3．遺伝子診断法に関する研究

Use of the *dnaJ* Gene for the Detection and Identification of All *Legionella pneumophila* Serogroups and Description of the Primers Used to Detect 16S rDNA Gene Sequences of Major Members of the Genus *Legionella*

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Running head: Identification of *L. pneumophila* serogroups using *dnaJ* gene

Key words: *Legionella pneumophila*, *dnaJ*, Identification, PCR

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Abstract:

We sequenced about 930 bp of the *dnaJ* gene from 15 *Legionella pneumophila* serogroups and some other members of the genus *Legionella*. As *L. pneumophila* 16S rDNA sequences could not discriminate between all subspecies and serogroups, we assessed the use of *dnaJ* gene sequences to differentiate between *Legionella* subspecies as well as between *L. pneumophila* serogroups. Phylogenetic analysis revealed that *dnaJ* gene sequences were more variable between the *L. pneumophila* serogroups than *mip* gene and 16S rDNA sequences. By studying 61 strains from 41 species of the genus *Legionella*, as well as other genera, we established a PCR method that could amplify 285 bp of *dnaJ* gene of all *L. pneumophila* serogroups. This primer set was more sensitive than *mip* gene primers and was able to detect 0.25 ng of purified DNA. We also describe the 16S rDNA primers that were used to detect most *Legionella* genus members.

Key words: *Legionella pneumophila*, *dnaJ*, Identification, PCR,

Legionella pneumophila, the causative agent of Legionnaires' disease and the type species of the genus *Legionella*, was first recognized in 1976 following an epidemic of acute pneumonia among veterans of the American Legion in Philadelphia, Pennsylvania, and led to the discovery of a new species and genus (7, 24). Since then, this genus has become very large with 47 species currently recognized (11, 20, 26). Nonetheless, the majority of Legionnaires' disease cases are still attributed to *L. pneumophila* (6, 23, 28).

Routine biochemical identification methods based on phenotypic properties (biochemical tests, serotyping) are not applicable to the identification of legionellae due to their poor biochemical activities. New methods based on molecular techniques have been developed that allow a more reliable characterization of *Legionella* strains. Genotyping methods such as ribotyping (9, 15), 16S-23S intergenic spacer region PCR (29), 16S or 5S rRNA gene sequencing (1, 4, 12, 17, 25), macrophage infectivity potentiator (*mip*) gene sequencing (27), and randomly amplified polymorphic DNA (RAPD) (21) have been shown to be useful in classifying isolated strains to the species level.

DnaJ, one of the members of the Hsp70 (Heat shock protein) family, co-regulates the activity of heat shock sigma factor σ^{32} (5, 8, 13, 14). The *dnaJ* gene has been used for species identification in genus *Mycobacterium* (3, 32), and may be a potentially useful gene for the detection and identification of other pathogenic bacteria.

Materials and Methods

Strains and cultivation. As shown in Table 1, 61 strains of *L. pneumophila* serogroups 1 – 15, 40 *Legionella* species and 6 other genera were used in this study. *Legionella* strains were incubated on buffered charcoal yeast extract agar plates supplemented with α-ketoglutarate and L-cysteine (α-BCYE) at 37°C in 5% CO_2 for 3 – 5 days. Bacteria from other genera were incubated on nutrient agar at 28 to 37°C depending on the species for 1 – 3 days.

***dnaJ* sequencing and phylogenetic analysis.** As only one *dnaJ* sequence from *L. pneumophila* was available when this study was initiated, we compared the *L. pneumophila dnaJ* sequence with sequences from other closely-related proteobacteria. Based on this analysis, we designed *dnaJ*-specific primers to allow

identification of *Legionella* species. After trying several primers, we finally selected the following primer set: DN 13 F (forward) primer: 5'- AGCGGGATTATTATGAAC-3', and New LG-R (reverse) primer: 5'-GACCGGTGTTTCTACAAC-3'. The amplicon is about 930 bp.

Amplicon sequences were determined using a DNA Sequencing Kit (BigDye™ terminator V3.0 cycle sequencing Ready Reaction) (Lot No. 0208111, Applied Biosystems, Tokyo) with forward and reverse primers and a 3100 genetic analyzer (Applied Biosystems, HITACHI, Japan). For sequencing reactions, 50 ng purified PCR product, 32 pmol primer, and 4 μl BigDye terminator Premix were mixed and adjusted to a final volume of 20 μl with distilled water. Reactions were run for 25 cycles of 10 seconds at 95°C, 5 seconds at 50°C, and 4 minutes at 60°C. As a double check, both strands were sequenced. *dnaJ* gene sequences from the 15 *L. pneumophila* serogroups and from some *Legionella* species were compared by multiple alignment using CLUSTAL W (31). Phylogenetic distances were calculated by the neighbor-joining method (31), with and without gaps, and their similarities calculated (Tables 2 and 3).

For comparative purposes, we obtained *mip* gene sequences from the *L. pneumophila* serogroups (27) and 16S rDNA sequences from some *Legionella* species as deposited in GenBank by Ratcliff (27), Fry (12), Hookey (17) and Park (26). After sequence alignment using CLUSTAL W (version 1.7) (31), phylogenetic distances and similarities were analyzed (Table 2 and Table 3).

Designing PCR detection primer for *Leginella* spp. and *L. pneumophila*. With compared sequences of *Legionella*, we designed the detective primer set of W 19 forward (*dnaJ*) primer, 5'-AGGTGGTTTTGGCGGATTTGG-3' and W 19 reverse (*dnaJ*) primer, 5'-TGAATTCTGACTTGCCCCATG-3' for *Legionella pneumophila*, which gave an amplicon of 285 bp. We also designed primers to amplify the *mip* gene and 16S rDNA sequences for comparison: W 3 forward (*mip*) primer, 5'-GATAAGTTGTCTTATAGCAT-3'; W 3 reverse (*mip*) primer, 5'-TTTCACTGAAATTAAGTGAA-3'; W 8 forward (16S rDNA) primer, 5'-AGCATKGTCTAGCTTGCTAG-3'; W 8 reverse (16S rDNA) primer, 5'-TCCTCCCCACTGAAAGTG-3'.

To assess primer sensitivity, purified DNA from *L. pneumophila* GTC 296^T (ATCC 33152) was used as template DNA. For sensitivity for living bacteria, fresh *L. pneumophila* GTC 296^T cells were cultured on α-BCYE agar plates for 3 days, suspended in distilled water and diluted using a 10-fold gradient. Live cells were counted by inoculation on α-BCYE agar plates. Dilutions were then used immediately as template DNA for PCR after boiling for 5 minutes.

Bacterial samples were boiled for 5 minutes and the crude DNA preparations then used as template DNA for PCR. PCR mixtures contained 0.1 μM each deoxynucleoside triphosphate (dNTP), 0.2 μM each primer, 1 U Taq™ polymerase (TAKARA SHUZO CO., LTD, Otsu, Japan) in 1× PCR buffer. Amplification was carried out in a thermal cycler (GeneAmp® PCR System 9700, PE Applied Biosystems) under the following conditions: samples were first incubated at 95°C for 5 min, then subjected to 40 cycles of 95°C for 30 sec, 50°C for 30 sec, 74°C for 60 sec, with a final extension at 74°C for 10 min. Amplified DNA was visualized on 1.5% agarose gels in TAE buffer (400 mM Tris, 10mM EDTA, 200 mM acetic acid) after electrophoresis at 150 V for approximately 25 min, ethidium bromide staining, and illumination with UV light (33).

Results

Sequence variation among the *dnaJ*, *mip*, and 16S rDNA sequences. As shown in Figs. 1 and 2, *dnaJ* and *mip* nucleotide sequences from the *L. pneumophila* serogroups were analyzed by the neighbor-joining

method. *dnaJ* gene variation among the 15 serogroups ranged from 88.9 to 99.0%, while *mip* gene variation ranged from 95.1 to 100%. In particular, *mip* gene sequences for serogroups 10 and 12, serogroups11, 13, 1and were identical, while the corresponding *dnaJ* sequences had 98.8%, 98.2% , 96.1%, 96.5% similarity.

dnaJ sequences from serogroups 4 and 5 that belong to *L. pneumophila* subspecies *pascullei* and *fraseri*, respectively, were apparently different from the other *L. pneumophila* subspecies *pneumophila* serogroups (Fig. 1). However, published *mip* gene sequences indicated that only serogroup 5, belonging to the subspecies *pascullei*, was different from the other *L. pneumophila* subspecies *pneumophila* serogroups. By *mip* gene sequence analysis, serogroup 4, belonging to subspecies *fraseri*, was within the group of remaining *L. pneumophila* subspecies *pneumophila* serogroups (Figs 1, and 2). This indicated that the *dnaJ* gene was more useful in differentiating between of *L. pneumophila* serogroups than the *mip* gene.

The *dnaJ* and 16S rDNA sequences from a number of *Legionella* species are compared in Table 2. *dnaJ* sequences were more variable than 16S rDNA sequences. Thus, our results suggested that the *dnaJ* gene was useful tool for species identification of members of the genus *Legionella*.

Primer specificity. To develop a rapid identification and detection method for *L. pneumophila* serogroups and members of the *Legionella* genus, we evaluated the W 19 (*dnaJ*) and W 3 (*mip*) primer sets for *L. pneumophila* and the W 8 (16S rDNA) primer set for genus *Legionella* members. As shown in Table 1, DNA template from 15 *L. pneumophila* serogroups were subjected to PCR using the W 19 (*dnaJ*) and W 3 (*mip*) primer sets. The 285 bp W 19 (*dnaJ*) and 597 bp W 3 (*mip*) amplicons are shown in Fig. 3. Other *Legionella* species and other water-borne pathogens, such as *Acinetobacter calcoaceticus* GIFU 11962^T, *Burkholderia cepacia* GTC 13^T, *Chryseobacterium meningosepticum* GTC 8^T, *Escherichia coli* GTC 503^T, *Pseudomonas aeruginosa* GTC 2^T, and *Sphingobacterium spiritivorum* GTC 120^T, were not amplified by the species-specific W 19 (*dnaJ*) and W 3 (*mip*) primer sets (Table 1).

The W 8 (16S rDNA) primer set was used to detect *Legionella* genus members. In this study, we used 51 *Legionella* strains, including 38 species and 2 subspecies of *Legionella*, as well as 6 species from other genera as targeting species. As shown in Table 1, the W 8 (16S rDNA) primer set specifically amplified most members of the *Legionella* genus (Fig. 3). *Acinetobacter calcoaceticus* GIFU 11962^T, *Burkholderia cepacia* GTC 13^T, *Chryseobacterium meningosepticum* GTC 8^T, *Escherichia coli* GTC 503^T, *Pseudomonas aeruginosa* GTC 2^T and *Sphingobacterium spiritivorum* GTC 120^T are commonly found in the water environment and produced no amplified PCR product using the W 8 (16S rDNA) primer set.

When *Legionella* species were amplified using the W 8 (16S rDNA) primer set, the species *L. brunensis* GTC515^T, *L. cherrii* GTC 374^T, *L. israelensis* GIFU 11367^T, *L. jamestowniensis* GIFU 10741^T, *L. londiniensis* GTC 741^T, *L. longbeachae* GIFU 9245^T, *L. oakridgensis* GIFU 10061^T, and *L. waltersii* GTC 1737^T, could not be amplified. Analysis of the corresponding 16S rDNA sequences deposited in GenBank showed that the 8 species differed at the primer binding sites by 1 to 2 bases. Therefore, new 16S rDNA-specific primer sets were designed for these species: W 8 forward 1, 5'-AGCACGGACTAGTTTACTAG-3' and W 8 reverse 1, 5'-TCCTCCCCAACAAAAGTG-3' for *L. oakridgensis*; W 8 forward 2, 5'-AGCATGACCTAGCTTGCTAG-3', for *L. longbeachae*; W 8 forward 3, 5'-AGCATGGTCCAGCTTGCTGA-3', for *L. israelensis*; W 8 forward 5, 5'-AGCATGGTCTAGCTTGCTAG-3', for *L. cherrii*; W 8 forward 6, 5'-AGCATTTTCTAGCTTGCTAG-3', for *L. jamestowniensis* and *L. brunensis*; W 8 forward 7, 5'-CGGCGCATTCTGCTTGCTAG-3', for *L. londiniensis*; and W 8 reverse 2, 5'-TTCGGGAACACTGATACAGGTG-3', for *L. waltersii*. Use of these primers allowed amplification of 16s rDNA from all species tested.

Primer sensitivity. While the minimal DNA concentration detected by the W 3 (*mip*) primer set was 2.5 ng, the minimal concentration detected by the W 19 (*dnaJ*) primer set was 0.25 ng (Fig 4). When live *L. pneumophila* GTC 296^T cells were used as template for PCR after 5 minutes boiling, the minimal colony forming unit (CFU) in the PCR solution detected by the W 3 (*mip*) primer set was 1000 compared to 100 CFU for the W 19 (*dnaJ*) primer set.

Discussion

Although 16S rDNA sequencing is one of the most common methods of bacterial identification, it cannot be used to differentiate between *L. pneumophila* serogroups 1 to 15 due to the very high degree of similarity between their 16s rDNA sequences.

The *dnaJ* gene is conserved in Eubacteria and Eucarya (2, 22). DnaJ, an Hsp 40 homolog, triggers the hydrolysis of DnaK-bound ATP, converting DnaK from its ATP-bound low-affinity state to its ADP-bound high-affinity state (16, 18). The *dnaJ* gene has been used as a highly effective tool to discriminate between species or subspecies. Bhattacharya (3) used part of the *dnaJ* gene to identify *Mycobacterium* species. In the present study, we first focused on *dnaJ* gene analysis of *L. pneumophila* serogroups 1 to 15. We also assessed the use of the *mip* gene that has been used to discriminate between *L. longbeachae* and some other *Legionella* species (10, 19, 30) and between *L. pneumophila* serogroups (27).

Analysis of DNA sequences from the 15 *L. pneumophila* serogroups revealed that the variation between the *dnaJ* genes was higher than between the *mip* gene sequences (Table 2). The average gene variation between genus *Legionella* species was 94.9% ± 4.9% (from 88.9% to 99.2%) for the *dnaJ* gene, but 98.6% ± 3.5% (from 95.1% to 100.0%) for the *mip* gene. Thus, it appeared that *dnaJ* gene sequences would more easily discriminate between the *L. pneumophila* serogroups 1 to 15 than the *mip* gene, and we hypothesized that the *dnaJ* gene was a suitable target for PCR-based identification of legionellae. Therefore, we designed species-specific and genus-specific primer sets to allow the identification of *Legionella* species, and demonstrated that the *dnaJ* gene was indeed a better target compared to the *mip* gene.

We designed the specific primer sets W 3 to amplify a 597 bp fragment of the *L. pneumophila mip* gene, W 8 to amplify a 380 bp fragment of *Legionella* genus 16S rRNA, and W 19 to amplify a 285 bp fragment of the *L. pneumophila dnaJ* gene. The W 3 (*mip*) and W 19 (*dnaJ*) primer sets specifically amplified *L. pneumophila* sequences. Both of *L. pneumophila*-specific primer set W 19 (*dnaJ*) and W 3 (*mip*) *L. pneumophila*-specific primer set (Fig. 3) didn't amplify *Legionella* species other than *L. pneumophila*. The *Legionella* genus-specific primer set W 8 (16S rDNA) also worked for most pathogenic *Legionella* (Table 1).

The annealing temperatures between the two *Legionella* species-specific primer sets were different, being 50 °C for the W 3 (*mip*) primer set compared to 57 °C for the W 19 (*dnaJ*) primer set. When the annealing temperature was decreased to 50 °C for amplification using the W 19 (*dnaJ*) primer set, *L. pneumophila* serogroups 4 (GIFU 9246), 5 (GTC 297), 14 (GTC 806), and 15 (GTC 807) were amplified as two amplicons.

Both the W 19 (*dnaJ*) and W 3 (*mip*) primer sets were designed to be *L. pneumophila*-specific. As shown in Fig 4, the W 19 (*dnaJ*) primer set was more sensitive than the W 3 (*mip*) primer set, such that the W 3 (*mip*) primer set could detect 2.5 ng purified DNA and 1000 CFU *L. pneumophila* GET 296^T in a PCR reaction, whereas the W 19 (*dnaJ*) primer set could detect 0.25 ng purified DNA and 100 CFU *L.*

pneumophila GET 296^T.

In conclusion, the species-specific W 19 (*dnaJ*) primer is recommended for the detection of serogroups of *L. pneumophila*. For the detection of the members of the genus *Legionella*, nine different primer mixtures to amplify 16S rDNA gene is recommended.

Because of their poor biological activities, biochemical identification of members of the genus *Legionella* is not possible. Molecular identification, such as,chromosomal DNA/DNA hybridization and 16S rDNA sequencing are only available methods. Chromosomal DNA/DNA hybridization is a demanding method and only used by taxonomists for classification. Identification by small ribosomal DNA sequencing is becoming a relatively easy method for microbiologists at research laboratories. However, the sequencing is still difficult to introduce as a routine method at clinical laboratories. Furthermore, the variation of 16S rDNA among members of the genus *Legionella* is not variable enough for their species identification. Variation found in *dnaJ* gene gives enough information for species identification of the members of the genus *Legionella*. In near future, OligoDNA microarray designed from *dnaJ* sequences would be a powerful candidate to identify species of the genus *Legionella* without sequencing.

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Table 1. Amplification of *Legionella* species with *mip, dnaJ* and 16S rDNA primers

Specie	Other name	W3 (*mip*)	W8 (16S rDNA)	W19 (*dnaJ*)
Legionella pneumophila Serogroup 1 GTC 745	ATCC 33153	+	+	+
Legionella pneumophila Serogroup 2 GTC 746	ATCC 33154	+	+	+
Legionella pneumophila Serogroup 3 GTC 747	ATCC 33155	+	+	+
Legionella pneumophila Serogroup 4 GIFU 9246	ATCC 33156	+	+	+
Legionella pneumophila Serogroup 5 GTC 297	ATCC 33216	+	+	+
Legionella pneumophila Serogroup 6 GTC 748	ATCC 33215	+	+	+
Legionella pneumophila Serogroup 7 GTC 750	ATCC 33823	+	+	+
Legionella pneumophila Serogroup 8 GTC 751	ATCC 35096	+	+	+
Legionella pneumophila Serogroup 9 GTC 754	ATCC 35289	+	+	+
Legionella pneumophila Serogroup 10 GTC 757	ATCC 43283	+	+	+
Legionella pneumophila Serogroup11 GTC 756	ATCC 43136	+	+	+
Legionella pneumophila Serogroup 12 GTC 804	ATCC 43290	+	+	+
Legionella pneumophila Serogroup 13 GTC 805	ATCC 45756	+	+	+
Legionella pneumophila Serogroup 14 GTC 806	ATCC 43730	+	+	+
Legionella pneumophila Serogroup 15 GTC 807	ATCC 35251	+	+	+
Legionella adelaidensis GTC 697^T	ATCC 35292	-	+	-
Legionella anisa GTC 464^T	ATCC 35292	-	+	-
Legionella birminghamensis GIFU 11749^T	ATCC 43702	-	+	-
Legionella bozemanii GIFU 9141^T	ATCC 33217	-	+	-
Legionella brunensis GTC 515^T	ATCC 43677	-	+a	-
Legionella cherrii GTC 374^T	ATCC 35252	-	+b	-
Legionella cincinnatiensis GTC 477^T	ATCC 43753	-	+	-
Legionella dumoffii GIFU 9244	ATCC 33343	-	+	-
Legionella erythra GIFU 11748^T	ATCC 35303	-	+	-
Legionella fairfieldensis GTC 698^T	ATCC 49588	-	+	-
Legionella feeleii GTC 322^T	ATCC 35072	-	+	-
Legionella geestiana GTC 703^T	ATCC 49504	-	+	-
Legionella gormanii GTC 300^T	ATCC 33297	-	+	-
Legionella gratiana GTC 699^T	ATCC 49413	-	+	-
Legionella hackeliae GIFU 10740^T	ATCC 35250	-	+	-
Legionella israelensis GIFU 11367^T	ATCC 43119	-	+c	-
Legionella jamestowniensis GIFU 10741^T	ATCC 35298	-	+d	-
Legionella jordanis GIFU 3193^T	ATCC 33623	-	+	-
Legionella londiniensis GTC 741^T	ATCC 49505	-	+e	-
Legionella longbeachae GIFU 9245^T	ATCC 33462	-	+f	-
Legionella micdadei GTC 299^T	ATCC 33218	-	+	-
Legionella moravica GTC 513^T	ATCC 49180	-	+	-
Legionella nautarum GTC 742^T	ATCC 49506	-	+	-
Legionella oakridgensis GIFU 10061^T	ATCC 33761	-	+g	-
Legionella parisiensis GTC 444^T	ATCC 35299	-	+	-
Legionella pneumophila subsp. *fraseri* GTC 302^T	ATCC 33156	+	+	+
Legionella pneumophila subsp. *pneumophila* GTC 296^T	ATCC 33152	+	+	+
Legionella pneumophila subsp.*pascullei* GTC 702^T	ATCC 33737	+	+	+
Legionella quateirensis GTC 743^T	ATCC 49507	-	+	-
Legionella quinlivanii GTC 512^T	ATCC 43830	-	+	-
Legionella rubrilucens GIFU 10743^T	ATCC 35304	-	+	-
Legionella sainthelensi GIFU 10392^T	ATCC 35248	-	+	-
Legionella santicrucis GIFU 11746^T	ATCC 35301	-	+	-
Legionella shakesparei GTC 701^T	ATCC 49655	-	+	-
Legionella spiritensis GIFU 11199^T	ATCC 35249	-	+	-
Legionella steigerwaltii GIFU 11747^T	ATCC 35302	-	+	-
Legionella tucsonensis GTC 516^T	ATCC 43878	-	+	-
Legionella wadsworthii GIFU 10062^T	ATCC 33877	-	+	-
Legionella waltersii GTC 1737^T	DSM 10501	-	+h	-
Legionella worsleiensis GTC 744^T	ATCC 49508	-	+	-
Acinetobacter calcoaceticus GIFU 11962^T	ATCC 23055	-	-	-
Burkholderia cepacia GTC 13^T	ATCC 25416	-	-	-
Chryseobacterium meningosepticum GTC 8^T	ATCC 13253	-	-	-
Escherichia coli GTC 503^T	ATCC 11775	-	-	-
Pseudomonas aeruginosa GTC 2^T	ATCC 10145	-	-	-
Sphingobacterium spiritivorum GTC 120^T	ATCC 33861	-	-	-

+: PCR positive
-: PCR negative
a: *L. bruensis* was amplified using W 8 forward 6 and W 8 reverse primers.
b: *L. cherrii* was amplified using W 8 forward 5 and W 8 reverse primers.
c: *L . israelensis* was amplified using W 8 forward 4 and W 8 reverse primers.
d: *L. jamestowniensis* was amplified using W 8 forward 6 and W 8 reverse primers.
e: *L. londiniensis* was amplified using W 8 forward 7 and W 8 reverse primers.
f: *L. longbeachae* was amplified using W 8 forward 2 and W 8 reverse primers.
g: *L. oakridgensis* was amplified using W 8 forward 1 and W 8 reverse 1 primers.
h: *L. waltersii* was amplified using W 8 forward and W 8 reverse 2 primers.

Table 2. Similarity between *dnaJ* sequences (lower left) and *mip* sequences (upper right) of *Legionella pneumophila* subspecies

Subspecies	% Nucleotide similarity														
	GTC296^T	GTC746	GTC747	GTC302	GTC702	GTC748	GIFU10064	GTC751	GTC754	GTC757	GTC803	GTC804	GTC805	GTC806	GTC807
L. pneumophila subsp. *pneumophila* serogroup1 GTC296^T		98.2	98.3	98.5	95.1	98.5	99.5	99.0	98.7	98.7	99.7	98.7	99.7	99.7	98.7
L. pneumophila subsp. *pneumophila* serogroup2 GTC746	96.3		98.5	99.0	95.9	99.0	98.4	98.5	99.2	99.2	98.2	99.2	98.2	98.2	99.5
L. pneumophila subsp. *pneumophila* serogroup3 GTC747	96.5	96.3		99.5	95.3	99.0	98.2	98.3	99.0	99.2	98.0	99.2	98.0	98.0	98.0
L. pneumophila subsp. *fraseri* Serogroup4 GTC302	91.6	93.1	93.1		95.6	99.0	98.4	98.5	99.2	99.2	98.2	99.2	98.2	98.2	98.5
L. pneumophila subsp. *pascullei* serogroup5 GTC702	90.5	92.0	91.2	94.3		96.2	95.6	95.7	96.2	96.1	95.1	96.1	95.1	95.1	95.4
L. pneumophila subsp. *pneumophila* serogroup6 GTC748	97.0	96.7	97.0	91.9	90.5		98.7	98.9	99.5	99.8	98.5	99.8	98.5	98.5	98.5
L. pneumophila subsp. *pneumophila* serogroup7 GIFU10064	97.5	97.9	98.5	93.2	91.9	97.3		99.2	98.9	98.9	99.5	98.9	99.5	99.5	98.9
L. pneumophila subsp. *pneumophila* serogroup8 GTC751	96.6	97.0	97.2	92.5	91.2	97.2	97.9		99.0	99.0	99.0	99.0	99.0	99.0	98.4
L. pneumophila subsp. *pneumophila* serogroup9 GTC754	96.3	97.6	95.6	92.5	91.3	95.8	96.9	96.0		99.7	98.7	99.7	98.7	98.7	98.7
L. pneumophila subsp. *pneumophila* serogroup10 GTC757	96.8	96.9	96.9	92.6	91.3	96.9	98.5	97.3	96.5		98.7	100.0	98.7	98.7	98.7
L. pneumophila subsp. *pneumophila* serogroup11 GTC803	96.4	97.8	96.4	93.0	91.8	96.3	97.2	96.0	97.3	96.4		98.7	100.0	100.0	98.7
L. pneumophila subsp. *pneumophila* serogroup12 GTC804	98.3	98.6	98.4	93.3	92.3	99.2	98.9	99.0	97.5	98.8	97.8		98.7	98.7	98.7
L. pneumophila subsp. *pneumophila* serogroup13 GTC805	96.3	98.0	95.8	93.2	92.1	96.0	97.4	96.2	98.4	97.2	98.2	97.9		100.0	98.7
L. pneumophila subsp. *pneumophila* serogroup14 GTC806	96.3	97.1	97.0	92.3	91.3	96.4	98.3	96.9	96.0	98.1	96.1	98.8	96.5		98.7
L. pneumophila subsp. *pneumophila* serogroup15 GTC807	88.9	91.1	91.1	97.5	92.3	90.9	91.3	91.0	90.8	90.0	91.1	92.7	90.1	89.5	

Sequenced length of *dnaJ* gene is about 930 bp and that of *mip* is about 700 bp.

Table 3. Similarity between *dnaJ* sequences (lower left) and 16S rDNA sequences (upper right) of *Legionella* species

Species	% Nucleotide similarity																				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
L. adelaidensis GTC697		95.6	94.9	94.9	94.8	95.0	92.5	95.4	95.4	93.9	94.8	95.0	95.2	95.2	93.6	94.9	93.8	93.4	94.9	95.0	94.9
L. brunensis GTC515	76.9		95.4	95.2	95.1	96.4	92.8	95.3	97.0	93.8	96.0	96.6	96.6	96.7	94.7	96.1	93.5	93.8	96.0	95.5	95.8
L. cherrii GTC374	76.2	76.3		96.4	93.6	95.6	92.2	96.9	95.2	93.0	97.0	97.0	97.1	96.9	94.8	97.5	94.7	95.1	97.1	98.8	96.0
L. cincinnatiensis GTC477	77.8	75.1	79.5		95.0	94.7	91.8	97.0	94.2	94.0	98.1	96.4	96.3	96.2	94.7	98.4	98.2	94.8	96.8	96.5	96.2
L. fairfieldensis GTC698	92.6	75.9	75.6	78.5		95.4	93.2	94.6	94.4	94.1	94.5	94.7	94.4	94.6	93.5	94.3	93.6	93.6	94.5	93.8	94.4
L. feeleii GTC322	73.1	72.0	71.2	71.9	71.9		93.6	95.3	95.5	93.1	95.0	95.4	95.4	95.1	93.9	95.4	93.0	93.8	95.6	95.7	94.5
L. geestiana GTC703	92.5	77.3	76.9	77.6	96.9	72.2		92.4	93.3	91.4	92.4	93.3	93.3	92.7	91.5	92.2	90.6	91.6	92.0	92.5	92.1
L. gratiana GTC699	77.2	75.8	80.7	88.6	77.8	72.6	77.7		94.4	92.8	97.4	96.8	96.7	96.7	95.3	97.3	95.6	94.8	97.1	96.7	96.2
L. hackeliae GIFU10740	73.7	70.7	72.4	71.3	72.9	75.4	72.1	71.8		93.3	95.6	95.7	95.5	95.0	93.1	94.6	92.8	93.5	94.9	94.4	94.6
L. israelensis GIFU11367	69.7	66.3	68.7	70.7	69.1	67.9	68.6	69.7	69.1		92.9	93.7	94.1	94.0	91.5	93.3	92.1	92.0	93.3	93.2	92.9
L. longbeachae GIFU9245	77.6	76.3	81.8	90.8	76.9	72.9	76.5	89.4	72.2	69.1		97.3	97.0	96.3	95.2	98.9	96.5	94.7	96.6	96.7	96.9
L.pneumophila subsp. *pneumophila* GTC296	90.7	75.2	74.4	76.8	88.7	71.5	88.8	76.2	71.6	68.8	75.7		99.3	99.1	95.5	97.0	94.9	94.8	96.9	97.0	96.7
L. pneumophila subsp. *fraseri* GTC302	98.3	77.5	76.9	77.3	92.6	73.2	93.1	76.9	73.1	69.1	76.8	90.4		99.6	95.6	96.8	94.8	94.8	96.6	97.4	96.9
L. pneumophila subsp. *pascullei* GTC702	94.1	76.7	76.6	78.7	98.2	72.5	97.7	78.7	72.5	69.3	77.9	90.1	93.6		95.7	96.7	94.8	94.8	96.7	97.1	97.0
L. quateirensis GTC743	77.6	84.8	76.4	76.6	78.0	71.9	77.0	76.7	72.3	70.6	76.4	76.0	77.1	77.4		95.6	93.6	96.0	94.8	95.3	97.5
L. sainthelensi GIFU10392	78.9	77.4	81.2	88.8	77.7	72.4	78.2	88.2	72.5	68.8	90.9	77.2	79.1	78.7	76.8		96.6	94.6	97.0	97.6	97.2
L. santicrucis GIFU11746	78.6	75.6	80.3	92.2	78.6	72.7	78.1	90.8	72.0	69.5	90.9	76.9	77.8	79.6	76.3	89.5		93.9	95.4	94.7	95.4
L. shakespearei GTC701	75.5	76.6	76.1	75.0	75.9	69.7	74.6	76.3	72.1	69.4	76.2	74.1	75.1	75.9	77.3	76.3	76.5		94.4	94.9	96.0
L. tucsonensis GTC516	75.1	72.2	70.7	73.1	72.7	77.5	73.8	73.9	79.0	69.3	73.1	72.8	74.7	73.8	74.2	73.8	74.7	71.9		96.8	95.7
L. wadsworthii GIFU10062	79.2	76.8	82.0	81.9	78.0	72.6	77.9	80.2	71.1	70.2	81.3	77.9	78.8	78.8	76.2	80.9	80.9	75.1	73.5		96.4
L. worsleiensis GTC744	77.5	80.0	75.5	75.1	77.2	72.0	76.7	76.2	73.1	70.5	76.2	75.7	76.7	77.5	83.9	76.6	75.5	76.2	73.8	76.3	

Sequenced length of *dnaJ* gene is about 930 bp and that of 16S rDNA is about 1300 bp.

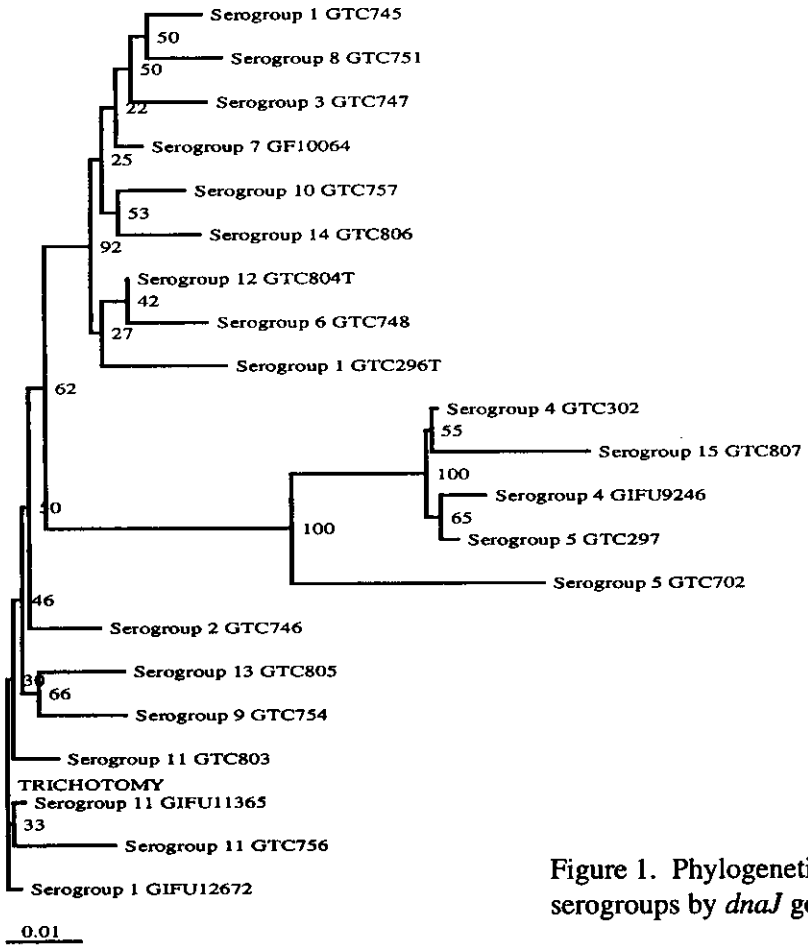

Figure 1. Phylogenetic tree of *L. pneumophila* serogroups by *dnaJ* gene nucleotide sequence.

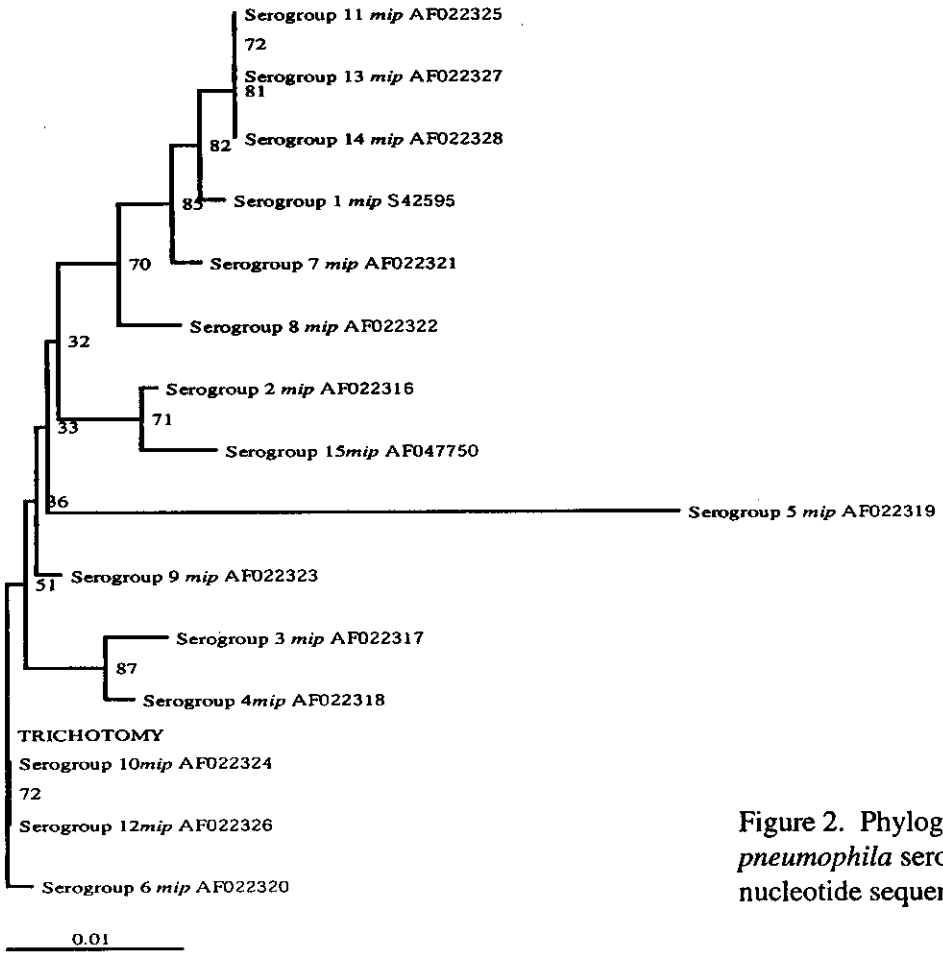

Figure 2. Phylogenetic tree of *L. pneumophila* serogroups by *mip* gene nucleotide sequence.

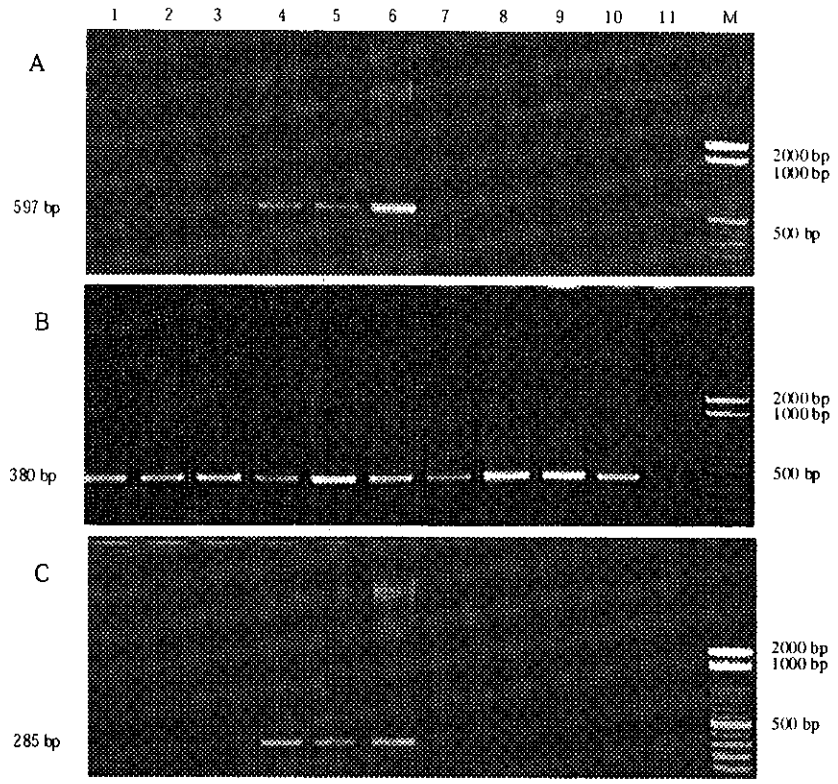

Figure 3. Primer specificity for *Legionella spp.*

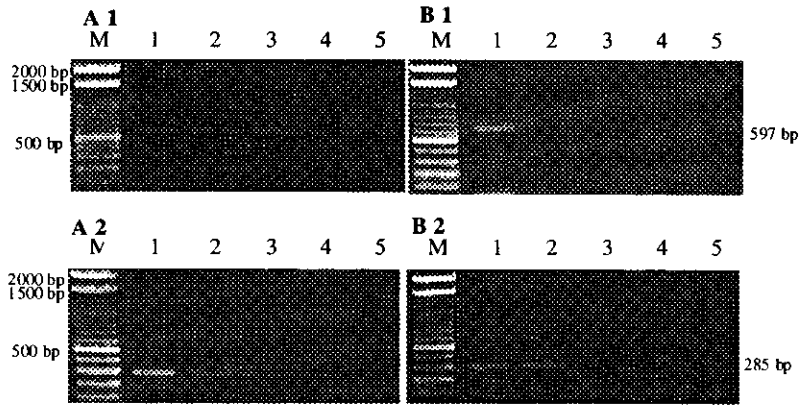

Figure 4. Primer sensitivity for *Legionella pneumophila* GTC 296^{T}

4．検査機関の精度管理に関する調査研究

平成14年度厚生労働科学特別研究
「生活環境におけるレジオネラ感染予防に関する研究」（H14—特別—047）

I. レジオネラ属菌検査方法精度管理

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はじめに

一般に多数の微生物が混在する環境水から特定菌種の細菌を定量培養するにはいろいろな制約が付きまとう。特に目標とする菌種の菌数が少なく検水の濃縮が必要な時、種々の要因によって濃縮操作後の菌の回収が不正確になる。遠心沈澱によって濃縮する時は遠心管の管壁に付着した菌を少量の液体で完全に回収するのは至難の技であり、ろ過膜でろ過した後にろ過膜上の菌を回収する際には、菌が膜の網目に捕捉されたままとどまり、決して100%の回収率は望めないという。しかも回収率は膜の種類や製造元によっても異なることが知られている。これら方法論の研究は概ね純培養菌の懸濁液を使って実施されており、実際の環境水を用いた場合には条件が一段と複雑になることは容易に推測出来る。更に酸性緩衝液を用いるなどの前処理に対する目的菌種・菌株の感受性は様々であり、目的菌たとえばレジオネラ属菌用に開発された選択培地の発育支持力も菌種・菌株によって異なる。

これら方法論上の問題点に加えて、検査担当者の意識と資質が検査成績に大きく影響する。厚生労働省健康局生活衛生課はこの点に着目し、平成14年度厚生労働科学特別研究「生活環境におけるレジオネラ感染予防に関する研究」で「レジオネラ属菌検査方法精度管理」をとりあげ、地方衛生研究所などの公的機関を対象に調査を実施することになった。

対象と方法

あらかじめ各都道府県・指定都市衛生主管部(局)長宛の平成15年1月31日付け健衛発第0131001号(厚生労働省健康局生活衛生課長名)の通知「レジオネラ属菌の検査方法の精度管理について」および精度管理参加の意志を問う連絡票に対する回答に基づいて厚労省健康局生活衛生課で作成された一覧表の参加機関72施設を対象とした。しかし送付試料の凍結などの不測の事態による検査実施日の変更のため実施施設数が減少し、または連絡がなかったり報告書が提出されなかった施設があったため、最終参加施設は69となった。対象施設はいずれも各都道府県・指定都市の地方衛生研究所、保健所等である(表1)。

1.　調査の方針と菌株の選定、3種検体の作成

検査方法調査は下記の目標達成を目指して立案した。

1)　レジオネラ属菌と他属菌との鑑別

擬似環境水に懸濁する菌株は、レジオネラ用の酸処理に耐えてGVPC培地に発育する菌株(表2)として *Achromobacter xylosoxidans*　GIFU 1050 = ATCC 9220を選定し、*L. pneumophila* SG1 GTC 745= GIFU 9135=ATCC 33153と併用した

2) *L. pneumophila* の血清群決定→常用抗血清の種類を知る

現在は *L. pneumophila* SGs 1-15の国産抗血清が市販されている。この抗血清を所有し常用しているか否かを知るため、*L. pneumophila* SG 7のパイロット株

GTC 750=GIFU　10064=ATCC 33823 を選定した。

3) レジオネラ属菌が推定され *L. pneumophila* の群別血清に凝集しない菌株の菌種の同定には DNA-DNA hybridization(DDH)が不可欠である。各施設での DDH 使用の有無を知るための菌株として、*L. longbeachae* の基準株 GTC　301^T = GIFU 9245^T = ATCC 33462^T を選定した。

これら 4 菌株を人工環境水由来株として用い、*L. pneumophila* SG1 GTC 745 と *Achromobacter xylosoxidans*　GIFU　1050 は混合菌液(検体 A)、*L. pneumophila*　SG7　GTC 750(検体 B)及び *L. longbeachae*　GTC 301^T(検体 C)は単独懸濁液とした、

2.　試料の配布

これら 3 種類の懸濁液(A, B および C)各 3ml ずつを、下記の添付書類 4 点とともに分担研究者研究施設から、平成 15 年 3 月 10 日に参加検査機関宛に日本通運(株)の宅配便(ペリカン便)の冷蔵保存で発送した。包装には日本細菌学会の教育用菌株搬送用の梱包材料を用いた。

添付書類：(1) レジオネラ属菌検査実施方法
(2) 成績表
(3) 試料受領と結果報告書
(4) 検査実施情報

3. 各検査機関での作業手順［上記(1)レジオネラ属菌検査実施方法より］

1)各容器に表記された試料番号を、報告書と成績表の所定欄に正確に転記する。試料番号は各施設の各試料に固有の番号で、他施設のどの試料とも共通しない。
2) 各容器の密栓を確認し、voltex mixer で 1 分間混和する。
3) 滅菌蒸留水 900μl に各菌液 100μl を加えてよく混和し、10 倍稀釈菌液 1ml 作製。
4) レジオネラ用選択培地平板 2 枚に、原液（未希釈菌液）と 10 倍希釈菌液をそれぞれ 100μl ずつ塗布する。
5) 原液（未希釈菌液） 1 ml を滅菌蒸留水 500ml に加え、よく混和し、これを擬似環境水とする。
6) 日常使用している方法で擬似環境水 100ml からレジオネラ属菌を検出する。
7) レジオネラ属菌陽性であれば、菌数を算出し、菌種、血清群を同定する。
8) 試料配付時に添付する報告書及び成績表に必要事項を記入し、速やかに郵送又は FAX で返送する。

(注意事項)
(1) 菌液試料到着後、できるだけ速やかに検査すること。
(2) 密栓した菌液容器を激しく震盪すると内部にエアロゾルが充満する可能性がある。開栓とその後の作業は安全キャビネット内で行うこと。

4. 研究の実施経過

3 月 10 日に精度管理参加各施設に対して菌液検体(3 種 1 組)を発送した。北海道から九州に至るまで搬送中の温度を一定に保つよう、民間宅配業者のクール宅配便を利用したにも拘わらず 3 月 14 日に隣県の参加施設から"検体凍結"の連絡を受けた。当日および翌日に全参加施設に検体到着時の状況について問い合わせたところ、20 施設から凍結/凍結疑いの報告を受けた。そこで当該施設に再検査実施の意

向を問い会わせ、3 月 17 日に再検査受諾 15 施設に再度菌液検体を送付した(表 1)。
各施設(1 施設を除く全参加施設)からは 3 月 28 日までに試験成績が報告されたので、それを解析し報告書をまとめた。

5. 研究成果の概要

Leginella pneumophila SG1 は調査に参加したすべての施設で検出されなかった。検体発送後に経日的に培養した検体 A では所期の菌数が回収でき、また *L. pneumophila* SG7(検体 B)と *L. longbeachae*(検体 C)は各施設で発育したのに検体 A のみが参加施設で発育しなかった理由は今後の検討に俟たねばならない。検体 A の擬似環境水中の *Achromobacter xylosoxidans* は、培養に成功したすべての施設(68/74 施設)がレジオネラ属菌種でないと報告した。
Legionella pneumophila 血清群 7 は、21 の施設が培養に成功したが、血清群を正しく同定したのは 9 施設に留まった。SG7 を含む混合血清群((SG2～14)であると報告してきた 2 施設を加えても 52%(11/21 施設)の施設のみが正しい血清群を決定できたにすぎなかった。残る 10 施設中 7 施設では血清群 7 を含まない抗血清セットを常用しており、3 施設は情報なし／未実施の報告であった。
Legionalla longbeachae を検体とした場合(検体 C)は、58 施設が集落を得ているが、正しい菌種名を報告したのは 18 施設に留まった(31%, 18/58 施設)。正しく同定できた施設は全て DNA-DNA- hybridization 法を実施していた。誤同定結果を報告した 5 施設は全て PCR や生化学性状により判定していた。残る 44 施設では型別不能、*L. pnemophila* 以外のレジオネラなどの報告であり、抗血清による判定のみを実施していると思われた。
汚染と思われる結果は全体で 6 例、2.7%(74 施設 x3 検体)であったが、当該菌株を回収・同定していないので菌種は不明である。当該菌の混入経路も不明であるが、爾後の確認培養のため検体発送時に手許に残しておいた検体からは目的菌以外の混入菌は発育しなかった。
検体輸送時の問題等があり、当初予定の通りの生菌数の菌液検体が届いていない事が考えられたので、各施設における検出率、定量数を調査できなかった。

5. 研究により得られた成果の今後の活用・提供

今回の精度管理調査の結果のまとめを参加各施設に送り、各施設での検査方法の妥当性等を再考する資料となることを期待する。*L. pneumophila* 血清群 7-15 に対する抗血清が入手可能であるにも拘わらず一部の施設でしか使用されていないこと、レジオネラ症防止指針に明記されている DNA-DNA hybridization を実施していない施設があることなどに着目し、これら方法の導入を啓蒙しなければならない。特に今回の対象施設は地方衛生研究所および保健所などの公的機関であり、各地域の中核～指導的立場にある。このような施設では抗血清や DDH レジオネラを常備し、その検査手技に熟達しておくことを啓蒙する必要がある。その上で、レジオネラ属菌と推定され、菌種・血清群が同定出来ない菌株は、地域の公的機関に依頼すれば同定可能であることを民間検査施設に周知徹底させねばならない。
今回の精度管理事業では検体輸送中に検体(菌液)が凍結するという事態が発生した。宅配業者により温度管理の考え方が異なるようなので、生物学的試料の輸送には注意が必要であることが判明した。今後同様の事業を行う際には、輸送業者とよく協議の上、委託をする必要がある。

添付書類（1）

レジオネラ属菌検査実施方法

平成１５年3 月10 日

（１）試料の配布（３月１０日に日通冷蔵宅配便で発送）

厚生労働科学研究分担研究者の研究施設から、参加検査機関宛に人工環境水由来の３菌株を含む懸濁液各３ml ずつをお送りします。

（２）各検査機関での作業手順

1. 各容器に表記された**試料番号**を、報告書と成績表の所定欄に正確に転記して下さい。各試料番号は貴施設の各試料に固有の番号で、他施設のどの試料とも共通しませんので、御注意下さい。
2. 各容器の密栓を確認したうえで、voltex mixer で１分間混和して下さい。
3. 滅菌蒸留水 900μl に各菌液 100μl を加えてよく混和し、10 倍稀釈菌液 1 ml を作ります。
4. レジオネラ用選択培地平板２枚に、原液（未希釈菌液）と 10 倍希釈菌液をそれぞれ 100μl ずつ塗布して下さい。
5. 原液（未希釈菌液）１ml を滅菌蒸留水 500ml に加え、よく混和し、これを**擬似環境水**とします。
6. 日常使用している方法で擬似環境水 100ml からレジオネラ属菌検出を試みて下さい。
7. レジオネラ属菌陽性であれば、菌数を算出し、菌種、血清群を同定して下さい。
8. 試料配付時に添付する回答用紙に必要事項を記入し、速やかに郵送又は FAX で返送して下さい。

（注意事項）

1. 菌液試料が到着すれば、できるだけ速やかに検査をして下さい。
2. 密栓した菌液容器を激しく震盪すると内部にエアロゾルが充満する可能性がありますので、開栓とその後の作業は安全キャビネット内で行って下さい。

（３）結果の評価と解析

各検査機関からの回答を評価解析の上、結果を各機関にお送りします。報告書を作成する際には、個別の検査機関名が明らかにならないように致します。

（３）事業実施責任者

「生活環境におけるレジオネラ感染予防に関する研究」分担研究者：

藪内　英子（前岐阜大学医学部微生物学講座）

添付書類 (2)　　　　　　　　成　績　表　　　　貴施設名＿＿＿＿＿＿＿＿＿

原液およびその10倍希釈液中のレジオネラ属菌とその他の菌の菌数				
受領した試料番号	原液100μl中生菌(集落)数		10倍希釈液100μl中生菌(集落)数	
	レジオネラ属菌	その他の菌	レジオネラ属菌	その他の菌

疑似環境水(500mlに希釈)中のレジオネラ属菌とその他の菌の菌数					
			前処理なし	酸 処 理	熱 処 理
試料番号＿＿＿＿ 遠心またはろ過による濃縮のために使用した擬似環境水の量 ＿＿＿＿＿ ml					
濃縮後液量＿＿＿ml	平板上集落数	レジオネラ属菌			
		その他の菌			
	原液中CFU/100ml	レジオネラ属菌			
		その他の菌			
塗布液量＿＿＿ml	レジオネラ菌種				
	血清群				

疑似環境水(500mlに希釈)中のレジオネラ属菌とその他の菌の菌数					
			前処理なし	酸 処 理	熱 処 理
試料番号＿＿＿＿ 遠心またはろ過による濃縮のために使用した擬似環境水の量 ＿＿＿＿＿ ml					
濃縮後液量＿＿＿ml	平板上集落数	レジオネラ属菌			
		その他の菌			
	原液中CFU/100ml	レジオネラ属菌			
		その他の菌			
塗布液量＿＿＿ml	レジオネラ菌種				
	血清群				

疑似環境水(500mlに希釈)中のレジオネラ属菌とその他の菌の菌数					
			前処理なし	酸 処 理	熱 処 理
試料番号＿＿＿＿ 遠心またはろ過による濃縮のために使用した擬似環境水の量 ＿＿＿＿＿ ml					
濃縮後液量＿＿＿ml	平板上集落数	レジオネラ属菌			
		その他の菌			
	原液中CFU/100ml	レジオネラ属菌			
		その他の菌			
塗布液量＿＿＿ml	レジオネラ菌種				
	血清群				

＊：実施していない方法の成績欄には斜線を引いて下さい．実施した検査結果が陰性の場合は“不検出”または“陰性”と記載して下さい．