Interpreting the Results of the Conventional Plate Culture and Gene Detection Methods for *Legionella* Detection in Environmental Water Samples

HIROAKI INOUE*

Tsukuba Research Laboratories, Aquas Corporation, 4-4 Midorigahara, Tsukuba, Ibaraki 300-2646, Japan

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The conventional plate culture method is widely used as a method for detection of *Legionella* in environmental water samples, but to obtain results takes more than a week. Because it is much quicker, the gene detection method has become widespread as an alternative detection method. However, the results of gene detection and plate culture methods may differ even when the same sample is examined; the gene detection method shows a higher detection ratio than the plate culture method. The reason for this difference is that the plate culture method detects *Legionella* cells that have the ability to form colonies on an agar plate, whereas the gene detection method detects any *Legionella* genes present regardless of the state of the *Legionella*. In this paper, we consider the factors that cause differences between the results of the plate culture and gene detection methods, and how to interpret the results of each.

**Key words**: Gene detection / *Legionella* / Plate culture / Viable but nonculturable (VBNC).

INTRODUCTION

*Legionella* species are Gram-negative bacteria found in artificial water environments such as cooling towers, whirlpool spas and hot water supplies. *Legionella* cause a serious form of pneumonia called Legionnaires' disease in humans if a susceptible host inhales aerosolized water containing the bacteria or aspirates water containing the bacteria (Fields et al., 2002). Therefore, *Legionella* detection and control in water systems are very important to public health. As a method for *Legionella* detection in environmental water, the conventional plate culture method is the most widely used. However, it takes more than a week to detect *Legionella*. On the other hand, gene detection methods have been used as an alternative to the culture method because *Legionella* genes can be detected within a few hours.

Whiley and Taylor (2016) summarized the results of 28 research studies comparing the results of the plate culture and the gene detection methods (quantitative PCR) that were reported during the 10 years from 2003 to 2013. The detection ratio of *Legionella* with the gene detection method was 72% (2856/3967), whereas the detection ratio of *Legionella* with the plate culture method was 34% (1331/3967). Thus, the results of the two detection methods often differ, and the detection ratio of *Legionella* is typically higher for the gene detection method compared with the culture method.

Here, we consider the factors that cause differences in the results of the plate culture and gene detection methods. In this paper, we describe how to interpret the results of both detection methods based on previous knowledge about the problems of the gene detection and plate culture methods and the factors that cause differences in the results of both detection methods.

PROBLEMS WITH THE PLATE CULTURE AND GENE DETECTION METHODS

As described following, several factors affect the accuracy of the plate culture and gene detection methods. Consequently, these factors may make a difference of results both methods.
Problems with the plate culture method

The standard detection method for Legionella from environmental water samples by the plate culture method is described in ISO 11731 (2017). The method has many steps to follow and it is more complicated than methods for the detection of many other bacteria. These steps include the following: 1) Concentration of water samples, 2) Pretreatment of concentrated samples, 3) Inoculation to selective agar plates, 4) Cultivation and counting, and 5) Identification of Legionella. In order to maintain the accuracy of Legionella detection, it is important to minimize the loss of Legionella cells in each step.

Concentration of water samples

Concentrating the water samples is necessary when detecting Legionella from environmental water samples, because low levels of Legionella populations need be detected. The methods for concentrating the water samples include a centrifugation method and a filtration method. In the centrifugation method, microorganisms in water samples are pelleted by centrifugal force and the supernatant is removed. The pellet is then resuspended in a small amount of sterilized water. On the other hand, in the filtration method a water sample is filtered using a membrane filter with pores smaller than Legionella cells (pore size: 0.2 to 0.45 µm), and the filtration residue is resuspended in a small amount of sterilized water.

According to Boulanger and Edelstein’s report (1995), the recovery ratio for the centrifugation method is about 30%, whereas the recovery ratio for the filtration method is about 50%. However, in the case of the centrifugation method, the recovery ratio varies depending on the centrifugal force, centrifugation time, supernatant removal method, and the method of resuspending the sediments. In the case of the filtration method, the differences in the recovery ratio and filtration time depend on the type and pore size of the membrane filter (Smith et al., 1993; Edagawa et al., 2013). Therefore, it is necessary to obtain the recovery ratio of Legionella by the concentration method before carrying out the process to detect Legionella. Obviously, the concentration methods used should be those that result in the highest possible recovery.

Pretreatment of concentrated samples

Microorganisms other than Legionella are generally present at higher concentrations than Legionella in environmental water samples. During the detection of Legionella in the samples, inhibiting the growth of nontarget microorganisms is necessary to facilitate the growth of Legionella. To inhibit nontarget organisms, acid treatment using 0.2 M HCl-KCl buffer (pH 2.2) (Bopp et al., 1981) and heat treatment at 50 °C for 30 minutes (Dennis et al., 1984b) are generally used as pretreatments. Citric acid buffer (Kasuga et al., 2002) and acid-phosphate buffer (Inoue et al., 2004a) have been proposed as acid treatment buffers, and their effectiveness has been reported. Since the ability to suppress contaminating microorganisms differs between acid treatment and heat treatment, a method suitable for examining water samples is desirable. Combining these pretreatments is also possible. In this case, heat treatment is performed first, and then samples are cooled to room temperature and treated with acid buffer (Kasuga et al., 1999; Inoue et al., 2014).

These pretreatments are carried out to reduce nontarget microorganisms. However, Legionella can also be damaged if the pH is too low, the acid treatment time is too long, or if the treatment temperature is too high or the heat treatment time is too long. Therefore, care should be taken when pretreating samples.

Types of selective agar plates

Selective agar plates contain media that inhibit the growth of nontarget microorganisms and facilitate the growth of target microorganisms. The selective agar plates for Legionella are generally non-selective BCYE-a (Edelstein, 1981) agar plates containing vancomycin and polymyxin B to inhibit the growth of bacteria other than Legionella, and cycloheximide (Dennis et al., 1984a), amphotericin B (Okuda et al., 1984) or natamycin (Edelstein and Edelstein, 1996) to inhibit the growth of fungi. Various selective agar plates are available as commercial products, but differences in the growth of Legionella often occur depending on the manufacturer even if the medium is supposedly the same. In addition, when the culture medium is prepared by oneself, it is desirable to evaluate the performance of the culture medium in advance because the growth ability of Legionella varies depending on the type of agar, yeast extract, and the activated charcoal powder used.

Effect of non-target microorganisms

When the population of nontarget microorganisms in the water samples is not sufficiently reduced by the pretreatment, the growth of Legionella is inhibited even when selective agar plates are used (FIG. 1). Various microorganisms inhabit environmental water sources, and the pretreatments will not be effective against nontarget microorganisms that are resistant to acid, heat, or antibiotics in the selective agar plates. When many contaminating microorganisms grow on the agar plates, Legionella colonies are not detected even if Legionella is present in the water samples (Inoue et al., 2014). In this case, the presence of Legionella in the sample water is unknown; it is not necessarily “not detected”. Therefore, when inhibitory effects of
nontarget microorganisms are clearly seen, to avoid misunderstanding of the examination results, one should not simply use “not detected”, but rather note that the presence of *Legionella* was not determined due to the effects of nontarget microorganisms. In this case, we use the notation “undetectable” and clearly distinguish it from “not detected”. As countermeasures for “undetectable” results, pretreatment using a combination of heat treatment and acid treatment, and the use of CAT α (Inoue et al., 2006; Inoue et al., 2014) agar plates containing selective agents are generally effective.

**Non-colony-forming Legionella**

The plate culture method is only capable of detecting *Legionella* that form colonies on agar plates. Therefore, *Legionella* that do not form colonies on agar plates, including not only dead cells but also *Legionella* in a viable but non-culturable (VBNC) state, cannot be detected by the plate culture method. Viable *Legionella* that cannot be detected by the plate culture method commonly inhabit environmental water.

**Problems with the gene detection methods**

Gene detection methods are used to amplify and detect *Legionella* genes using polymerase chain reaction (PCR) or loop-mediated isothermal amplification (LAMP). In the quantitative PCR method using real-time PCR, *Legionella* genes can be quantified from a calibration curve created using a positive control. The gene detection method is specific for detecting genes from *Legionella*, so if a gene is present, it is detected regardless whether the bacteria are alive or dead.

**Primer specificity**

For the PCR and LAMP methods, short oligonucleotide primers that serve as the starting point for gene amplification have been designed to amplify the *Legionella* 16S rRNA gene, 5S rRNA gene, *mip* gene and other relevant genes. Since different *Legionella* species have slightly different gene sequences, the species of *Legionella* that can be detected differ depending on the primers used. That is, when using a single set of primers, the PCR (include real-time PCR) method and the LAMP method do not detect all *Legionella* genes with the same sensitivity, and depending on the species of *Legionella*, some will not be detected at all or the detection sensitivity will be very low. For example, the instructions for the CycleavePCR® *Legionella* 16S rRNA Detection Kit (Takara Bio, Japan) state that when the PCR amplification efficiency for *L. pneumophila* is 100%, the amplification efficiencies for *L. cherrii* and *L. oakridgensis* are less than 1%. In addition, the Loopamp® *Legionella* detection kit E (Eiken Chemical, Japan), which uses the LAMP method, cannot detect *L. londiniensis*, and a primer set (Nippon Gene, Japan) for detecting *L. londiniensis* by the LAMP method is sold separately. Similarly, *L. thermali* (Ishizaki et al., 2016), which was isolated from hot spring water, could not be detected by the Loopamp® *Legionella* detection kit E. Thus, all species of *Legionella* will be difficult to detect by a single gene detection method.

**Effect of enzyme inhibitors**

PCR and LAMP methods detect target genes by amplifying DNA with a DNA polymerase, but
amplification of DNA is inhibited if the sample contains amplification reaction inhibitors. Various amplification reaction inhibitors are present in environmental water samples. Humic acid and fulvic acid (Watson et al., 2000), which are common in the soil, strongly inhibit PCR amplification. Hot spring waters can also contain these substances, so care must be taken when examining hot spring water. Some commercially available detection kits for *Legionella* contain an internal control for detecting reaction inhibitors; their use can confirm the presence or absence of reaction inhibitors. When preparing PCR reaction solutions by oneself or when using a LAMP kit, the presence or absence of reaction inhibitors can be confirmed by adding a positive control to the sample (Edagawa et al., 2009). Reaction inhibitors can be removed from the extracted DNA using a commercially available purification kit. In particular, kits designed for extraction and purification of DNA from soil are generally effective. It is desirable to verify the removal of inhibitors and the recovery ratio of the DNA prior to carrying out *Legionella* detection assays.

**Selective detection of viable *Legionella* by the gene detection methods**

Here we describe a technique which is closely related in the differences between the results of the plate culture method and the gene detection method.

In order to specifically detect only viable *Legionella*, methods to suppress the amplification of DNA from dead bacteria have been developed (Nogva et al., 2003). Ethidium monoazide (EMA) and propidium monoazide (PMA) are selective membrane-permeable dyes that penetrate into dead cells (more precisely, cells with damaged cell membranes) and bind to DNA to inhibit DNA amplification by PCR. On the other hand, in the case of viable cells with undamaged membranes, DNA amplification by PCR is not inhibited because EMA cannot penetrate into cells (Fig. 2). The use of EMA (Chang et al., 2009; Chang et al., 2010; Chen et al., 2010; Delgado-Viscogliosi et al., 2009; Qin et al., 2012; Inoue et al., 2015a) and PMA (Chang et al., 2010; Yáñez et al., 2011; Slimani et al., 2012) for the PCR quantification of *Legionella* have been described in a number of studies. In evaluations of bath water, EMA treatment halved the discrepancy between the PCR method detection and the plate culture method non-detection (Inoue et al., 2015a). However, countermeasures such as excluding water samples with high turbidity are required since EMA treatment will be inhibited by turbidity in the sample water.

**FACTORS THAT CAUSE DIFFERENCES IN THE RESULTS BETWEEN GENE DETECTION AND PLATE CULTURE METHODS**

The major factors that result in differences between the results of the gene detection method and the plate culture method are the presence of residual *Legionella* genes in the dead *Legionella* cells and the presence of unculturable *Legionella*. Another factor is the variability of the results when the *Legionella* levels are close the
detection limit of each method.

**Presence of the dead Legionella**

Dead *Legionella* cannot be detected by the plate culture method. In contrast, the gene detection method detects not only dead *Legionella* but also residual *Legionella* genes. The present of a certain level of the dead *Legionella* cells in the water systems are expected, and it is consider that the dead *Legionella* cells increase by the active sterilization. For example, DNA will be degraded by the strong oxidizing power of chlorine (Bej et al., 1991; Prince and Andrus, 1992). However, a time lag exists between the time necessary for *L. pneumophila* to be sterilized with chlorine and the time for *L. pneumophila* genes to be degraded with chlorine (Inoue et al., 2004b) (TABLE 1). In addition, the detection ratio of *L. pneumophila* from cooling tower water samples using the gene detection method is higher than the detection ratio obtained with the plate culture method, since organic biocides used for disinfecting cooling towers have no gene degrading action (TABLE 2).

### TABLE 1. Effect of free residual chlorine on *L. pneumophila* detectable by the PCR and plate culture methods (Inoue et al., 2004b).

| Concentrations of free residual chlorine: | PCR<sup>a</sup> | Culture<sup>b</sup> | PCR<sup>c</sup> | Culture<sup>d</sup> | PCR<sup>e</sup> | Culture<sup>f</sup> |
|------------------------------------------|----------------|----------------|----------------|----------------|----------------|----------------|
| 10 mg/L                                  | +++            | 1.7 × 10<sup>7</sup> | +++            | 1.7 × 10<sup>7</sup> | +++            | 1.7 × 10<sup>7</sup> |
| 1 mg/L                                   | +              | ND             | +++            | ND             | +++            | ND             |
| 0.2 mg/L                                  | −              | ND             | ++             | ND             | +++            | ND             |
| 0.1 mg/L                                  | −              | ND             | −              | ND             | +++            | ND             |
| 0.01 mg/L                                 | −              | ND             | −              | ND             | ++             | ND             |
| 0.001 mg/L                                | −              | ND             | −              | ND             | +              | ND             |
| 0.0001 mg/L                               | −              | ND             | −              | ND             | −              | ND             |

<sup>a</sup> *L. pneumophila* ATCC33152 cells were suspended in 5 L of tap water to a concentration of approximately 10<sup>7</sup> CFU/100 mL, and appropriate concentrations of 12% sodium hypochlorite solution were added at room temperature (25°C). After 10, 30, 60, 120, 360, 720 and 1440 min, 500 mL of the mixture were dispensed into a sterile 500-mL polypropylene bottle with sodium hyposulfite. Collected samples were concentrated 100-fold by filtration, and *L. pneumophila* were detected by both the PCR and plate culture method.

<sup>b</sup> Intensity of amplified DNA band on the gel image is indicated at four levels: ++++, bright; ++, clear; +, dim but visible; −, not visible.

<sup>c</sup> CFU/100 mL; ND, not detected (less than 10 CFU/100 mL)

### TABLE 2. Effect of organic biocides on *L. pneumophila* detection by the PCR and plate culture methods (Inoue et al., 2004b).

| GA<sup>a</sup> | CMI<sup>b</sup> | BNPD<sup>c</sup> |
|----------------|----------------|----------------|
| PCR<sup>d</sup> | Culture<sup>e</sup> | PCR<sup>f</sup> | Culture<sup>g</sup> |
| PCR<sup>h</sup> | Culture<sup>i</sup> | PCR<sup>j</sup> | Culture<sup>k</sup> |
| Before treatment | +++ | 1.7 × 10<sup>7</sup> | +++ | 1.7 × 10<sup>7</sup> | +++ | 1.7 × 10<sup>7</sup> |
| After 24 h | +++ | ND | +++ | ND | +++ | 6 × 10 |
| 48 h | +++ | ND | +++ | ND | +++ | ND |

<sup>a</sup> Glutaraldehyde (20 mg/L)

<sup>b</sup> 5-Chloro-2-methyl-4-isothiazolin-3-one (2 mg/L)

<sup>c</sup> 2-Bromo-2-nitropropane-1,3-diol (10 mg/L)

<sup>d</sup> see TABLE 1 footnote
of a water system and the basis for differences between the gene detection and plate culture method results in water systems treated with biocides.

In the case of the heat treatment that circulates hot water in hot water supply systems, even if Legionella is sterilized, the genes are not degraded by heat. As a result, a discrepancy occurs such that the gene detection method is positive for the presence of Legionella, but the culture method is negative.

Presence of unculturable Legionella

Legionella have the ability to transition to a viable but non-culturable (VBNC) state under certain conditions. For example, L. pneumophila usually forms colonies on agar plates quite easily, but they can enter into a VBNC state when left for a long period of time under oligotrophic conditions (Steinert et al., 1997) or in response to low-concentration chlorination (Dusserre et al., 2008). When in the VBNC state, no colonies are formed on agar plates. However, if L. pneumophila in the VBNC state is infected with an amoeba, growth is observed within the amoeba, and colonies can subsequently form on agar plates. Edagawa et al. (2015) reported that Legionella was not detected by the standard plate culture method in bath water samples, but viable L. pneumophila was detected by the amoebic coculture method from the same samples. In addition, Ducret et al. (2014) reported that L. pneumophila in the VBNC state grew on BCYE agar plates supplemented with sodium pyruvate and glutamate. This medium may therefore be able to detect Legionella in the VBNC state.

On the other hand, several species of Legionella cannot be cultured in any known artificial media. L. lytica (Drożniński et al., 1991) and L. drancourtii (La Scola et al., 2004) are obligate intracellular parasites that cannot grow on BCYE agar plates, but grow within amoebae. In addition, some species such as L. drozanskii (Adeleka et al., 2001) cannot grow at culture temperatures of more than 35 °C and cannot be detected by the standard plate culture method. However, we detected L. lytica and L. drozanskii from cooling tower water samples by a combination of amoebic coculture and quantitative PCR (Inoue et al., 2019). Therefore, viable Legionella that cannot be detected by the standard plate culture method can exist in environmental water.

Wéry et al. (2008) showed the diversity of Legionella spp. in cooling tower waters by the single-strand conformation polymorphism and clone library analysis. We reported that Legionella spp. in cooling tower water samples were more diverse than in bath water samples based on EMA-PCR and clone library analysis (Inoue et al., 2015b).

FIG. 4 shows a comparison of the species of Legionella detected by the plate culture and EMA-PCR method in cooling tower water samples. Based on the culture method, L. pneumophila was the dominant
species (82%), but EMA-PCR showed only a small amount (3.4%). On the other hand, EMA-PCR was dominated by various uncultured Legionella species. Consequently, the presence of various uncultured Legionella groups that did not fall into existing species was shown.

These results suggest that many uncultured Legionella groups are present in the environment. Importantly, these groups are difficult to detect by the plate culture method.

**Effect of dispersion**

Even if the water sample is well agitated and uniformly dispersed during examination, the results of Legionella detection can vary when the number of Legionella cells is low because Legionella in the water sample are present as suspended particles (FIG. 5). Therefore, differences can occur between the results of the plate culture and gene detection method due to the effects of dispersion when the number of bacteria is near the limit of detection and the lower limit of detection for both methods is the same.

The detection results of Legionella were compared by the gene detection and plate culture methods. In this case, the colony forming units of Legionella detected in the plate culture method were close to the detection limit, and detection of Legionella was negative by the gene detection method and positive in the culture method (Furuhata et al., 2005; Inoue et al., 2015a).

**WHAT THE RESULTS OF THE CULTURE AND GENE DETECTION METHODS MEAN**

TABLE 3 shows the relationship between the state of Legionella in environmental water and whether it is detectable by the culture and gene detection methods. However, it is not a simple story, because various states and types of Legionella are mixed in actual environmental water samples. Therefore, understanding the Legionella state detected by each detection method is very important when interpreting the results of each detection method.
method. The culture method selectively detects only viable *Legionella* with the ability to form colonies on agar plates. In contrast, the gene detection method detects the presence of *Legionella* regardless of whether it is alive or dead. That is, both detect *Legionella*, but the range of *Legionella* detected actually differs greatly (Fig. 6). This is a major factor contributing to the higher detection ratio for the gene detection method compared to that of the plate culture method.

If *Legionella* is detected by the plate culture method, it is a direct indication that the water system is currently contaminated with viable *Legionella* with the ability to infect humans. On the other hand, if *Legionella* is only detected by the gene detection method, it indicates that *Legionella* genes are present in the water system. Since the genes are not necessarily derived from viable *Legionella*, their presence does not indicate an immediate danger. However, the presence of *Legionella* genes is important evidence that the current (or past) water system is (or was) contaminated with *Legionella*. In addition, since the presence of VBNC *Legionella* cannot be detected by the plate culture method, if the gene detection method is positive, not only dead *Legionella* but also VBNC *Legionella* can be present in the water systems of the plate culture method-negative. So further public health surveillance is required when the gene detection method-positive.

In this way, the results of the gene detection and the plate culture methods have different meanings, so the results cannot be simply compared (Whiley and Taylor, 2016). Therefore, we must understand and carefully interpret the meaning of the results of the gene detection and the plate culture methods.

**CONCLUSIONS**

To date, over 60 species of *Legionella* have been described (Euzéby 2019), but there is currently no method to detect all of them with the same sensitivity. Although the plate culture method is the simplest and will continue to be an important detection method in the future, elucidating overall *Legionella* contamination of environmental water using only the plate culture method is impossible. Comprehensive analysis combining the plate culture and gene detection methods is needed to elucidate the actual state of *Legionella* contamination.

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