主アメーバの衛生管理手法の確立に関しては、モノクロラミンの暴露実験より安全性が明らかでかつ実用濃度とされる 3mg/L の条件で、*Acanthamoeba* 栄養体に加え従来薬剤消毒が困難であったシストに対しても、24 時間の作用、Ct 値 3700 が維持される条件で 3log 程度の不活化が期待できる結果を得た。実際の浴槽環境ではバイオフィルムの問題があり、今回の実験結果と同様のモノクロラミンの有効性を見込むことは難しいが、浴槽中でのアメーバ抑制と遊離する菌の消毒により、根本的なレジオネラ属菌汚染の抑制が期待される。モノクロラミンの試験導入を行っている施設でアメーバの検出率が極めて低いという調査データ等を考慮すると、モノクロラミン消毒方法が今後の浴槽衛生管理の有効な手段となるのではないかと考えられる。

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限らず、様々な微生物の宿主として *Acanthamoeba* を始めとする自由生活性アメーバが存在し、レジオネラ感染症のみならずこれまで明らかとなっていない疾患に関わっている可能性もあり、今後の研究が必要である。これらの宿主アメーバを消毒コントロールする手法が重要となるが、浴槽水環境ではこれを根本的に除去することが重要であり、モノクロラミンにその実用的な作用が認められた点は今後の衛生管理の改善につながる結果である。

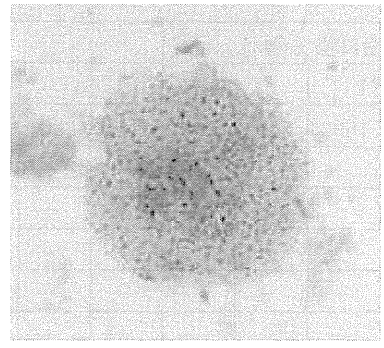

図-１、浴槽水より分離された *Acanthamoeba* sp.細胞内に
検出された共生体（T49 symbiont）

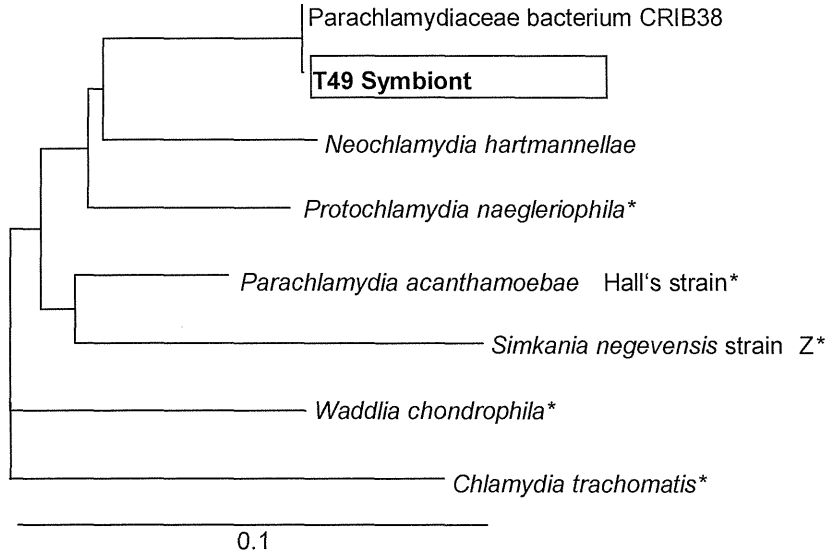

図-2、16SrRNA 遺伝子解析に基づくクラミジア関連微生物の系統関係

＊ これまでにヒトへの健康影響が知られるもの

表-1、モノクロラミン 24 時間作用試験結

	実 Ct 値	シスト数(プレート当たり接種量)			
消毒条件		10	100	1000	
0mg/L 0 分	0	* 3 / 3	3 / 3	3 / 3	対照
0mg/L 24 時間	0	3 / 3	3 / 3	3 / 3	
3mg/L 5 時間	810	3 / 3	3 / 3	3 / 3	
3mg/L 24 時間	3700	0 / 3	0 / 3	0 / 3	Ct 値 3700 で 3-log 以上の不活化

＊アメーバ増殖プレート数 / 試験プレート数

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 (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	—	—	—	—	—	—	—	—	—	—	+

—：アメーバ増殖なし、±：アメーバ増殖するが菌感染認める、

＋：アメーバ増殖、菌感染認めず

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	78(12)	4263-4270	2012
Taguri T, Oda Y., Sugiyama K., Nishikawa T, Endo T, Izumiyama S, Yamasaki M., and Kura F	A rapid detection method using flow cytometry to monitor the risk of legionellosis in bath water.	Journal of Microbiological Methods	86(1)	25-32	2011
Matsui M, Fujii S, Shiroiwa R, Amemura-Maekawa J, Chang B, Kura F, Yamauchi K	Isolation of *Legionella rubrilucens* from a pneumonia patient co-infected with *Legionella pneumophila.*	Journal of Medical Microbiology	59(10)	1242-1246	2010
Amemura-Maekawa J, Kura F, Helbig JH, Chang B, Kaneko A, Watanabe Y, Isobe J, Nukina M, Nakajima H, Kawano K, Tada Y, Watanabe H, and Working Group for *Legionella* in Japan	Characterization of *Legionella pneumophila* isolates from patients in Japan according to serogroups, monoclonal antibody subgroups and sequence types.	Journal of Medical Microbiology	59(6)	653-659	2010
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前川純子、倉　文明	レジオネラ感染の分子機構と診断法の進歩	呼吸	30(2)	124-128	2011
倉　文明、常　彬、前川純子	レジオネラの環境中での生態とその迅速検出	化学療法の領域	26(12)	2385-94	2010
杉山寛治、小坂浩司、泉山信司、縣　邦雄、遠藤卓郎	モノクロラミン消毒による浴槽レジオネラ属菌の衛生対策	保健医療科学	59(2)	109-115	2010

IV. 研究成果の刊行物・別刷

J Infect Chemother
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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

Jun-ichi Kanatani · Junko Isobe · Keiko Kimata · Tomoko Shima · Miwako Shimizu · Fumiaki Kura · Tetsutaro Sata · Masanori Watahiki

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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

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

J. Kanatani · J. Isobe · K. Kimata · T. Shima · M. Shimizu · T. Sata · M. Watahiki (✉)
Department of Bacteriology, Toyama Institute of Health, 17-1 Nakataikoyama, Imizu, Toyama 939-0363, Japan
e-mail: masanori.watahiki@pref.toyama.lg.jp

F. Kura
Department of Bacteriology I, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

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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 %.

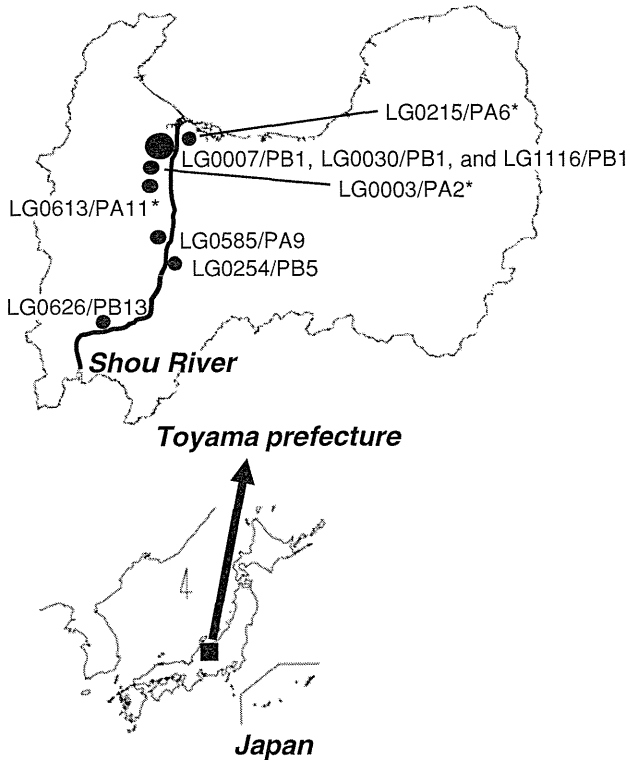

Fig. 1 Geographic distribution of ST505 strain. *Isolate name* indicates the strain/origin as described in Table 1. The *size of the circle* indicates the number of isolates. *Asterisk* indicates the clinical isolates associated with bathwater by epidemiological investigation

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

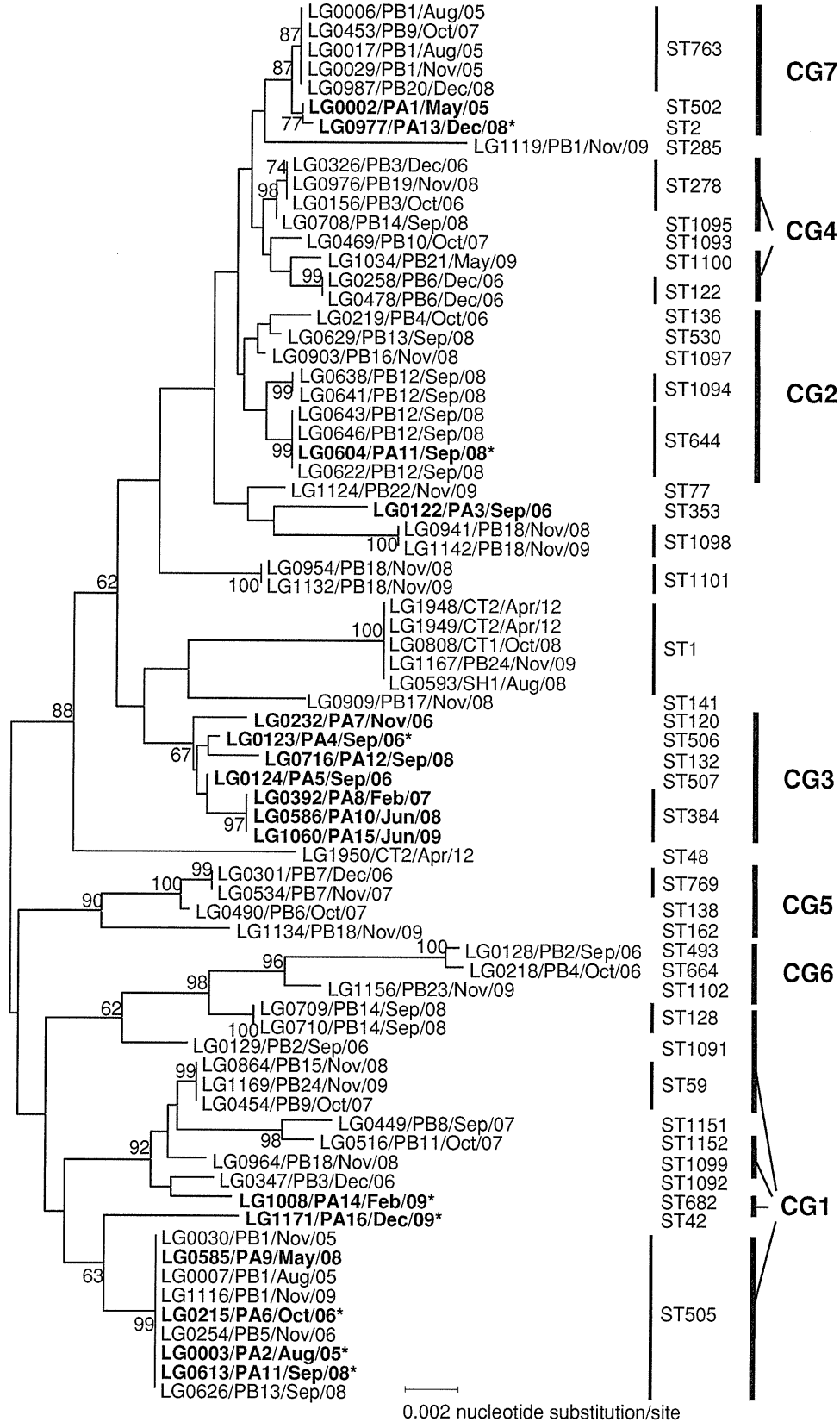

Fig. 2 Phylogenetic analysis of the concatenated sequences (*flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, *neuA*) of *Legionella pneumophila* SG 1 isolates in this study. *Isolate name* indicates the strain/origin/month/year as described in Table 1. *Isolates in boldface* are from patients. *Asterisk*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

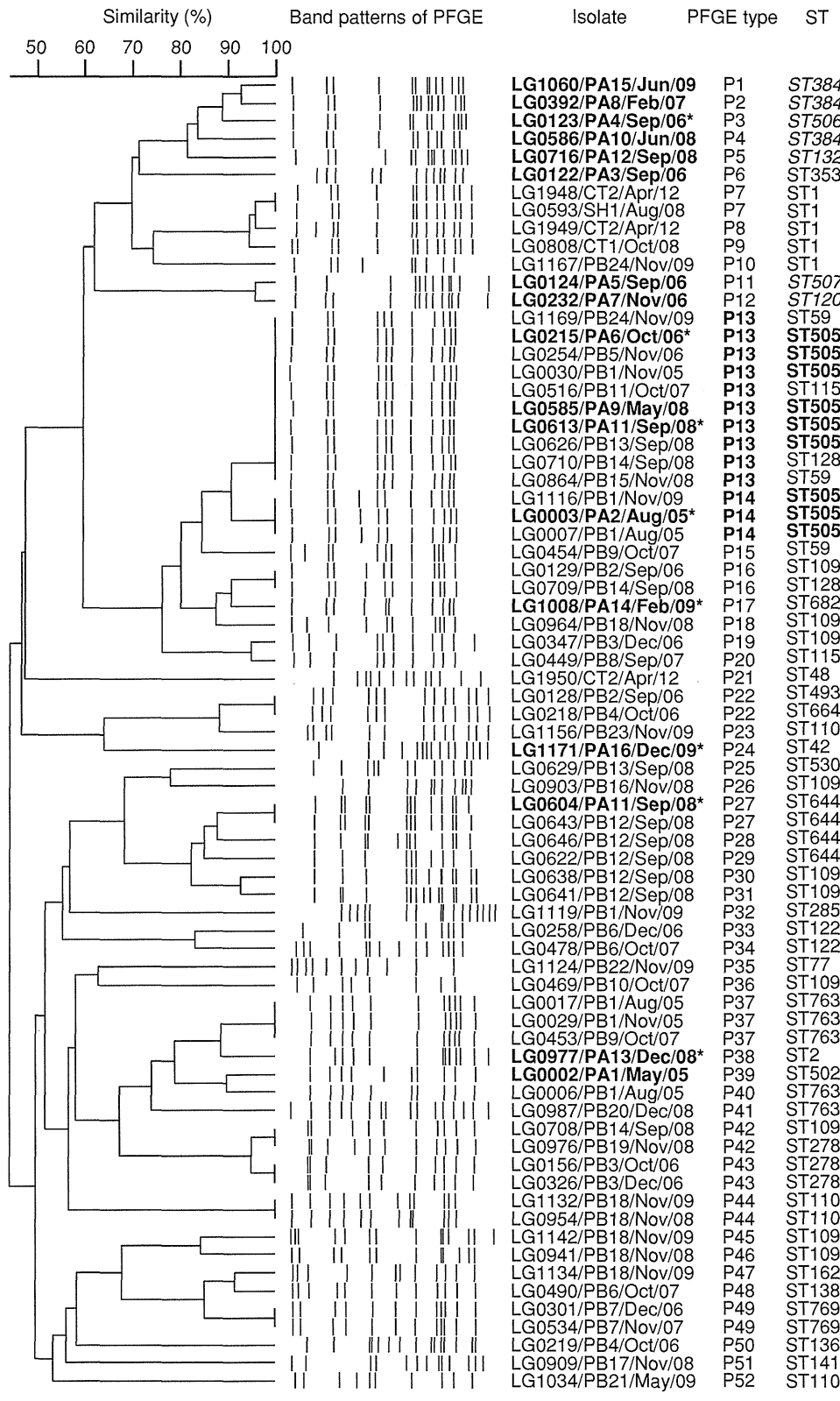

Fig. 3 *Dendrogram* of the pulsed-field gel electrophoresis (PFGE) pattern constructed from *L. pneumophila* SG 1 isolates in this study. *Isolate name* indicates the strain/origin/month/year as described in Table 1. *Isolates in boldface* are from patients. *Asterisk* indicates the clinical isolates 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

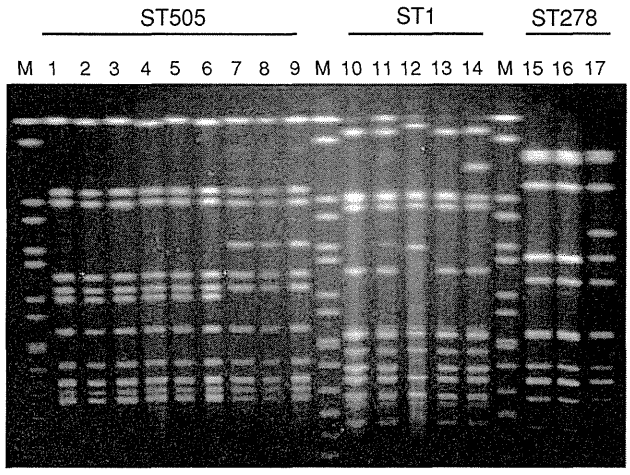

Fig. 4 PFGE patterns with *SfiI* digestion of *L. pneumophila* SG 1 isolates. *Lanes*: *M Salmonella enterica* serovar Braenderup H9812 strain digested with *XbaI* as a size marker, *1* LG0215, *2* LG0254, *3* LG0030, *4* LG0613, *5* LG0585, *6* LG0626, *7* LG0003, *8* LG0007, *9* LG1116, *10* LG0593, *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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