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# *Legionella* Contamination and Molecular Identification in Water Systems of the Public Facilities in Korea

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**With the increase in population visiting hot water systems such as spas and water parks, the opportunity to be exposed to the risk of legionellae's infection is rising in Chungnam, Korea. So, we investigated *Legionella* contamination and its population on environmental sources for assessing the relationships in the types of facilities and water sources. PCR and culture method were used to detect and isolate *Legionella* spp. Partial 16s rRNA and rpoB gene sequences were used to identify the isolates and determine subspecies of *Legionella pneumophila* isolates. The detection rate (29%) of *Legionella* spp. from public bathes was the highest and *L. pneumophila* was the dominant species in Chungnam, Korea. *L. pneumophila* isolates used in this study were divided into subsp. *pneumophila* and subsp. *fraseri*. All of *Legionella* species isolated in this study except for *L. nautarum* were pathogenic to human. *L. pneumophila* isolates used in this study were divided into subsp. *pneumophila* and subsp. *fraseri*. Results of identification between rpoB and 16s rRNA trees were nearly consistent, except for some isolates. Our findings will be helpful to create a better understanding of the molecular basis of *Legionella* and further preventing and controlling outbreaks by legionellosis.**

**Key words:** legionella, identification, subtyping, spa, cooling tower.

*Legionella* species known for the causative agent of Legionnaire's disease are ubiquitous in natural freshwater environments and artificial water systems such as cooling towers, treated sewage and public baths[1,2,3,4]. More than 40 species of legionellae have been identified and *L. pneumophila* is the most prevalent human pathogen among *Legionella* species. But, the non-*pneumophila* legionellae of more than 20 species are also pathogenic to humans[5].

Legionellosis is commonly transmitted by the inhalation of aerosols from water systems contaminated by *Legionella* species[6,7]. Major clinical symptoms by legionellae's infection are acute pneumonia and nonpneumonic pontiac fever[5]. Legionnaires' disease by acute pneumonia showed a lower incidence of 0.5 to 5%, but there are many cases either not diagnosed or not

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reported[8]. It has been that the mortality rate ranges from 15 to 30% due to complications of pneumonia if not properly treated as well[6].

Also, in the outbreak of legionellosis at a hot spring spa in Japan in 2002, 295 peoples including seven deaths were infected[9]. With the increase in population visiting hot water systems such as spas, springs and water parks which are easy to multiply legionellae, the opportunity to be exposed to the risk of legionellae's infection is rising in the country, although a few sporadic cases are reported in Korea.

A contemporary assessment of *Legionella* species causing legionellosis in the country would be important, so we investigated the distribution of *Legionella* species from various environmental water sources such as cooling tower water systems and hot or cold water systems in spas and decorative fountain using conventional PCR methods as well as culture methods seen as 'gold standard'. Furthermore, we performed molecular typing of 16s rRNA and rpoB genes to determine genetic diversity between the isolates and *Legionella* reference strains and to find out the molecular relationships in the types of facilities and of water sources and the epidemiology of *Legionella* infection. Also, in general, it was reported that *L. pneumophila* species is divided into three subspecies (*L. pneumophila* subsp. *pneumophila*, subsp. *fraseri* and subsp. *pascullei*)[10]. So, to subgroup *Legionella pneumophila* isolates analyzed in this study, we constructed a phylogenetic tree with rpo B sequences except for 16s rRNA gene which unfitted for the population study on a subspecies level because of its high similarity[5,11].

## MATERIALS AND METHODS

### Environmental water sampling

In total, 777 water samples were studied in sixteen cities in Chungnam from June to September 2010. Samples were collected from various environmental water sources such as cooling tower water systems and hot or cold water systems in buildings, schools, hotels, marts, public bathes and decorative fountains.

### Isolation for *Legionella* species

Samples were cultivated onto BCYE-± medium supplemented with GVPC (ammonia-free glycine, vancomycin, polymyxin B and cycloheximide), selective antimicrobial agents. For the isolates exhibiting *Legionella*-like morphology in this medium, subcultivation onto BCYE-± medium with and without L-cysteine, and blood agar not to be supplemented with iron and L-cysteine was implemented. The colonies that only grow on BCYE-± medium with L-cysteine were reconfirmed for PCR assay[12].

### Identifying *L. pneumophila* and other-*Legionella* species using PCR

Two pairs of primers were used to amplify 16s rRNA, rpoB and mip (macrophage infectivity potentiator) genes for detection of *Legionella* isolates. 16s rRNA and rpoB genes are specific for *Legionella* genus and mip genes for *L. pneumophila* (Table 1).

Template DNA(ca. 50 ng) and 20 pmol of each primer were added to a PCR mixure tube (AccuPower PCR PreMix; Bioneer, Daejeon, Korea), which contained 1 U of *Taq*DNA polymerase, each deoxyribonucleoside triphosphate at a concentration of 250 ¼M, 50 mM Tris-HCl (pH 8.3), 40 mM KCl, 1.5 mM $MgCl_2$, and gel loading dye[13].

Amplification was carried out in AB thermal DNA cycler (Applied Biosystems, Foster City, CA) heated to 95°C for 5min before 30 cycles of 95°C for 1min, 60°C for 1min and 72°C for 1min, followed by extension at 72°C for 5min. The amplicons were visualized by a QiaXel multi-capillary electrophoresis system (Qiagen, Hilden, Germany).

### Sequencing and phylogenetic analysis

The amplicons were purified using the AccuPrep® PCR purification kit (Bioneer, Daejeon, Korea) prior to DNA sequencing. The primers used for amplification of 16s rRNA and rpoB genes in this study are directly sequenced with Big Dye terminator reaction mix (Applied Biosystems, Foster City, CA, USA). All sequences of each isolate determined and the reference strains (Table 2) were aligned with Megalign (DNASTAR) and CLUSTAL W (version 1.81)[14]. The genotypes of the isolates were determined as the types of the highest scoring strain using the Basic Local Alignment Search Tool (BLAST) in Genbank. Phylogenetic trees were constructed using the neighbor-joining method (NJ) in MEGA version 5 and branch bootstrap values were evaluated with 1000 replications[15].

**Nucleotide sequence accession numbers**

The sequences from 16s rRNA and rpoB genes determined in this study have been deposited in GenBank database under accession numbers KC464647 to KC464704 and KC464705 to KC464762, respectively.

## RESULTS

***Legionella* Detection**

*Legionella* species were isolated from 86 samples collected from a total of 777 environmental water samples by standard culture method and PCR (Table 3). Especially, it could be differentiated from *L. pneumophila* and other *Legionella* spp. by PCR assay using 16s rRNA and rpoB genes specific for *Legionella* genus and mip genes for *L. pneumophila*, which resulted in 66 isolates of *L. pneumophila* and 20 isolates of non-*L. pneumophila*.

The environmental water samples collected in this study were classified broadly into four types; cooling tower water, hot water system, cold water system and decorative fountain. Of these, the detection rate of *Legionella* spp. was the highest in hot water system (17.3%), followed by cooling tower water (11.7%), cold water system (2.4%) and decorative fountain (0%).

Cold water system and decorative fountain have lower detection rate than hot water system or cooling tower water which are at relatively high temperature. In cooling tower, hospitals-nursing homes (18.2%) were most frequent, and schools (16.7%) showed relatively high detection rate, although the number of samples in schools was less than that in other facilities.

The samples in hot water system indicated overall high positive rates. In particular, hot bathtubs in public bathes were positive in 29% of the samples.

**16s rRNA and rpoB DNA sequencing**

Amplicons of all isolates were implemented by 16s rRNA and rpoB DNA sequencing. Each of the sequences acquired was identified as the highest scoring reference strain by BLAST search in GenBank and this identification was supported by a phylogenetic tree that incorporated all of the isolates including 59 reference strains (Table 2). In 16s rRNA sequence analysis, all of isolates had 98~100% similarities, and 9 isolates (10.4%) of them had 100% similarity, whereas, in rpoB sequence analysis, 95-100% similarities, and 18 isolates (20.8%) had 100% similarity to each of the GenBank reference sequences (data not shown).

Identification results between 16s rRNA and rpoB gene analyses in most of the isolates were consistent, but some of isolates did not match or were not amplified.

**Distribution of *Legionella* spp**

Regarding the four different water systems, the distribution of the isolates was similar, but slightly different (Table 4). *L. pnemophila* was prevalent regardless of facilities, temperatures and geographic regions (Fig 1), except for sample types (cold water systems). Among *L. pneumophila* isolates, two isolates were *L. pneumophila* subsp. *fraseri* and isolated from school and building, respectively.

Non- *L. pneumophila* species isolated from cooling tower water were *L. anisa* (21.3%) and *L. wadsworthii* (1.7%). *L. anisa* was only isolated from cooling tower, except for one strain isolated from cold water systems, and *L. wadsworthii* was restricted to Geumsan city, south of Chungnam. In hospitals, there were *L. anisa* (6.7%) and *L. oakridgensis* (6.7%). Those from public bathes were *L. pneumophila* and *L. dumoffii*. Interestingly, all of *L. dumoffii* strains in this study

**Table 1.** Primer sets for gene amplification of *Legionella* spp.

| Primer | Sequence | Region | Size of products |
|---|---|---|---|
| p1.2 | 5'-AGG GTT GAT AGG TTA AGA GC-3' | 16s rRNA | 386bp |
| cp3.2 | 5'-CAA CAG CTA GTT GAC ATC G-3' | | |
| ML1 | 5'-GAT AAG TTG TCT TAT AGC ATT GG-3' | mip | 630bp |
| ML2 | 5'-TCT GTC CAT CCT GGG ATA ACT TG-3' | | |
| RL1 | 5'-GAT GAT ATC GAT CAY CTD GG-3' | rpoB | 369bp |
| RL2 | 5'-TTC VGG CGT TTC AAT NGG AC-3' | | |

**Table 2.** *Legionella* reference strains included in this study

| Taxon | Sero group | Strain no.(designation) | Accession no. | |
|---|---|---|---|---|
| | | | rpoB | 16s rRNA |
| *L. pneumophila* subsp. *pneumophila* | 1 | ATCC 33152(philadelphia 1) | af377748 | m36023 |
| *L. pneumophila* subsp. *pneumophila* | 1 | ATCC 33153(Knoxville-1) | ay036036 | |
| *L. pneumophila* subsp. *pneumophila* | 1 | (SF9) | ay036037 | |
| *L. pneumophila* subsp. *pneumophila* | 1 | ATCC 43109(OLDA) | ay036038 | |
| *L. pneumophila* subsp. *pneumophila* | 2 | ATCC 33154(Togus-1) | ay036039 | |
| *L. pneumophila* subsp. *pneumophila* | 3 | ATCC 33155(Bloomington-2) | ay036040 | |
| *L. pneumophila* subsp. *fraseri* | 4 | ATCC 33156(Los Angeles-1) | ay036041 | |
| *L. pneumophila* subsp. *fraseri* | 5 | ATCC 33216(Dallas 1E) | ay036042 | |
| *L. pneumophila* subsp. *pascullei* | 5 | ATCC 33737(U8W) | aj746049 | |
| *L. pneumophila* subsp. *pascullei* | 5 | ATCC 33736(U7W) | aj746050 | |
| *L. pneumophila* subsp. *pascullei* | 5 | ATCC 33735(MICU B) | aj746051 | |
| *L. pneumophila* subsp. *pascullei* | 5 | ATCC 33737(gifu13567) | ab107779 | |
| *L. pneumophila* subsp. *pneumophila* | 6 | ATCC 33215 (Chicago 2) | ay036043 | |
| *L. pneumophila* subsp. *pneumophila* | 7 | ATCC 33823(Chicago 8) | ay036044 | |
| *L. pneumophila* subsp. *pneumophila* | 8 | ATCC 35096(Concord 3) | ay036045 | |
| *L. pneumophila* subsp. *pneumophila* | 9 | ATCC 35289(IN-23-G1-C2) | ay036046 | |
| *L. pneumophila* subsp. *pneumophila* | 10 | ATCC 43283(Leiden 1) | ay036047 | |
| *L. pneumophila* subsp. *pneumophila* | 11 | ATCC 43130(797-PA-H) | ay036048 | |
| *L. pneumophila* subsp. *pneumophila* | 12 | ATCC 43290(570-CO-H) | ay036049 | |
| *L. pneumophila* subsp. *pneumophila* | 13 | ATCC 43736(82A3105) | ay036050 | |
| *L. pneumophila* subsp. *pneumophila* | 14 | ATCC 43703(1169-MN-H) | ay036051 | |
| *L. pneumophila* subsp. *fraseri* | 15 | ATCC 35251(Lansing3) | ay036052 | |
| *L. adelaidensis* | | UOEH 13562 | af367721 | z49716 |
| *L. anisa* | | ATCC 35292(WA-316-C3) | af367722 | z32635 |
| *L. dumoffii* | | ATCC 33279(NY-23) | af367728 | z32637 |
| *L. londiniensis* | | ATCC 49505 | af367740 | z49728 |
| *L. micdadei* | | ATCC 33218(TATLOCK) | af367743 | m36032 |
| *L. nautarum* | | ATCC 49506 | af367745 | z49730 |
| *L. oakridgensis* | | (HM 7002) | af367746 | z32643 |
| *L. wadsworthii* | | ATCC 33877 | af367757 | z49738 |
| *L. birminghamensis* | | UOEH11749 | af367723 | z49717 |
| *L. bozemanii* | 1 | ATCC33217 | af367724 | z49719 |
| *L. brunensis* | | UOEH12655 | af367725 | z32636 |
| *L.* cherrii | | UOEH10742 | af367726 | z49720 |
| *L. cincinnatiensis* | | UOEH12201 | af367727 | z49721 |
| *L. erythra* | 1 | ATCC35303 | af367729 | m36027 |
| *L. fairfieldensis* | | UOEH13563 | af367730 | z49722 |
| *L. feeleii* | 1 | ATCC35072 | af367731 | z49740 |
| *L. geestiana* | | ATCC49504 | af367732 | z49723 |
| *L. gormanii* | | ATCC33297 | af367733 | z32639 |
| *L. gratiana* | | ATCC49413 | af367734 | z49725 |
| *L. hackeliae* | | ATCC35250 | af367735 | m36028 |
| *L. israelensis* | | ATCC43119 | af367736 | z32640 |
| *L. jamestowniensis* | | ATCC35298 | af367737 | z49726 |
| *L. jordanis* | | HM7000 | af367738 | z32667 |
| *L. lansingensis* | | ATCC49751 | af367739 | z49727 |
| *L. longbeachae* | 1 | ATCC33462 | af367741 | m36029 |
| *L. maceachernii* | | ATCC35300 | af367742 | z32641 |
| *L. moravica* | | ATCC43877 | af367744 | z49729 |
| *L. parisiensis* | | UOEH11745 | af367747 | z49731 |
| *L. quinlivanii* | 1 | ATCC43830 | af367749 | z49733 |
| *L. rubrilucens* | | ATCC35304 | af367750 | z32643 |
| *L. sainthelensi* | 1 | ATCC35248 | af367751 | z49734 |
| *L. santicrucis* | | UOEH11746 | af367752 | z49735 |
| *L. shakespearei* | | ATCC49655 | af367753 | z49736 |
| *L. spritensis* | 1 | UOEH11199 | af367754 | m36030 |
| *L. steigerwaltii* | | UOEH11747 | af367755 | z49737 |
| *L. tucsonensis* | | ATCC49180 | af367756 | z32644 |
| *L. worsleiensis* | | ATCC49508 | af367758 | z49739 |

were only isolated from public bath.

Hot water systems took on a wide variety of types of *Legionella* species; *L. pneumophila* (81.8%), *L. oakridgensis* or *L. pneumophila* (4.5%), *L. dumoffii* (9.2%) and *L. nautarum* or *L. londiniensis* (4.5%). *L. pneumophila* (33.3%), *L. anisa* (33.3%) and *L. dumoffii* (33.3%) were isolated from cold water systems.

**Phylogenetic analysis**

77 partial sequences of rpoB and 16s rRNA genes obtained in this study were used to construct a phylogenetic tree with 59 reference strains deposited in the Genbank database (Fig 2). But, 18 isolates possessing identical sequences and 21 reference strains are not shown in this phylogenetic tree because of space restrictions. This phylogenetic tree showed the same overall topology compared to the original tree including all of the isolates, and 21 reference strains were not related to the all of isolates obtained in this study. The topologies of 16s rRNA and rpoB trees were partially similar but incongruent in many respects; both of the trees were divided into Group 'N' (*L. pneumophila*) and Group 'S' (non- *L. pneumophila* species), but the rpoB tree had longer terminal branches than those of the 16s rRNA.

The 16s rRNA and rpoB sequences were segregated into distinct four subgroups (N-I and S-I to III) and eight subgroups (N-I to IV and S-I to IV), respectively. Subgroups in each tree were limited to the strains with sequence similarity values equal to or above 97%[16,17].

The overall similarities within Genus *Legionella* of the 16s rRNA and rpoB sequences analyzed in this study ranged from 93.5-100% and 69.1-100%, respectively. But, there was no difference between protein sequences deduced from each gene in *Legionella* strains. N-I, the major subgroup in 16s rRNA tree, was belonged to *L. pneumophila*. The nucleotide sequences similarity within N-I was nearly identical (99.6 to 100%) and showed 1.5-2.6%, 3.7-4.5%, and 4.5-5.3% nucleotide divergence from subgroup S-I, S-II, and S-III, respectively. The reference strains belonging to *L. dumoffii*, *L. anisa* and *L. wadsworthii* were comprised in a subgroup, S-I, which was supported by high bootstrap values. The nucleotide similarities of the sequences within S-I ranged from 99.6-100%, whereas the three reference strains in the rpoB phylogeny were distinctly divided into each subgroup having above 3% nucleotide divergence (Fig 2).

In the rpoB phylogeny, group N (*L. pneumophila*) was divided into four subgroups (N-I to IV). The similarity of the nucleotide sequences within subgroup N-I was 97.8-100%

**Table 3.** Investigation results for potential sources of *Legionella* infection

| Sample type | Facility | No.samples/No.isolate (%)of facility | No.samples/No.isolate (%)of sample type |
|---|---|---|---|
| Cooling tower water | Building | 49/436(11.2) | 61/520(11.7) |
| | Hospitals/Nursing home | 6/33(18.2) | |
| | Hotel | 0/3(0) | |
| | Mart/Department store | 4/36(11.1) | |
| | School | 2/12(16.7) | |
| Hot water system | Bathtub in Public bath | 9/31(29.0) | 22/127(17.3) |
| | Shower in public bath | 5/30(16.7) | |
| | Shower in Nursing home | 0/15(0) | |
| | Shower in hospital | 4/26(15.4) | |
| | Faucet in hospital | 4/25(16.0) | |
| Cold water system | Bathtub in Public bath | 1/28(3.6) | 3/127(2.4) |
| | Shower in public bath | 1/27(3.7) | |
| | Shower in Nursing home | 1/16(6.3) | |
| | Shower in hospital | 0/27(0) | |
| | Faucet in hospital | 0/21(0) | |
| | Faucet in building | 0/8(0) | |
| Decorative fountain | Decorative fountain | 0/3(0) | 0/3(0) |
| Total | 86/777(11.1) | | |

**Table 4.** Distribution of *Legionella* spp. isolated in this study

| Facility | Water source | Total | Species | | | | |
|---|---|---|---|---|---|---|---|
| | | | *L. pneumophila* [a] | *L. anisa* [a] | *L. wadsworthii* [a] | *L. dumoffii* [a] | Not determined [b] |
| Hospital | Cooling Tower | 6 | 6 | | | | |
| | Shower (hot) | 4 | 4 | | | | |
| | Faucet (hot) | 4 | 3 | | | | 1 |
| | Shower (cold) | 1 | | 1 | | | |
| Public bath | Bathtub (hot) | 9 | 8 | | | | 1 |
| | Shower (hot) | 5 | 3 | | | 2 | |
| | Bathtub (cold) | 1 | 1 | | | | |
| | Shower (cold) | 1 | | | | 1 | |
| Building | Cooling Tower | 49 | 36 | 11 | 1 | | 1 |
| Mart/Department store | Cooling Tower | 4 | 2 | 2 | | | |
| School | Cooling Tower | 2 | 2 | | | | |
| Total | 86 | 65 | 14 | 1 | 3 | 3 | |

[a] Species associated with disease [b] Isolates not consistent for identification

**Table 5.** Comparison of identification with partial rpoB and 16s rRNA gene sequences of 86 isolates in Chugnam

| Isolate | No. of isolates | The closest reference strain in rpoB(Similarity, %) | The closest reference strain in 16s rRNA(Similarity, %) |
|---|---|---|---|
| S5/HW-Hos/Asan/kor/10 | 1 | *L. pn* subsp. *pn* (100) | *L. nautarum* (96) |
| S15/HW-Bath/Yesan/Kor/10 | 1 | *L. londiniensis* (98) | *L. nautarum* (99) |
| N41/CT-Buil/Yesan/Kor/10 | 1 | *L. pn* subsp. *fraseri* (99) | *L. pn* subsp. *pn* (99) |
| N50/HW-Hos/Asan/Kor/10 | 1 | *L. pn* subsp. *fraseri* (93)*L. pn* subsp. *pascullei* (94) | *L. pn* subsp. *pn* (99) |
| N55/CT-Buil/Yesan/kor/10 | 1 | *L. pn* subsp. *pn* (99) | *L. oakridgensis* (99) |

* *Pn, pneumophila.*

except for N60/CT-Buil/Asan/Kor/10 strain showing 1.8-3.6% nucleotide divergence with subgroup N-I. Subgroup N-I showed 3.2-4.4%, 9.8-11.6% and 12.3-14.5% nucleotide divergence from subgroup N-II, N-III and N-IV, respectively and the nucleotide divergence between N-III and N-IV accounted for 5.1%.

Group S (Non-*L. pneumophila*) in rpoB tree was segregated into distinct four subgroups (S-I to IV) and the overall nucleotide divergence within subgroups in Group S was 19.6-27.8%.

**Subtyping of *L. pnemophila* isolates based on rpoB sequences**

49 *Legionella pneumophila* isolates were dispersed over four distinct subgroups (P-I to II, subsp. *pascullei* and subsp. *fraseri*) and three subspecies on *Legionella pneumophila* are completely distinguished, which were supported by high bootstrap values (Fig 3).

*L. pneumophila* subsp. *pneumophila* including P-I to II subgroups showed the sequence dissimilarities of 8.9-12.6% and 11.3-16.7% from subsp. *pascullei* and subsp. *fraseri*, respectively. The sequence dissimilarities between subsp. *pascullei* and subsp. *fraseri* ranged from 3.8-5.2%. *L. pneumophila* subsp. *pneumophila* was divided into two subgroups (P-I to II) and intra-subgroup homology except for N60/CT-Buil/Asan/Kor/10 strain was ranged from 97.1- 100% and 100%, respectively. N60 strain showed the nucleotide divergence of 3.5-6.0% with P-I and the divergence between P-II and P-I was 3.8-5.2%. N50/HW-Hos/Asan/Kor/10 strain did not belong to any subgroup of *L. pneumophila* which showed the nucleotide divergence of 4.8-5.6%, 6.3% and 9.3-13.0% from subsp. *fraseri*, subsp. *pascullei* and subsp. *pneumophila*, respectively.

**Comparison of identification between rpoB and 16s rRNA trees**

The closest reference strains of most of the sequences in 16s rRNA tree were consistent with the results in rpoB tree, but not consistent in a few sequences (Table 5).

N55/CT-Buil/Yesan/kor/10 strain was closest to *L. oakridgensis* (99.6%) in 16s rRNA tree, but to *L. pneumophila* subsp. *pneumophila*(1)/knoxville-1 (99.7%) in rpoB tree. The closest reference strain of the subgroup including S15/HW-Bath/Yesan/Kor/10 and S5/HW-Hos/Asan/kor/10 strains in 16s rRNA tree was *L. nautarum* (99.6% and 96.9%, respectively), but the strains in rpoB tree were *L. londiniensis* (98.9%) and *L. pneumophila* subsp. *pneumophila*(14)/1169-MN-H (100%), respectively. N50/HW-Hos/Asan/Kor/10 strain did not belong to any subgroup in rpoB tree, whereas belonged to *L.*

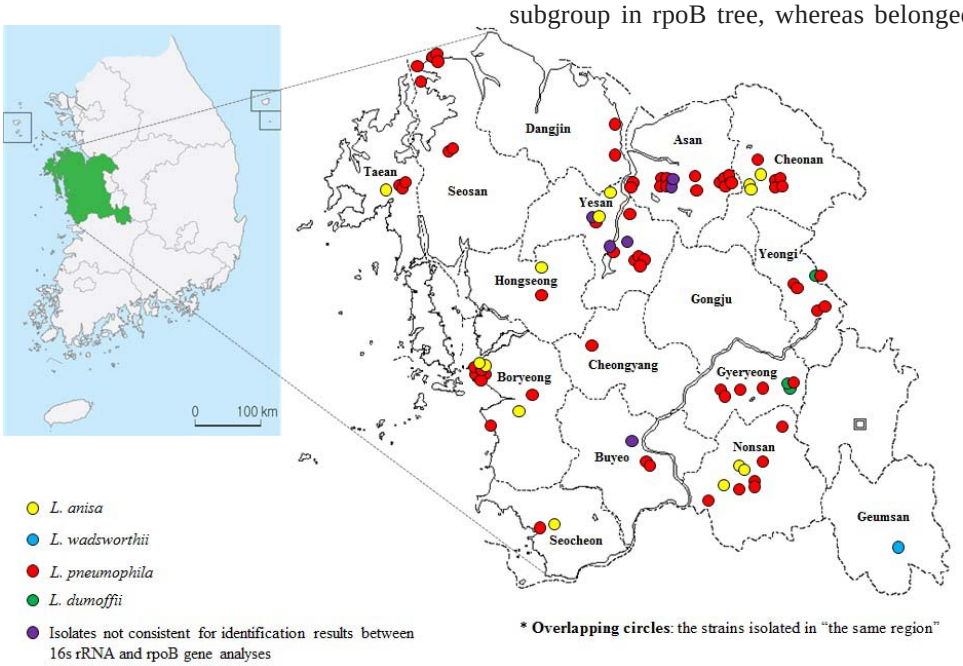


**Fig. 1.** Geographic distribution of Legionella isolates in sixteen cities in Chungnam, 2010 (n=86)

*pneumophila* subsp. *pneumophila*(1)/philadelphia (99.6%) in 16s rRNA tree.

## DISCUSSIONS

The aim to this study is to investigate the diversity of *Legionella* spp. distributed in various man-made water sources in Chungnam, Korea. Culture and PCR assay were used to identify *Legionella* spp. Many genes including 16s rRNA, 5S rRNA, 23S-5S spacer region, mip, dotA and rpoB (RNA polymerase subunit ²) etc. were used to detect *Legionella* spp. in PCR assay. 16s rRNA, mip and rpoB genes were utilized to detect *Legionella* spp. in this study. 16s rRNA sequences have been widely used for many genotypic schemes of bacteria, as it is highly conserved among species[18]. But, 16s rRNA sequence-based classification lacked to discriminate clearly between species or subspecies because of its lower variation[5]. For example, the variation of the 16s rRNA(23%) within most *Legionella* species at DNA level was lower than that of mip gene(56%), which related to the infection of macrophage[19, 20]. So, rpoB locus is added to overcome these limitations, which codes for the ² subunit of the RNA polymerase. Ko et al.

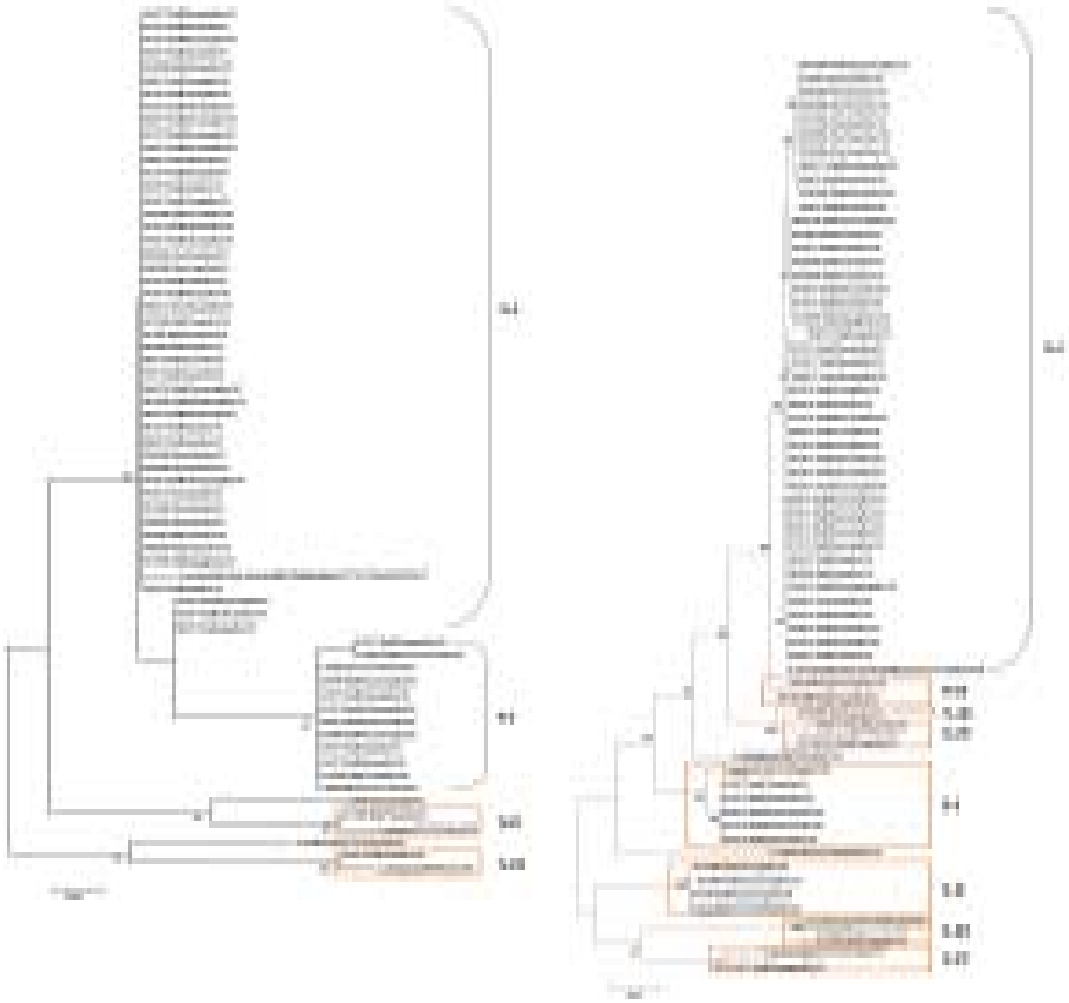

**Fig. 2.** Dendrogram showing phylogenetic relationships of *Legionella* species by maximum likelihood method. The tree is derived from the sequences of partial 16s rRNA (a) and rpoB gene sequences (b). The numbers at the branches indicate the bootstrap values at the node for 100 replicates. Bootstrap values below 60% were omitted. CT, Cooling Tower; HW, Hot Water; CW, Cold Water; Buil, Building; HoNu, Hospital/Nursing home; Hotl, Hotel; MaDe, Mart/Department; Scho, School

demonstrated that rpoB-based analysis was not only able to discriminate *Legionella* spp. but also three subspecies of *L. pneumophila*[3].

Samples investigated in this study were collected from the facilities related to the occurrence of *Legionella* outbreaks worldwide. The detection rate of *Legionella* spp. was the highest from hot water from bathtubs in public bathes (29.0%), followed by cooling tower water from hospitals/nursing homes (18.2%) and hot water from showers in public bathes (16.7%). Public bathes and hospitals were the highest in the frequency of *Legionella* spp. among facilities. The reasons that Public bathes, especially the bath tubs in public bathes, are chief reservoirs of *Legionella* spp. are as follows; first, the water temperature in hot tub is kept at 30-50°C for them to grow and water is not usually drained throughout the year, whereas cooling tower is only kept at 30-40°C during summertime that air-conditioner is operated. Second, calcium and magnesium salts (resulted from lime scale), iron compounds (derived from corrosion products in baths) and dirt, dust and dead skin cells in hot tubs may provide a source of nutrients in multiplying *Legionella* spp.

**Fig. 3.** Dendrogram showing phylogenetic relationships between reference strains and isolates of *Legionella pneumophila* by maximum likelihood method. The tree is derived from the sequences of partial rpoB gene sequences. The numbers at the branches indicate the bootstrap values at the node for 100 replicates. Bootstrap values below 60% were omitted. CT, Cooling Tower; HW, Hot Water; CW, Cold Water; Buil, Building; HoNu, Hospital/Nursing home; Hotl, Hotel; MaDe, Mart/Department; Scho, School

So, this result suggests that special cautions are required to prevent and control the occurrence of legionellosis in public bathes by state or local health authorities, as the recreational facilities such as hot spring or spa have become popular in Korea and the customers using regularly these site are old people, who were at greater risk of legionellosis than the other ages. Furthermore, aerosols resulted from the *Legionella*-contaminated agitated water in bathes can be easily penetrated in body via inhalation.

In this study, the most dominant species was *L. pneumophila*, regardless of facilities and sample types. This result was consistent with those previously reported[2, 6, 21, 22]. In addition, it was reported that *L. pneumophila* causes most of outbreaks or cases of legionellosis in aquatic environments[23]. But this species is not the most predominant in all of the aquatic environments. In fresh water environments such as lakes, rivers and groundwater, the various non- *L. pneumophila* species such as LLAP species, *L. oakridgensis*, *L. worsleiensis* and *L. bozemanii* were common members[24,25,26]. There are some possible reasons that *L. pneumophila* was the most predominant species in many aquatic environments, especially man-made water sources. I) It suggested that *L. pneumophila* could have better ability to survive and proliferate in man-made environments than that of non- *L. pneumophila* species[27]. By Wery et al.,

*legionellae* were initially diverse in cooling tower, but non-*L. pneumophila* species were dramatically reduced as *L. pneumophila* increases[27]. Furthermore, some of non-*L. pneumophila* species do not multiply easily under the presence of biofilms or sediment [28] and within protozoa; *L. micdadei* does not proliferate rapidly within *Acanthamoeba* species[29, 30]. II) It was believed that *L. pneumophila* could survive a wide range of temperatures. In general, *L. pneumophila* grows at 25-42°C and does not multiply at temperatures of less than 20°C but can survive for a long time at temperatures below freezing[31,32]. In addition, *L. pneumophila* can survive at higher temperature. By Nathalie et al., re-colonization of *L. pneumophila* could have confirmed despite heat shock treatment of 70°C, whereas *L. anisa* was no longer detected after heat shock treatment[33]. III) *L. pneumophila* may have a resistance to the chemicals such as disinfectants[34]. Garcia et al. demonstrated that *L. pneumophila* could persist for many years despite regular hyperchlorination in different facilities[35]. This resistance may have emerged resulting from repeated treatment but actually, a strain of *L. pneumophila* never exposed to chlorine had a resistance[35]. By Thomas et al., most of disinfectants used in the study were not effective for controlling *L. pneumophila* except chlorine-dioxide. But, even chlorine-dioxide did not eliminate amoebae and re-colonization of *L. pneumophila* was observed after interrupting chlorine-dioxide treatment[36]. So, this resistance may be related to protection by amoebae[21]. Non-*L. pneumophila* species isolated in this study were *L. anisa, L. wadsworthii, L. oakridgensis/L. pneumophila, L. dumoffii* and *L. nautarum/L. londiniensis.* Among species isolated, the other species except for *L. nautarum* and *L. londiniensis* were associated with human disease. *L. anisa,* one of the dominant species isolated in this study, was known for the pathogen causing pontiac fever and rarely reported in hospital-acquired cases[37, 38]. Each species showed its own distinct characteristic with regard to sample location or sample type. Especially, *L. dumoffii* strains were only isolated from public bath in this study. We confirmed that *L. dumoffii* strain is most common in public bath or spa bath worldwide; in environmental investigations related to the outbreak of legionellosis occurred in a spa bath in Japan, the dominant species isolated from bathtub water were *L. londiniensis, L. dumoffii* and *L. pneumophila* SG1[39]. Furthermore, Hurn's investigation demonstrated that *L. dumoffii, L. maceachernii,* and *L. micdadei* were isolated from a spa area associated with travel-related pontiac fever[40]. It was confirmed that *L. dumoffii* could persist at low temperature in this study, given that *L. dumoffii* strains (S2/HW-Bath/Gyeryeong/kor/10 and S3/CW-Bath/Gyeryeong/kor/10) collected in bath tubs in a public bath were detected in cold water as well as hot water, although accurate temperature of cold water in the bath tub was unknown.

Partial 16S rRNA and rpoB gene sequences were used to identify 77 *Legionella* isolates and construct each phylogenetic tree. 16s rRNA sequences of the isolates (93.5-100%) showed higher similarity than rpoB sequences of those (69.1-100%) within Genus *Legionella*. Consequently, in 16s rRNA phylogeny, the reference strains belonging to *L. dumoffii, L. anisa* and *L. wadsworthii* were comprised in a subgroup, S-I, whereas the three reference strains in rpoB phylogeny were distinctly divided into each subgroup having above 3% nucleotide divergence. Moreover, such a difference of sequence similarity based on two genes had an influence on identification of the *Legionella* isolates (S5/HW-Hos/Asan/kor/10, S15/HW-Bath/Yesan/kor/10, N50/HW-Hos/Asan/kor/10 and N55/CT-Buil/Yesan/kor/10). S5 isolate could be initially identified to Non-*L. pneumophila* spp., which was positive for 16s rRNA and rpoB genes specific for *Legionella* genus and negative for mip gene specific for *L. pneumophila.* But, it belonged to *L. pneumophila* in the rpoB phylogeny, whereas was closer to *L. nautarum* than *L. pneumophila* in the 16s rRNA phylogeny. So, it could be speculated that the discrepancy between different trees of S5 isolate resulted from lateral gene transfer between species[5,41,42], and in fact, evidences that rRNA genes subject to lateral gene transfer have been steadily increasing[43,44,45,46].

N50 isolate was identified to *L. pneumophila* in both 16s rRNA and rpoB phylogenies, but in subtyping based on rpoB sequences, it did not belong to any subgroup of *L. pneumophila*. In this study, the strains with sequence similarity values equal to or above 97%

organized each subgroup. N50 isolate showed the nucleotide divergency of 4.8-5.6%, 6.3% and 9.3-13.0% from subsp. *fraseri*, subsp. *pascullei* and subsp. *pneumophila*, respectively and therefore it suggested lateral transfer of rpoB gene between three *L. pneumophila* subspecies. But, unfortunately, it was unable to prove as there was no other gene phylogeny comparable to rpoB phylogeny used in this study. Despite of sequence disimilarities of two genes, there was no difference between protein sequences deduced from each gene in *Legionella* isolates. Base substitutions in 16s rRNA and rpoB genes are limited not allowing for amino acids substitution because such amino acid substitutions affected their primary and secondary structures related to maintaining its functions[5,47]. For establishing a polyphasic approach to taxonomy in *Legionella* species, more markers including existing 16s rRNA and rpoB genes are needed as these gene exchanges complicate the classification of *Legionella* species.

## CONCLUSIONS

Our study revealed *Legionella* contamination and identification by two genes analysis on public facilities in Korea. Public bathes represented high frequency (29%) of *Legionella* contamination and *L. pneumophila* was the dominant species in Korea. All of *Legionella* species isolated in this study except for *L. nautarum* were pathogenic to human. The comparison of phylogenetic analysis based on partial 16s rRNA and rpoB gene sequences of the isolates showed a high degree of diversity in isolates, suggesting lateral gene transfer between *Legionella* species.

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