一致した環境由来の菌は，ミルクの調製に使用した給水機からのみ分離され，粉ミルクから調整したミルクの誤嚥による肺炎と考えられた。菌数はそれぞれ22,000cfu/L，200cfu/Lであった。その後，給水／給湯機は，給湯機に置き換えられ，感染は起きていない。

その他，散発感染事例（セルフサービスの自動車の洗浄設備，電話線のマンホール）とその対策は別の文献を参照されたい[10]。なお，国内では1999年に家庭における循環式浴槽利用の水中出産による新生児感染事例があったが[9]，海外では，同年1999年のイタリアの病院における水中出産感染事例の他，2014年に，英国や米国の家庭での感染事例[15]が報告された。英国では，水中出産において，ヒーターとポンプ付きの浴槽の使用停止が勧告された。

6.2 集団感染事例

海外の大規模な集団感染事例では，入浴設備による感染は少数で，冷却塔を感染源とするものが多い。50名以上の患者の報告された事例は少なくとも34件あり，感染源は，冷却塔・空調関連16件（47%），渦流浴等循環式浴槽6件（18%），噴水と掘削各2件，冷却潤滑液，加湿器，給湯設備各1件，不明が5件となっている。冷却塔・空調関連が多いのが海外の特色である。ポンティアック熱の集団発生も8件あった。起因菌が特定され報告された集団発生のうち，24/29（83%）が*L. pneumophila* SG1による。

表7には2000年以降の事例を示した。17事例の内，12事例が冷却塔や空調設備が感染源として報告されている。確定症例数の多かった上位4つの集団感染事例は1事例当り179症例～449症例でスペイン，ポルトガル，カナダ，英国で発生した。

一例として，最近のポルトガルVila Franca de Xiraの事例を詳しく紹介する[16]。11月7日に，24時間以内に2つの病院に18人の患者が入院したという届出があり集団感染が検知された。緊急対応として翌8日に消毒のための塩素濃度が上げられ，公衆プール，循環風呂，噴水の稼働が停止された。確定症例334例，内10人死亡（さらに2死亡例を調査中）でポルトガルで最大の集団感染事例となった。患者の67%が男性で，年齢中央値58歳（25-92歳），すべて尿中抗原陽性であった。Sequence-based typingによる起因菌の遺伝子型はST1905（我々の分類で冷却塔C2グループ，文献17）が患者12例の検体から分離され，環境分離株とST（sequence type）が一致した。可能性の高い感染源は，産業用の水冷式冷却塔であった。最初の患者は，2週間前に冷却塔の維持管理に従事していた。この地域では，10月18日～11月1日

表7　最近の海外のレジオネラ症大規模集団感染事例

	年月	国名	施設	感染源	患者数	確定症例数	死亡者数	
1	2000	オーストラリア	水族館	冷却塔		125	4	LpSG1
2	2000	スペイン		冷却塔		54	3	LpSG1
3	2001	スペイン	病院	冷却塔	>800	449	6	LpSG1
4	2002	米国	レストラン	装飾用噴水	117	11	0	L.anisa
5	2002	米国	ホテル	渦流浴(循環式)	50	0	0	L.micdadei
6	2002	英国	娯楽センター	空調設備		179	7	LpSG1
7	2002	スペイン	製氷工場	冷却塔	151	113	2	LpSG1
8	2003-2004	フランス	工場	冷却塔		86	18	LpSG1
9	2004	米国	ホテル	(循環式)	107	>30	0	LpSG1
10	2005	ノルウェー	リグニン製造工場	空気洗浄のための冷却設備		55	10	LpSG1
11	2006	スペイン	市センター	冷却塔		146	0	LpSG1
12	2006	英国	レジャー施設	渦流浴(循環式)	118	5	0	LpSG1
13	2007	ロシア	町の給水設備	給水設備	130	74	5	LpSG1
14	2009-2010	ドイツ	醸造排水処理プラント	冷却塔		65	5	LpSG1
15	2012	英国	蒸溜所？	冷却塔？	101	53	3	LpSG1
16	2012	カナダ	事務所用ビル	冷却塔		182	13	LpSG1
17	2014	ポルトガル	？	冷却塔	417	334	10+2？	LpSG1

Pontiac fever，少数の肺炎も

には2～3m/sの北東の風が吹いていて，それに沿って患者が発生分布した。

その他，集団感染事例とその対策事例のうち，米国のレストランの噴水，スペインの道路の切削機と散水，ノルウェーの木材の生物処理工場の酸化池と河川の汚染については，文献10で紹介した。キプロスの病院で発生した超音波加湿器による集団感染事例，英国の小売店に展示された循環式浴槽による集団感染事例，米国の病院玄関ロビーの修景水による感染事例，英国エディンバラで冷却塔が感染源と疑われる集団感染事例，2012年カナダ最大の冷却塔による集団感染事例については文献13を参照されたい。

7. 外部精度管理

培養法による水中のレジオネラ属菌の検査は行政検査として保健所等で，また自主検査として民間検査機関等で実施されている。適切に検査されず非検出あるいは検出される菌数が少ない検査機関に検体が集まることがないように，検査機関の外部精度管理が必要であるが，日本では今のところ研究班以外にレジオネラの外部精度管理は実施されていない。一方，ヨーロッパ等ではHealth Protection Englandにより，米国では米国CDCにより実施されている。研究班では，レジオネラ属菌の培養検査法の安定化に向けた取り組みとして，1）精度管理，2）標準的検査法，3）研修システムの3点を柱とし，レジオネラ属菌検査精度管理ワーキンググループ（以下WG）内で検討を行ってきた。平成25年度から微生物定量試験用標準菌株の販売を行っているシスメックス・ビオメリュー社のBioBall（特注品）を利用することにより，配付試料にメーカー保証が得られ，また，メーカーによる商品が発送されることから，多施設へ安定した輸送が可能となった。平成26年度は，全国41の地方衛生研究所を対象として，WG推奨法を指定して実施した[18]。非濃縮試料及び，未処理による検査工程を加えたことにより全体として菌の検出数の良好目標範囲に入る割合が増加した。

一方で，供試菌は，酸処理や熱処理，さらには選択分離培地により発育が抑制される場合があった。今後の外部精度管理においては，検査工程のどの部分に重きを置くかの定義付けを適切に行い，実検体検査に対する注意点等については研修会で対応することを想定している。

8. 分子疫学

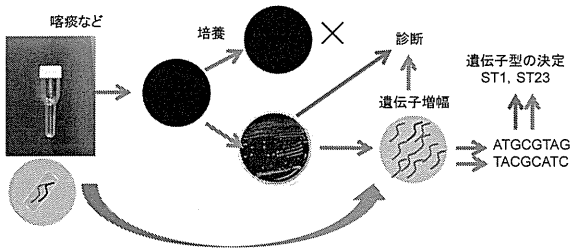
*L. pneumophila*の生息環境により，菌の遺伝子型に違いがあることが明らかになってきた[17]。7つの遺伝子の一部領域をPCRで増幅し，遺伝子配列を決定し型別する。MLST（multilocus sequence typing）法と同一の手法だが，病原性に関係する遺伝子も用いているため，SBT（sequence-based typing）法と称している。The European Working Group for Legionella Infectionsにより検査法が設定されている（http://www.hpa-bioinformatics.org.uk/legionella/legionella_sbt/php/sbt_homepage.php）。たとえば，（*flaA, pilE, asd, mip, mompS, proA, neuA*）が（2,3,9,10,2,1,6）の場合，ST23と名称がつけられている。

この手法を用いると，これまでパルスフィールドゲル電気泳動（PFGE）の泳動パターンで菌株が比較されてきたところ，数字で分類できるようになり，世界中の施設で型別された菌株が容易に比較できるようになった。

我々は，SBTにより，血清群1の国内分離株を遺伝子型でグループ分けすると，浴槽水分離株が多く含まれるB1，B2，B3，冷却塔水分離株が多く含まれるC1，C2，土壌・水溜まり分離株が多く含まれるS1，S2，S3，感染源不明の臨床分離株が多いUグループの大きく9つに分かれることを見出した。これらのグループは感染源の推定に利用できる。冷却塔水分離株は，浴槽水分離株や土壌分離株とは異なり，STが比較的均一であった[17]。臨床分離株のminimum spanning treeについての最新情報はレジオネラ・レファレンスセンター報告として，衛生微生物技術協議会の毎年の年次大会で報告されている。平成26年度は*L. pneumophila*の臨床分離株308株が151のSTに分かれたこと，多く分離されたST，集団感染との関係，日本固有のST，296株の*L. pneumophila* SG1のSTの関係と分離数を示すminimum spanning treeが記載されている（http://www.nih.go.jp/niid/images/lab-manual/reference/H26_Legionnaires.pdf）。現在では，水溜まり[19]，シャワー水由来の菌のSTも調査されている[2]。一方，修景水，給水/給湯水，加湿器由来株のSTは今後の調査が待たれる。

感染源の調査には，これまで環境由来と患者由来の菌株を培養法によって分離し，パルスフィールドゲル電気泳動で比較する必要があった。しかし，現在では

ST の多様性が明らかとなり，菌株識別率がパルスフィールドゲル電気泳動と同程度であることから，菌株を分離しないで直接感染源を推定する方法が，実施されるようになった（図 5）。菌株が得られない場合や，迅速な集団感染の予測のために有用である。



（レジオネラ肺炎の診断と疫学におけるレジオネラ・ニューモフィラの遺伝子増幅と遺伝子型の決定）

図 5　菌を分離しなくてもできる新しい感染源の調査法

9. 防止対策

遊離塩素消毒を行っているにも関わらず浴槽からレジオネラ属菌が検出されることがあり，遊離塩素消毒が全ての浴槽の安全を担保するとは言い難い。その原因として，井水や温泉水など多様な水質の存在，高 pH の条件下では遊離塩素消毒の効果が期待できないこと，アンモニアを含む温泉水に遊離塩素を添加した場合に遊離塩素濃度は速やかに低下する，等が考えられる。そこで，研究班では，米国の水道で実用化されているモノクロラミン（結合塩素の一種）消毒に着目し，モノクロラミン消毒の入浴施設への応用について検討を行なった。施設における実施例を含めたモノクロラミン消毒の有用性については総説にまとめられているので参照されたい[20]。

レジオネラ症対策のまとめを図 6 に示した。「つけない，増やさない，吸い込ませない」という基本が重要である。増やさないという点については，改正された「循環式浴槽におけるレジオネラ症防止対策マニュアル」で，モノクロラミン消毒が公知のこととなったことは特筆される[4]。今後，全国でモノクロラミン消毒を取り入れた条例に改定されていくものと思われる。さらに，「レジオネラ症対策のてびき」[21]は，環境衛生監視員や旅館業・公衆浴場業等の衛生管理担当者双方にとって入浴施設の日常の衛生管理や，保健所の開催する衛生管理講習会の資料として有用である。また，レジオネラ症防止指針も基本的な文献である[22]。

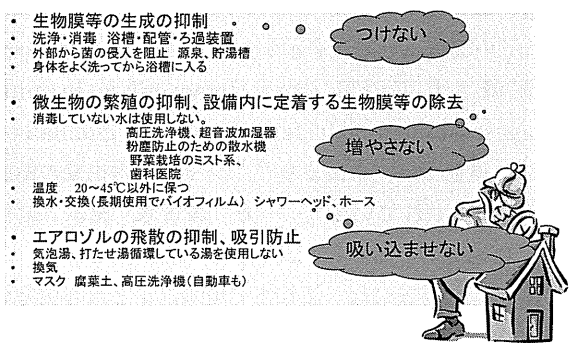


図 6　レジオネラ症防止対策の基本　まとめ

10. 謝辞

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Note

Detection of *Legionella* Species in Environmental Water by the Quantitative PCR Method in Combination with Ethidium Monoazide Treatment

HIROAKI INOUE[1]*, TOMOKO TAKAMA[1], MIWA YOSHIZAKI[2], AND KUNIO AGATA[1]

[1] *Tsukuba Research Laboratories, Aquas Corporation, 4-4 Midorigahara, Tsukuba, Ibaraki 300-2646, Japan*
[2] *SCM Center, Takara bio Inc., 3-4-1 Seta, Otsu, Shiga 520-2193, Japan*

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We detected *Legionella* species in 111 bath water samples and 95 cooling tower water samples by using a combination of conventional plate culture, quantitative polymerase chain reaction (qPCR) and qPCR combined with ethidium monoazide treatment (EMA-qPCR) methods. In the case of bath water samples, *Legionella* spp. were detected in 30 samples by plate culture, in 85 samples by qPCR, and in 49 samples by EMA-qPCR. Of 81 samples determined to be *Legionella*-negative by plate culture, 56 and 23 samples were positive by qPCR and EMA-qPCR, respectively. Therefore, EMA treatment decreased the number of *Legionella*-positive bath water samples detected by qPCR. In contrast, EMA treatment had no effect on cooling tower water samples. We therefore expect that EMA-qPCR is a useful method for the rapid detection of viable *Legionella* spp. from bath water samples.

Key words : Bath / Cooling tower / Ethidium monoazide (EMA) / *Legionella* / Quantitative polymerase chain reaction (qPCR).

Legionella species are Gram-negative bacteria that inhabit man-made water environments such as bath and cooling tower water. Inhalation of aerosolized water from *Legionella* contaminated sources can result in a severe form of pneumonia called Legionnaires' disease (Vogel and Isberg, 1999). Therefore, the control of *Legionella* contamination in water systems is very important, and the effectiveness of the treatment to control it is evaluated by monitoring for the presence of *Legionella*.

In general, the conventional plate culture method has been used for the detection of *Legionella* spp. from environmental water samples. However, more than 1 week is needed to obtain results because growth of *Legionella* spp. on the selective agar is very slow. In contrast, gene amplification methods such as polymerase chain reaction (PCR) or loop-mediated isothermal amplification (LAMP) detect *Legionella* spp. within a few hours (Furuhata et al., 2005; Inoue et al., 2004a). However, the number of *Legionella*-positive samples detected by the PCR and LAMP methods is higher than that by the plate culture method, because gene amplification methods detect not only viable, but also dead *Legionella* (Ng et al., 1997).

Ethidium monoazide (EMA) treatment is known to

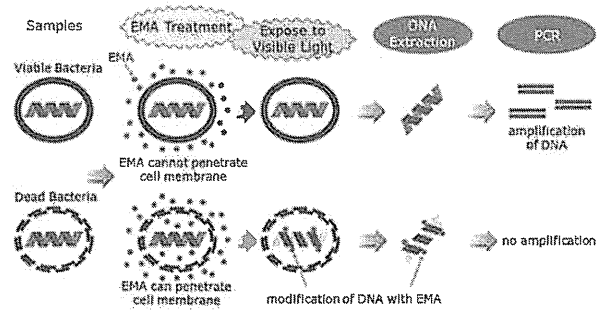

FIG. 1. The principle of ethidium monoazide (EMA) treatment on bacterial cells. This figure was modified and reprinted by courtesy of Takara Bio Inc. from the catalogue of the company.

*Corresponding author. Tel: +81-29-847-6000, Fax: +81-29-847-6080, E-mail : h_inoue0417(a)aquas.co.jp

eliminate the PCR amplification of DNA in dead cells (Nogva et al., 2003). The principle of EMA treatment is shown in FIG. 1. EMA is a DNA-intercalating dye that can penetrate membrane-damaged cells such as dead cells, form covalent links with DNA, and cleave to genomic DNA into pieces following exposure to visible light. EMA-treated DNA is not a target for amplification by PCR, and therefore viable *Legionella* are selectively detected by PCR when combined with EMA treatment. The effect of EMA treatment on *Legionella* detection by PCR has been published (Chang et al., 2009; Chang et al., 2010; Chen et al., 2010; Delgado-Viscogliosi et al., 2009; Qin et al., 2012).

In this paper, we report a comparison of the detection of *Legionella* spp. by the plate culture method, quantitative PCR (qPCR) method and qPCR method in combination with EMA treatment (EMA-qPCR).

All water samples from different baths (111 samples) and cooling towers (95 samples) were collected between September and December 2012. These samples were collected in sterile 500-ml polypropylene bottles with sodium thiosulfate, and were examined as soon as possible.

Legionella spp. were detected according to the standard method (ISO11731, 1998). That is, collected water samples were concentrated 100-fold by centrifugation (6400×g, 30 min) or filtration (pore size: 0.45 μm, cellulose acetate, Advantec, Japan). A portion of the concentrated samples (500μl) was pretreated with acid-phosphate buffer (Inoue et al, 2004b), and inoculated onto GVPC selective agar plates (Merck, Japan). The plates were incubated at 37℃ for 6 to 8 days, and the colonies of *Legionella* spp. that grew on GVPC selective agar plates were enumerated. The detection limit of this method is 10 CFU/100 ml. The species of the isolated *Legionella* strains (up to 10 strains per sample) were identified by using the immune serum aggregation assay (Denka Seiken, Japan) and the DNA-DNA hybridization assay (Kyokuto Pharmaceutical Industrial, Japan). *Legionella* strains that could not be identified by the DNA-DNA hybridization assay were identified by evaluating the 16S rRNA gene partial sequences.

Viable *Legionella* Selection Kit for PCR Ver. 2.0 (Takara Bio, Japan) was used as an EMA reagent. EMA treatment was carried out according to the manufacturer's instructions. The 100-fold concentrated 1 ml samples were further concentrated to a final volume of 40μl. To these samples were added 10μl of reaction buffer, 2.5μl of dilution buffer and 2.5μl of EMA reagent. The samples were then mixed gently using a vortex type mixer, and incubated in the dark for 15 min at room temperature. Subsequently, the samples were exposed to visible light for 15 min using a LED Crosslinker 12 (Takara Bio, Japan). After EMA treatment, *Legionella* DNA was extracted and purified using NucleoSpin Tissue XS (Takara Bio, Japan) according to the manufacturer's instructions. For each sample, 20 μl of purified DNA solution was obtained. In addition, purified DNA solutions that had not been treated with EMA were prepared as controls.

Cycleave PCR *Legionella* (16S rRNA) Detection Kit (Takara Bio, Japan) was used as a qPCR reagent. Reaction mixtures for qPCR were prepared in 0.2 ml PCR tubes according to the manufacturer's instructions. Purified DNA template solutions (5μl) were added to the prepared reaction mixtures (20μl), and were subject to PCR amplification in the Thermal Cycler Dice Real Time System *II* (Takara bio, Japan). The PCR program parameters were: initial denaturation step of 10 s at 95℃ followed by 45 cycles of denaturation for 5 s at 95℃, annealing for 10 s at 55℃, and extension for 20 s at 72℃. DNA amplification was detected by monitoring the fluorescence at 2 wavelengths (FAM and ROX). The amplified 16S rRNA gene of *Legionella* and the internal control gene were detected by FAM and ROX, respectively. The samples containing no amplified DNA or late amplified internal control DNA due to the

TABLE 1. Distribution of *Legionella* counts by the plate culture method, and the detection results by qPCR and EMA-qPCR methods from bath water samples.

Legionella counts [a] (CFU/100ml)	No. of samples	qPCR		EMA-qPCR	
		Positive	Negative	Positive	Negative
Less than 10	81(73.0%)	56(65.9%)	25(96.2%)	23(46.9%)	58(93.5%)
10-40	14(12.6%)	13(15.3%)	1(3.8%)	10(20.4%)	4(6.5%)
50-90	2(1.8%)	2(2.4%)	0(0%)	2(4.1%)	0(0%)
100-990	11(9.9%)	11(12.9%)	0(0%)	11(22.4%)	0(0%)
1000-9900	3(2.7%)	3(3.5%)	0(0%)	3(6.1%)	0(0%)
Subtotal	30(27.0%)	29(34.1%)	1(3.8%)	26(53.1%)	4(6.5%)
Total	111(100%)	85(100%)	26(100%)	49(100%)	62(100%)

[a] Measured by the plate culture method

TABLE 2. Distribution of *Legionella* counts by the plate culture method, and the detection results by qPCR and EMA-qPCR methods from cooling tower water samples.

Legionella counts [a] (CFU/100ml)	No. of samples	qPCR		EMA-qPCR	
		Positive	Negative	Positive	Negative
Less than 10	55(67.1%)	53(66.2%)	2(100%)	53(66.2%)	2(100%)
10-100	13(15.9%)	13(16.2%)	0(0%)	13(16.2%)	0(0%)
100-990	7(8.5%)	7(8.8%)	0(0%)	7(8.8%)	0(0%)
1000-9900	4(4.9%)	4(5.0%)	0(0%)	4(5.0%)	0(0%)
10000-99000	3(3.7%)	3(3.8%)	0(0%)	3(3.8%)	0(0%)
Subtotal	27(32.9%)	27(33.8%)	0(0%)	27(33.8%)	0(0%)
Total	82(100%)	80(100%)	2(100%)	80(100%)	2(100%)

[a] Measured by the plate culture method

presence PCR inhibitors were eliminated from the data analysis.

TABLE 1 shows the results of *Legionella* spp. detection from bath water samples by the plate culture, qPCR and EMA-qPCR methods. *Legionella* spp. were detected in 30 (27%) of 111 samples by the plate culture method, and all strains tested were identified as *Legionella pneumophila* (30/30 samples, 100%). In the case of bath water samples, the internal control gene was amplified from all samples tested, indicating the absence of PCR inhibitors. Of 81 samples that were *Legionella*-negative by the plate culture method, 56 and 23 samples were positive by the qPCR and EMA-qPCR method, respectively. That is, EMA treatment prior to qPCR reduced the number of *Legionella*-positive bath water samples by half. Out of 30 *Legionella*-positive samples identified by the plate culture method, one sample (10 CFU/100 ml) and four samples (10, 20, 20 and 30 CFU/100 ml) were judged *Legionella*-negative by the qPCR and EMA-qPCR method, respectively. In addition, one *Legionella*-negative sample by the qPCR method changed to *Legionella*-positive by the EMA-qPCR method. It was thought that these inconsistencies were errors involving *Legionella* counts near the detection limit of the qPCR and EMA-qPCR methods.

TABLE 2 shows the results of *Legionella* detection from cooling tower water samples by the plate culture, qPCR and EMA-qPCR methods. We tested 95 samples, but were unable to detect *Legionella* in one sample due to the overgrowth of non-target microorganisms on GVPC agar plates, and were unable to amplify internal control DNA from 12 samples due to the presence of residual PCR inhibitors. Therefore, these 13 samples were eliminated from the data analysis. *Legionella* spp. were detected in 27 (33%) of 82 samples by the plate culture method, and these strains tested were identified as *L. pneumophila* (20/27 samples, 74%), *L. pneumophila* and *L. quinlivanii* (1/27, 4%), *L. pneumophila* and *L. gratiana* (1/27, 4%), *L. feeleii* (2/27, 7%), *L. busanensis* (1/27, 4%), and *Legionella* sp. (2/27, 7%). Of 55 samples that were found to be *Legionella*-negative by the plate culture method, 53 samples were positive in both the qPCR and EMA-qPCR methods. That is, no effect of EMA treatment was observed.

Legionella counts by the plate culture method and *Legionella* 16S rRNA gene copies by the qPCR method were plotted as scatter diagrams (FIG. 2 and FIG. 3). No correlation for both the bath water and cooling tower water samples was seen. Therefore, it seems that

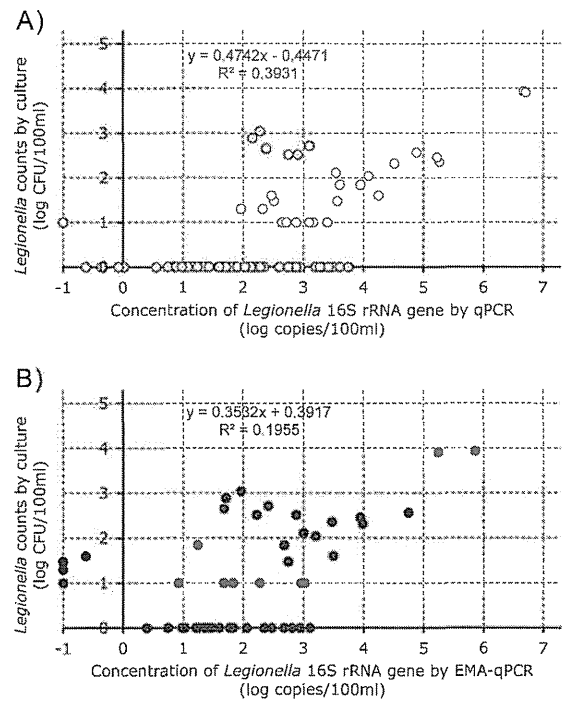

FIG. 2. Comparison of the results of the detection of *Legionella* by the plate culture and qPCR (A) or EMA-qPCR (B) methods from bath water samples.

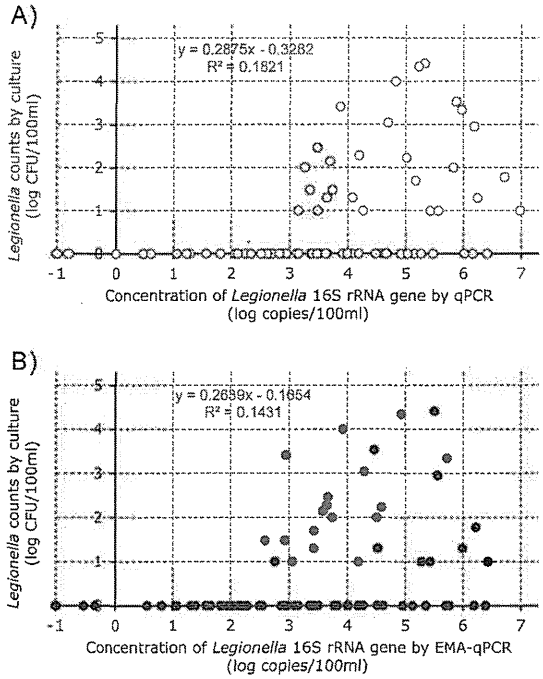

FIG. 3. Comparison of the results of the detection of *Legionella* by the plate culture and qPCR (A) or EMA-qPCR (B) methods from cooling tower water samples.

Legionella counts by the plate culture method cannot be expected based on the results of EMA-qPCR method.

In this study, our experimental data indicate that the EMA-qPCR method is useful for the rapid detection of viable *Legionella* spp. from bath water samples. In contrast, the effect of EMA treatment was not recognized in cooling tower water samples. The cause of the lack of effect of EMA treatment on cooling tower water samples is unknown, but we propose that the inhibition of EMA treatment could have been due to the water quality and/or the sludge in samples, and/or the presence of viable but nonculturable (VBNC) *Legionella*. Further research will be necessary to characterize the inhibition of EMA treatment in cooling tower water samples by using Control Test Kit (Viable Bacteria Selection, Takara Bio, Japan), because the sludge in the samples will especially inhibit transmission of the visible light. We expect that the presence of VBNC *Legionella* in environmental water is an important factor in the difference in the results between EMA-qPCR and the plate culture method, because VBNC *Legionella* are detected by the EMA-qPCR method but not by the plate culture method. The analysis of VBNC *Legionella* in environmental water is now in progress.

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MICROBES AND ENVIRONMENTS

Short Communication

Molecular Characterization of Viable *Legionella* spp. in Cooling Tower Water Samples by Combined Use of Ethidium Monoazide and PCR

HIROAKI INOUE[1,2]*, REIKO FUJIMURA[2], KUNIO AGATA[1], and HIROYUKI OHTA[2]

[1]*Tsukuba Research Laboratories, Aquas Corporation, 4–4 Midorigahara, Tsukuba, Ibaraki 300–2646, Japan; and*
[2]*Ibaraki University College of Agriculture, 3–21–1 Chuou, Ami-machi, Ibaraki 300–0393, Japan*

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Viable *Legionella* spp. in environmental water samples were characterized phylogenetically by a clone library analysis combining the use of ethidium monoazide and quantitative PCR. To examine the diversity of *Legionella* spp., six cooling tower water samples and three bath water samples were collected and analyzed. A total of 617 clones were analyzed for their 16S rRNA gene sequences and classified into 99 operational taxonomic units (OTUs). The majority of OTUs were not clustered with currently described *Legionella* spp., suggesting the wide diversity of not-yet-cultured *Legionella* groups harbored in cooling tower water environments.

Key words: clone library, Ethidium monoazide (EMA), *Legionella*, quantitative polymerase chain reaction (qPCR), Viable but nonculturable (VBNC)

Legionella species are Gram-negative bacteria that are ubiquitously found in natural and man-made water systems. In aquatic environments, *Legionella* spp. can proliferate as the intracellular parasites of free-living protozoa (8, 19). The human inhalation of aerosols from *Legionella*-contaminated waters, mainly from cooling tower waters and bath waters, often results in a severe form of pneumonia called Legionnaires' disease (legionellosis) (22). Therefore, the control of *Legionella* populations in water systems and monitoring for *Legionella* contamination are very important areas in public health microbiology.

The populations of *Legionella* spp. in environmental water samples have so far been estimated by culture-based plate counting and culture-independent molecular methods using the quantitative polymerase chain reaction (qPCR). Many attempts to grow environmental strains of *Legionella pneumophila*, the main causative agent of legionellosis, on plate media have been successful, and have provided insights into the ecology of *L. pneumophila* in natural environments (9). Regarding molecular methods, *Legionella* genus-specific (14) and *L. pneumophila* species-specific (13) PCR assays have been developed and proven to be valuable tools for investigating *Legionella* contamination in water systems. The molecular detection of *Legionella* spp. by conventional PCR methods could not previously distinguish viable bacterial cells from viable but nonculturable (VBNC) and dead cells (15, 24). However, the use of DNA-intercalating dyes such as ethidium monoazide (EMA) and propidium monoazide (PMA) before PCR was recently found to be effective for the specific amplification of DNA from *Legionella* cells maintaining membrane integrity (17). EMA and PMA can penetrate membrane-damaged cells and form covalent links with DNA, and such labeled genomic DNA within damaged cells is degraded upon exposure to visible light. The use of EMA (2–4, 6, 11, 18) and PMA (3, 20, 25) for the PCR quantification of *Legionella* has been described previously. However, to the best of our knowledge, the *Legionella* groups detected by the EMA- or PMA-treated PCR method have not yet been fully characterized.

Therefore, the aim of the present study was to determine whether the *Legionella* groups detectable by EMA-treated PCR belonged to known *Legionella* spp.. We compared the diversities of viable *Legionella* groups in six cooling tower water samples and three bath water samples by constructing clone libraries. As a result, 617 clones from *Legionella* spp. were recovered and their sequences determined from the water samples.

Water samples were collected from six different cooling towers (sample ID; CTW-A, -B, -C, -G, -H, and -I) and three different baths (BW-D, -E, and -F) between November 2012 and January 2014. Water quality control management prior to our water sampling is described in Supplementary Table S1. Samples were taken in sterile 500-mL polypropylene bottles with 0.05% (w/v) sodium thiosulfate, kept in the dark at 4°C until microbiological plating and DNA extraction, and used for these analyses within 3 d.

Legionella populations in water samples were enumerated according to the standard culture method (12). Briefly, water samples were subjected to centrifugation at 6,400×*g* for 30 min and the precipitate was suspended in one-100th the volume of the initial water sample. A portion of the suspension was mixed with the same volume of acid-phosphate buffer (10), and after 10 min, inoculated onto GVPC selective agar plates (Merck, Tokyo, Japan). These plates were incubated at 37°C for 8 d. Isolates of *Legionella* from the GVPC selective agar plates (50 to 100 strains per sample, except for the very low-CFU sample [four strains, CTW-G] and the sample with CFUs below the detection limit [CTW-H]) were tested by the immune serum aggregation assay (Denka Seiken, Niigata, Japan) and DNA-DNA hybridization assay (Kyokuto Pharmaceutical Industrial, Tokyo, Japan). 16S rRNA gene sequences were determined for strains that were negative for both assays, as described below.

* Corresponding author. E-mail: h_inoue0417@aquas.co.jp;
Tel: +81–29–847–6000; Fax: +81–29–847–6080.

L. pneumophila Phladelphia-1 (ATCC 33152) was used as the standard in qPCR assays, and cultured on a buffered charcoal yeast extract medium supplemented with 2-ketoglutarate (BCYEα) (7) at 30°C. The genomic DNA of *L. pneumophila* cells was extracted by the alkaline-boil method of Beige *et al.* (1) and purified using a NucleoSpin gDNA Clean-up kit (TaKaRa Bio, Otsu, Japan) according to the manufacturer's instructions. The copy number of *Legionella* 16S rRNA genes was calibrated using a Cycleave PCR *Legionella* (16S rRNA) Detection Kit (TaKaRa Bio).

A Viable *Legionella* Selection Kit for PCR ver.2.0 (TaKaRa Bio) including the EMA treatment was used for the clone library construction, as described by the manufacturer. Briefly, 1 mL of the 100-fold concentrated water sample was further concentrated to a final volume of 40 μL and mixed with 10 μL of the kit reaction buffer, 2.5 μL of the kit dilution buffer, and 2.5 μL of EMA reagent. After gently mixing using a vortex mixer and incubating in the dark for 15 min at room temperature, the samples were exposed to visible light for 15 min in a LED Crosslinker 12 (TaKaRa Bio). Thereafter, DNA was extracted and purified from each sample as described above.

Populations of *Legionella* spp. and *L. pneumophila* were quantified by qPCR using the primer pairs LEG-225F (5'-AAG ATT AGC CTG CGT CCG AT-3') and LEG-858R (5'-GTC AAC TTA TCG CGT TTG CT-3') (14), and *Lmip*L920 (5'-GCT ACA GAC AAG GAT AAG TTG-3') and *Lmip*R1548 (5'-GTT TTG TAT GAC TTT AAT TCA-3') (13), respectively. PCR reaction mixtures (30 μL) contained 5 μL of template DNA, 1 μL of 10 μM forward primer, 1 μL of 10 μM reverse primer, 0.15 μL of Ex *Taq* polymerase, 2.4 μL of dNTPs, 3 μL of 10×Ex buffer (TaKaRa Bio), and 1 μL of 1,000 dilutions of SYBR Green I dye (Lonza, ME, USA) with dimethyl sulfoxide in a Thermal Cycler Dice Real Time System *II* (TaKaRa Bio). The PCR program parameters were: an initial denaturation step of 2 min at 95°C followed by 45 cycles of denaturation for 15 s at 95°C, annealing for 30 s at 65°C (LEG primer pair) or 50°C (*Lmip* primer pair), and extension for 60 s at 72°C. A melting curve analysis was performed to detect the presence of primer dimers after the final extension by increasing the temperature from 50 to 95°C in 0.5°C increments every 10 s. The calibration qPCR was performed using *L. pneumophila* DNA, and the copy numbers of *Legionella* 16S rRNA genes were quantified as described previously: PCR performance was confirmed to be reproducible at the threshold cycles (Ct) <37 (11). Furthermore, the ratios of *L. pneumophila* were calculated from the amounts of *Legionella* 16S rRNA genes and *L. pneumophila mip* genes. To construct clone libraries, PCR using primers LEG-225F and LEG-858R was carried out according to the protocol of Nishizawa *et al.* (16) to minimize PCR bias: an initial denaturation step of 2 min at 95°C followed by each threshold cycle as determined by qPCR, denaturation for 15 s at 95°C, annealing for 30 s at 65°C, and extension for 60 s at 72°C. The reaction mixture (30 μL) was composed of 5 μL of template DNA, 1 μL of 10 μM LEG-225F primer, 1 μL of 10 μM LEG-858R primer, 0.15 μL of Ex *Taq* polymerase, 2.4 μL of dNTPs, and 3 μL of 10×Ex buffer (TaKaRa Bio) in a Thermal Cycler SP (TaKaRa Bio). The PCR products were purified by using a QIAquick PCR purification kit (Qiagen, CA, USA), ligated with the vector pMD20-T using a Mighty TA-cloning kit (TaKaRa Bio), and the ligation products were used to transform *E. coli* DH5α Competent Cells (TaKaRa Bio) according to the manufacturer's instructions. The nucleotide sequences of clones were determined with a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, CA, USA) using M13 primer RV (5'-CAG GAA ACA GCT ATG ACC-3') or M13 primer M4 (5'-GTT TTC CCA GTC ACG AC-3') according to the manufacturer's instructions and were read on an Applied Biosystems 3130xl genetic analyzer. Operational taxonomic units (OTUs) were defined as sequences with at least 99% similarity of all clones based on an analysis using Mothur platform software (http://www.mothur.org). The phylogenetic tree was constructed by the neighbor-joining method using MEGA5 software. Diversity indices (Chao 1, Simpson, Shannon-Wiener, and Good's coverage) were calculated on Mothur platform software at a cut-off level of 0.01 (99% sequence identity with gaps) in the average neighbor method.

The 16S rRNA gene partial sequences were deposited in DDBJ with accession numbers AB857847 to AB858225 and AB933772 to AB934017.

Fig. 1 shows a graphical representation of the relationships between viable population densities of *Legionella* spp. in the tested water samples determined by the standard culture method (horizontal axis) and those by the EMA-qPCR method targeting the *Legionella* 16S rRNA genes (vertical

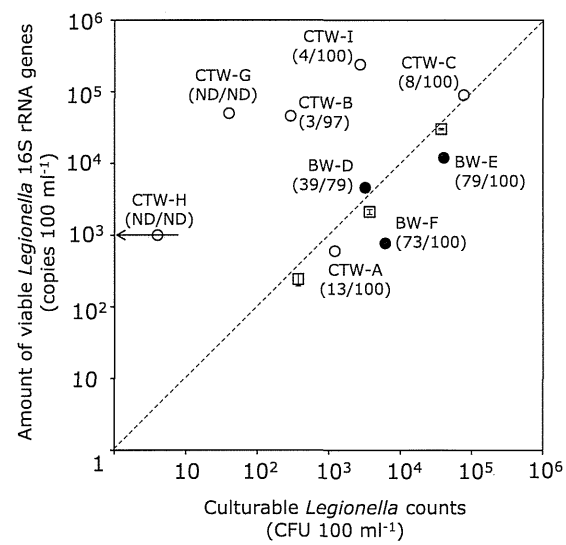

Fig. 1. Viable population densities of *Legionella* spp. in tested cooling tower (open circles), bath (solid circles) water samples, and cell suspensions of *L. pneumophila* ATCC33152 (open squares) determined by the standard culture method (horizontal axis) and EMA-qPCR targeting 16S rRNA genes (vertical axis). CTW-A to -C and -G to -I, and BW-D to -F represent the sample ID. The number pair in parentheses under the sample ID shows the percentage of the *Legionella pneumophila* population density relative to the total *Legionella* spp. population density, which was estimated by qPCR specific for *L. pneumophila* (left figure) and the identification of isolates (right figure); ND, not detected. Arrow on the sample CTW-H symbol shows that the sample harbored <10 CFU 100 mL^{-1} of *Legionella* spp. The relationship between CFU 100 mL^{-1} (x) and the 16S rRNA gene copy number 100 mL^{-1} (y) in *L. pneumophila* ATCC33152 suspensions was approximated as a dotted straight line, $y = 0.45x^{1.05}$ ($r^2 = 0.996$) (duplicate determinations).

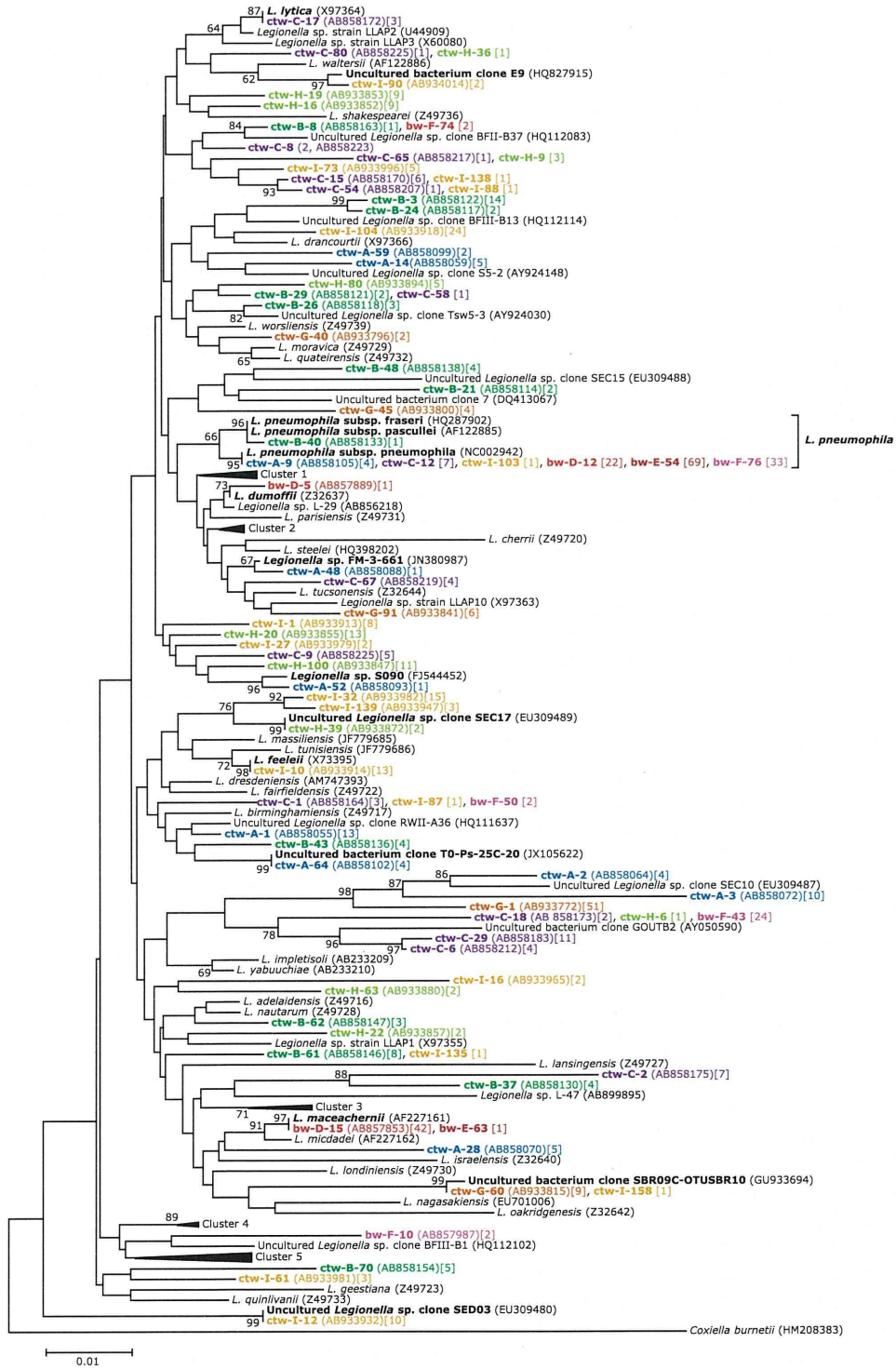

Fig. 2. Neighbor-joining tree based on the alignment of 616-bp 16S rRNA gene sequences of 79 major representative *Legionella* clones, the *Legionella* spp. that have been described to date, and the related known uncultured *Legionella* clones. Numbers at nodes are bootstrap percentages (based on 1,000 resamplings); only values above 60% are shown. The sources of the clones were expressed in different color letters: CTW-A, blue; CTW-B, green; CTW-C, purple; CTW-G, orange; CTW-H, lime green; CTW-I, yellow; BW-D, red; BW-E, brown; BW-F, pink. After the representative clone, the accession number of the representative clone and the number of similar sequences (based on a 1% cut-off) are given in parentheses and square brackets, respectively. Cluster 1 includes *L. anisa* (Z32635), *L. bozemanii* (Z49719), *L. wadsworthii* (Z49738), *L. gormanii* (Z32639), and *L. steigerwaltii* (Z49737). Cluster 2 includes *L. sainthelensi* (Z49734), *L. santicrucis* (HF558374), *L. longbeachae* (AY444740), *L. cicinatiensis* (Z49721), and *L. gratiana* (Z49725). Cluster 3 includes *L. beliardensis* (AF122884), *L. busanensis* (AF424887), and *L. gresilensis* (AF122883). Cluster 4 includes *L. rubrilucens* (Z32643), *L. taurinensis* (DQ667196), and *L. erythra* (Z32638). Cluster 5 includes *L. brunensis* (Z32636), *L. cardiac* (JF831047), *L. hackliae* (M36028), *L. jamestownensis* (Z49726), *L. jordanis* (Z32667), and *L. spintensis* (M36030).

axis). When *L. pneumophila* ATCC33152 was used as a positive control, the relationship between CFU 100 mL^{-1} (x) and the 16S rRNA gene copy numbers 100 mL^{-1} (y) was approximated as an equation, $y = 0.45x^{1.05}$ ($r^2 = 0.996$). Culturable *Legionella* counts ranged from <10 to 7.6×10^4 CFU 100 mL^{-1} and the copy numbers of *Legionella* 16S rRNA genes from viable cells were between 6.0×10^2 and 2.4×10^5 100 mL^{-1}. Four (CTW-B, -C, -G, and -I) of the six cooling tower water samples contained approximately 10^5 100 mL^{-1} *Legionella* 16S rRNA gene copies, which was approximately 100-fold higher than that in the other samples (CTW-A and -H) and 10- to 100-fold higher than that in the bath water samples. *Legionella* viable counts for all bath water samples and the three cooling tower water samples (CTW-A, -C, and -I) were $>10^3$ CFU 100 mL^{-1}, which was higher than that in the other cooling tower water samples. Four sample plots (CTW-A, CTW-C, BW-D, and BW-E) were close to the positive control line, while the plots of the other cooling tower water samples (CTW-B, -G, -H, and -I) deviated markedly upward from the line, suggesting that these samples contained larger *Legionella* populations that were unable to grow under the tested culturing conditions than the culturable ones, which was also found in our recent study (11). The identification of isolates by the immunoassay and the DNA-DNA hybridization assay revealed the dominance of *L. pneumophila*, accounting for >79% of the total *Legionella* populations, except for the very low-CFU sample (only four isolates for CTW-G) and one sample that was below the detection limit (CTW-H). The sequences of the 16S rRNA genes from all four isolates from CTW-G and one from CTW-B were 100% identical to those from *Legionella* sp. LC2720 and *Legionella* sp. L-29, respectively. Approximately 20% of the isolates from BW-D were identified as *Legionella dumoffii*.

Fig. 2 shows the neighbor-joining tree based on the *Legionella* 16S rRNA gene partial sequence (616 bp) from the cooling tower and bath water samples. A total of 617 clones (cooling tower waters: 417 clones, bath waters: 200 clones) were recovered from the water samples and classified into 99 OTUs at a cut-off level of 0.01 (99% sequence identity). Good's coverages of these libraries were 82.9% to 96.0% (cooling tower waters) and 96.9% to 98.6% (bath waters). The most abundant OTU, represented by clone ctw-A-9 (137 clones, 22% of all clones), clustered with the *L. pneumophila* group (Fig. 2). The dominance of *L. pneumophila* in BW-E and BW-F was confirmed by the clone library analysis, accounting for 99% and 51% of clones, respectively. In the other bath water sample (BW-D), *L. pneumophila* was also the main member (34%) of the clone library. In contrast, the percentage of *L. pneumophila* clones was very low in the cooling water samples: less than the detection limit for CTW-G and CTW-H and 1 to 11% for the other cooling tower water samples. The second most abundant OTU, represented by clone bw-D-15 (43 clones, 7% of all clones), was affiliated with the *L. maceachernii* cluster and accounted for 65% of the clones from BW-D and 1% of those from BW-E. The other clones that clustered with known *Legionella* spp. were *L. feeleii* (13 clones, 2% of all clones), *L. lytica* (three clones, 0.5% of all clones), and *L. dumoffii* (one clone, 0.2% of all clones).

Although the clone sequences that clustered with the *L. maceachernii* sequence were abundant in BW-D, this organism was not detected by the plate culture method. These results may be explained by either its VBNC state or a failure to outcompete *L. pneumophila* in the culture. On the other hand, *Legionella* sp. L-29 and *Legionella* sp. LC2720 were not detected by the clone libraries from CTW-B and -G, respectively. It is likely that although the plate culture method detected their very low population densities, the coverage of our clone library was too low to detect them.

Diversity indices were calculated and are summarized in Supplementary Table S2. The Chao1 values of these libraries were 10 to 67 (cooling tower water) and 2 to 7 (bath water). The Simpson ($1/\lambda$) values of these libraries were 2.08 to 13.32 (cooling tower water) and 1.03 to 2.58 (bath water). The Shannon-Wiener (H') values of these libraries were 1.14 to 2.67 (cooling tower water) and 0.07 to 1.06 (bath water). All these indices suggested that the diversity of *Legionella* communities present in cooling tower water was higher than that in bath water, and may be explained by differences in water treatments. Bath water was cleaned with a higher concentration of chlorine for a shorter period of time than cooling tower water, which may have resulted in the selective survival of chlorine-resistant strains. Further studies will be needed to clarify the relationship between the diversity of *Legionella* floras and the treatment of water systems.

A number of clones (390 clones, 63% of all clones) showed less than 99% similarity to the sequences of the known culturable *Legionella* spp. strains or uncultured *Legionella* clones. Thirty clones (7 OTUs) were closely related to the uncultured *Legionella* sp. clone SEC03 (10 clones) from the cooling tower water (23), the uncultured bacterium clone SBR09C-OTUSBR10 (10 clones), the uncultured bacterium clone T0-Ps-25C-20 (21) (four clones), the uncultured *Legionella* sp. clone SEC17 (23) (two clones), the uncultured bacterium clone E9 (two clones), *Legionella* sp. FM-3-661 (one clone), and *Legionella* sp. S090 (5) (one clone).

In conclusion, our results showed that the EMA-PCR method was capable of revealing more diverse *Legionella* groups than the standard culture method and is, thus, a better tool for monitoring *Legionella* contamination in various environments.

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We express our sincere thanks to Dr. Fumiaki Kura and Dr. Junko Amemura-Maekawa of the National Institute of Infectious Diseases for reviewing our experimental data. We thank Ms. Tomoko Takama and Ms. Asako Morishita in our laboratory for the plate culture data collection and excellent laboratory assistance in the molecular biological experiments, respectively. This work was partially supported by the Health and Labor Science Research Grants (H25-kenki-009).

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Supplementary tables

Table S1. Water quality control management before water sampling

Sample ID	Sampling site	Treatment condition	Concentration of biocide	Sampling date
Cooling tower waters				
CTW-A	a building in Niigata	no treatment	none	November 6, 2012
CTW-B	a plant in Tokyo	continuous supply of CMI [a]	<0.1 mg L^{-1} (CMI)	February 7, 2013
CTW-C	a factory in Yamanashi	intermittent supply [b] of CMI	<0.1 mg L^{-1} (CMI)	February 14, 2013
CTW-G	a factory in Saitama	continuous supply of CMI	<0.1 mg L^{-1} (CMI)	December 3, 2013
CTW-H	a building in Kanagawa	continuous supply of CMI	<0.1 mg L^{-1} (CMI)	January 17, 2014
CTW-I	a factory in Fukuoka	continuous supply of stabilized chlorine	2.0 mg L^{-1} (total chlorine)	November 29, 2013
Bath waters				
BW-D	a sports club in Kanagawa	continuous supply of free chlorine	1.0 mg L^{-1} (free chlorine)	March 21, 2013
BW-E	a hotel in Miyagi	continuous supply of free chlorine	no data (free chlorine)	April 23, 2013
BW-F	a sports club in Osaka	continuous supply of free chlorine	0.5 mg L^{-1} (free chlorine)	May 15, 2013

[a] 5-Chloro-2-methyl-4-isothiazolin-3-one

[b] The water treatment chemicals including CMI were batch fed as necessary.

Table S2. Diversity indices of *Legionella* communities

Sample	No. of clones analyzed	No. of OTUs obtained	Chao1	Simpson (1/λ)	Shannon-Wiener (*H'*)	Good's coverage (%)
CTW-A	51	11	15	7.87	2.16	92.2
CTW-B	58	17	23	10.60	2.53	87.9
CTW-C	62	16	28	13.32	2.63	87.1
CTW-G	75	8	10	2.08	1.14	96.0
CTW-H	66	19	30	9.71	2.45	84.8
CTW-I	105	29	67	9.91	2.67	82.9
BW-D	65	3	3	1.90	0.71	98.5
BW-E	70	2	2	1.03	0.07	98.6
BW-F	65	7	7	2.58	1.16	96.9

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Case report

A case of pneumonia caused by *Legionella pneumophila* serogroup 12 and treated successfully with imipenem

Midori Nishizuka [a], Hiroki Suzuki [a,*], Tomoka Ara [a], Mari Watanabe [a], Mami Morita [a], Chisa Sato [a], Fumihiro Tsuchida [a], Junji Seto [b], Junko Amemura-Maekawa [c], Fumiaki Kura [c], Hiroaki Takeda [a]

[a] *Department of Respiratory Medicine, Saiseikai Yamagata Saisei Hospital, Japan*
[b] *Department of Microbiology, Yamagata Prefectural Institute of Public Health, Japan*
[c] *Department of Bacteriology I, National Institute of Infectious Diseases, Japan*

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ABSTRACT

The patient was an 83-year-old man hospitalized for *Haemophilus influenzae* pneumonia, who developed recurrent pneumonia after improvement of the initial episode. *Legionella pneumophila* serogroup 12 was isolated from the sputum, accompanied by increased serum antibody titers to *L. pneumophila* serogroup 12. Therefore, the patient was diagnosed as having *Legionella* pneumonia caused by *L. pneumophila* serogroup 12.

Case reports of pneumonia caused by *L. pneumophila* serogroup 12 are rare, and the case described herein is the first report of clinical isolation of this organism in Japan. When the genotype was determined by the protocol of The European Working Group for *Legionella* Infections (Sequence-Based Typing [SBT] for epidemiological typing of *L. pneumophila*, Version 3.1), the sequence type was ST68. Imipenem/cilastatin therapy was found to be effective for the treatment of *Legionella* pneumonia in this patient.

1. Introduction

Legionella infection is caused by organisms of the genus *Legionella*, which are Gram-negative bacilli. *Legionella pneumophila* strains are the predominantly isolated in clinical practice. *L. pneumophila* has been classified into 15 serogroups, of which serogroup 1 is most frequent cause of *Legionella* pneumonia, whereas *L. pneumophila* serogroup 12 is rarely responsible. Only four cases of pneumonia caused by *L. pneumophila* serogroup 12 have been reported to date [1–4]. Herein, we report a case of pneumonia caused by *L. pneumophila* serogroup 12, which is the first report of clinical isolation of this organism in Japan.

2. Case report

The patient was an 83-year-old man with a 3-day history of cough, sputum expectoration and anorexia, who was brought to our hospital by ambulance because of weakness of both legs in the beginning of January, 2012; he was then admitted to the hospital with the diagnosis of pneumonia. The patient had underlying diabetes mellitus, and had been on treatment with oral prednisolone 10 mg/day for the interstitial pneumonia. He gave no history of visits to hot spring facilities or circulation-type baths. Gram staining of the sputum on admission revealed phagocytosed Gram-negative bacilli, and sputum culture grew *Haemophilus influenzae*. The patient improved after 10 days of treatment with piperacillin/tazobactam (TAZ/PIPC) (12.5 g/day). Because the patient also had concomitant asthmatic symptoms, the steroid dose was increased to 80 mg of methylprednisolone/day (Fig. 1). Chest radiography carried out on the 13th hospital day revealed infiltrative opacities in the right upper lung field, with deterioration of the infiltrative opacities in the lower lung fields bilaterally that had improved once with TAZ/PIPC (Fig. 2A). Chest computed tomography (Fig. 2B and C) also confirmed the presence of infiltrative opacities in the right upper and both lower lobes. Anorexia was the only subjective symptom, and there was no diarrhea. The patient was recorded to have fever (body temperature, 38 °C) only on the 18th hospital day. The patient received oxygen supplementation at 2 L/min by nasal

* Corresponding author. Department of Respiratory Medicine, Saiseikai Yamagata Saisei Hospital, 79-1, Okimachi, Yamagata-city, Yamagata 990-8545, Japan. Tel.: +81 023 682 1111; fax: +81 023 682 0123.
E-mail address: hiroki-s@ma.catvy.ne.jp (H. Suzuki).

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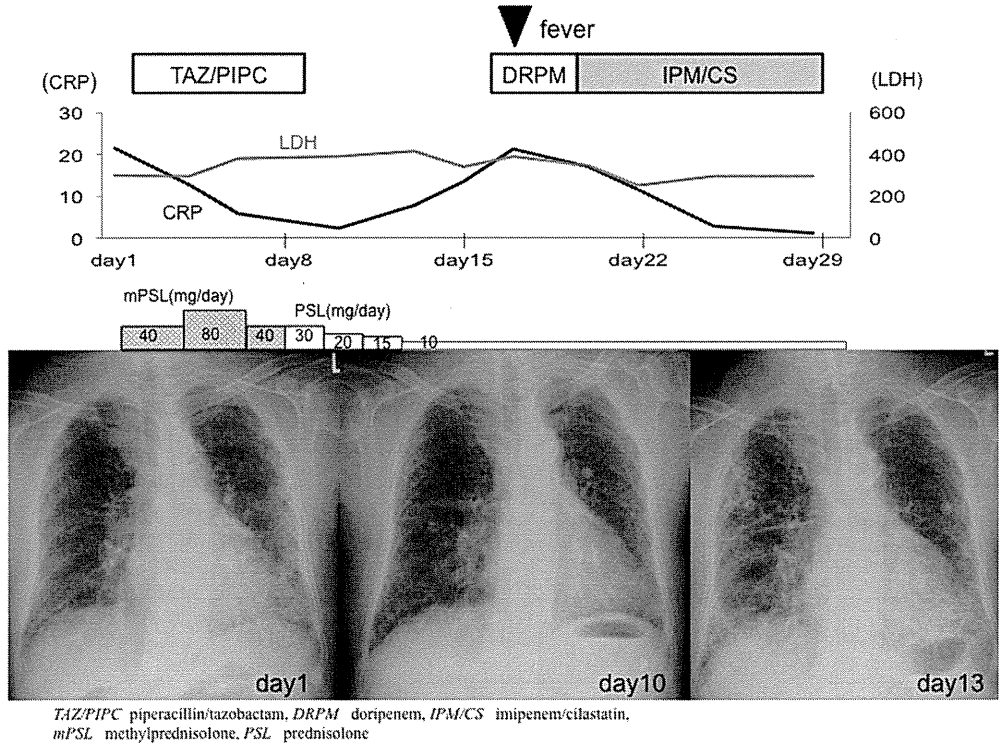

TAZ/PIPC piperacillin/tazobactam, *DRPM* doripenem, *IPM/CS* imipenem/cilastatin, *mPSL* methylprednisolone, *PSL* prednisolone

Fig. 1. Clinical course.

cannula; however, the oxygen flow needs to be increased temporarily to 3 L/min from 17th hospital day to 26th hospital day. Laboratory data obtained on the 13th hospital day were as follows: WBC count 17,400 cells/μL (stab cells 2.0%, segmented cells 89.0%), and serum CRP 7.63 mg/dL, revealing a tendency towards increase in the values of these parameters. Serum LDH was increased to 416 IU/L. The other parameters were BUN 24.0 mg/dL, serum creatinine 1.08 mg/dL, Na 136 mEq/L, K 3.8 mEq/L, Cl 98 mEq/L, fasting blood glucose 113 mg/dL, and HbA1c 6.2%. Gram staining of the sputum on the 13th hospital day showed phagocytosed Gram-negative bacilli. Doripenem (750 mg/day) therapy was initiated on the 17th hospital day. The

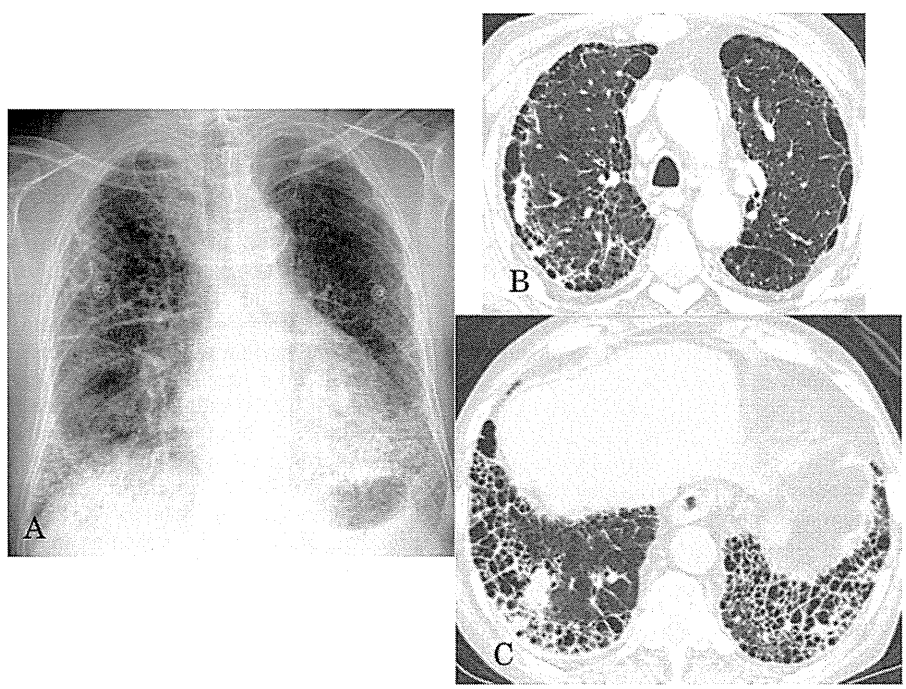

Fig. 2. Chest radiography and chest computed tomography on the 13th hospital day demonstrates the infiltrative opacities in the right upper and both lower lobes.

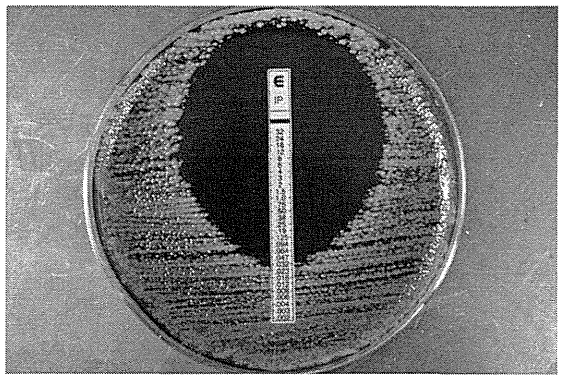

Fig. 3. Result of imipenem Etest for *Legionella pneumophila*.

doripenem therapy was switched to imipenem/cilastatin (1.5 g/day) therapy on the 20th hospital day because of elevation of the hepatic enzyme levels, and the patient's clinical condition improved thereafter. Because sputum obtained on the 13th hospital day cultured by the conventional method did not reveal any growth of bacteria, BCYE agar culture was carried out, which grew *Legionella* colonies on the 32nd hospital day. The MIC of imipenem for the isolated strain using Etest (AB Biodisk, Solna, Sweden) was 0.047 μg/ml when measured by the method of Murakami et al. [5] (Fig. 3). The slide agglutination test carried out using monovalent immune sera (Denka Seiken, Tokyo, Japan) identified the *Legionella* bacterium isolated from the sputum as *L. pneumophila* serogroup 12. The serum antibody titer on the 32nd hospital day was determined using microplate agglutination test kit (Denka Seiken, Tokyo, Japan) and in-house heat-killed *L. pneumophila* antigens (serogroups 7–15) [6]. The serum antibody titer determined was 1:512 for *L. pneumophila* serogroup 6 and 1:8192 for *L. pneumophila* serogroup 12, showing a distinct single high-titer. The serum antibody titers against *L. pneumophila* serogroups 1 to 5 and 7 to 11 and 13 to 15 were <1:16. A definitive diagnosis of infection caused by *L. pneumophila* serogroup 12 was made in our patient because *L. pneumophila* serogroup 12 was isolated from the sputum, accompanied by an increase of the serum antibody titer to *L. pneumophila* serogroup 12. Urinary *Legionella* antigen (BinaxNOW®) was negative throughout the course of the illness. The genotype was determined according to the protocol of the European Working Group for *Legionella* Infections (EWGLI; http://www.ewgli.org/) (Sequence-Based Typing (SBT) for epidemiological typing of *L. pneumophila*, Version 3.1). The results showed (3, 13, 1, 28, 14, 9, 3) for (*flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, *neuA*), and the sequence type was ST68.

Because the possibility of nosocomial infection was considered to be highly likely in the patient, testing of swabs obtained from 13 sites in the hospital, including faucets and showerheads in the bathrooms of the ward, and environmental culture tests, including of the shower water, were performed. None of the cultures grew *Legionella* organisms, and the source of infection in this patient remained unclear.

3. Discussion

Fifty-seven species of *Legionella* are currently known (http://www.bacterio.net/legionella.html, as of January 22, 2014), among which *L. pneumophila* is the most frequently encountered causative organism of *Legionella* pneumonia. Among the 15 serogroups of *L. pneumophila*, serogroup 12 was first identified in 1987 [1]. Among the 5370 clinical strains whose genotypes are registered in the EWGLI database, there are 11 strains of serogroup 12 including our case as of January 21, 2014. Our present case is the first report of clinical isolation of this serogroup in Japan. Recently, 2 new cases of infection with *L. pneumophila* serogroup 12 including our case were recorded in the surveillance data of legionellosis in Japan [7]. A review of the literature collected within the scope of our search yielded 4 cases of pneumonia caused by serogroup 12 [1–4]. Clinical data were available for 2 of these patients, both of whom were cases of nosocomial infection occurring in immunosuppressed patients, just as in our patient [1,2].

The SBT proposed by the EWGLI is a technique to determine the base sequence by PCR amplification of some parts of 7 genes, i.e., *flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, and *neuA*. The genotype of *L. pneumophila* in this case was ST68, which was found in 18 clinical strains in the EGWLI database as of January 21, 2014. Of these 18 strains, serogroup 6 accounted for the majority (11 strains), followed by serogroup 12 (4 strains). ST68 was previously isolated in 1 case of serogroup 6 in Japan, and therefore our case represents the second case of isolation of the ST68 genotype.

Our patient showed an increase of the serum antibody titer to *L. pneumophila* serogroup 6. Because cross-reactions between serogroups 6 and 12 have been reported previously [1], a cross-reaction in our patient also may occur. However, the case of infection with more than one *L. pneumophila* serogroup cannot be ruled out.

In a study by Murakami et al., the MIC of imipenem was 0.023–0.064 μg/ml for all 23 clinical isolates of *Legionella*, indicating good sensitivity of this organism to imipenem. The MIC for the isolate in our patient reported here was also within the above range. In general, it is considered that imipenem exerts no effect on *Legionella* bacteria *in vivo* because of its poor transfer into macrophages [8,9]. On the other hand, the efficacy of this drug for this infection has also been occasionally reported [10–15].

Ramirez JA et al. reported imipenem showed superior bactericidal activity against intracellular *L. pneumophila* compared to erythromycin in an *in vitro* model [13].

Our patient with *Legionella* pneumonia reported here improved with imipenem/cilastatin therapy, imipenem/cilastatin may be effective for the treatment of *Legionella* pneumonia in some cases.

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話題の環境衛生

モノクロラミン消毒による浴槽水の衛生対策

株式会社マルマ　研究開発部長
杉山　寛治

1．はじめに

循環式浴槽では肺炎の起因菌であるレジオネラ属菌の汚染が問題となっており，死亡例を含む集団感染が繰り返されたことから[1, 2]，厚生労働省の指導のもとで緊急避難的に遊離塩素消毒による管理が行われた。しかし，今日に至るまで，遊離塩素消毒を行っているにも関わらず浴槽からレジオネラ属菌が検出されることがあり[3]，遊離塩素消毒が全ての浴槽の安全を担保するとは言い難い状況にある。その原因として，井水や温泉水など多様な水質の存在，高 pH の条件下では遊離塩素消毒の効果が期待できないこと等が考えられる。また塩素臭などが敬遠されて遊離塩素の使用が必ずしも徹底されない恐れもあり，多方面から代替消毒方法が求められている。そこで，厚生労働科学研究班では，米国の水道で実用化されているモノクロラミン（結合塩素の一種）消毒[4, 5]に着目し，モノクロラミン消毒の入浴施設への応用について検討を行なった。本稿では，浴槽水のモノクロラミン消毒について解説するとともに，実際の入浴施設への導入事例等を紹介したい。

2．遊離塩素消毒の問題点

都道府県や政令市の浴槽水等の衛生管理を定めた条例では，循環式浴槽の消毒に使われる遊離塩素の濃度を 0.2 または 0.4mg/L 以上に保ち，かつ最大 1.0mg/L を超えない範囲で適切に管理することが求められている。しかし，実際の入浴施設では，アルカリ泉質の影響や温泉の泉質による遊離塩素の消失によって十分な消毒効果が得られない場合もあり，レジオネラ属菌が検出されることがある。

遊離塩素の殺菌力は，次亜塩素酸（HClO）と，それより殺菌力が落ち同じ殺菌速度を得るために約 80 倍の濃度が必要と言われる次亜塩素酸イオン（ClO^-）の存在比により決まる[6]。アルカリ泉質の温泉に次亜塩素酸ナトリウムを注入した場合は，殺菌力の劣る次亜塩素酸イオンの存在割合が高くなるため，遊離塩素の殺菌力は大きく低下する。

また，アンモニアを含む温泉水に遊離塩素を添加した場合，遊離塩素濃度は速やかに低下する。**図 1** に，アンモニア態窒素（アンモニウムイオン中の窒素の量）1mg/L を含む工業用水（pH7.5）に遊離塩素を 1.95 ～ 11.7mg/L（0.5 ～ 3.0 モル当量）となるように添加した後，40 ℃，1 時間静置後の各塩素濃度とアンモニア態窒素濃度を示した。アンモニア態窒素 1mg/L を含む水において，遊離塩素を検出するのに必要な遊離塩素の投入量は 8mg/L 以上（不連続点塩素処理に相当）という配管洗浄濃度に匹敵する高濃度なものであることがわかる。また，やっと検出された遊離塩素もアンモニアを含む新たな水が追加されると，遊離塩素と速やかに反応するため，遊離塩素は再度検出されなくなることが容易に推測できる。このようにアンモニアを含む水で遊離塩素濃度を一定に保つことは非常に困難

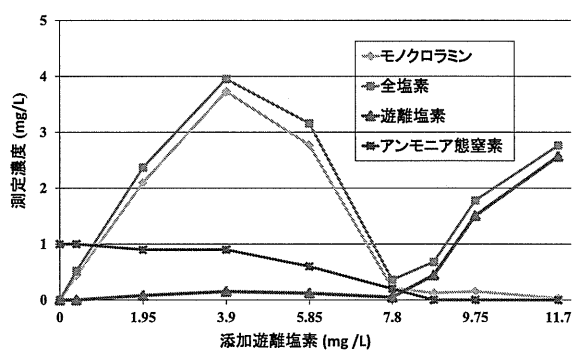

図 1　アンモニア態窒素を含む工業用水に遊離塩素を添加した時の各塩素濃度