

## Simultaneous detection of *Legionella* species and *Legionella pneumophila* by duplex PCR (dPCR) assay in cooling tower water samples from Jakarta, Indonesia

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### Abstrak

**Tujuan:** Metode kultur memiliki sensitivitas yang rendah dan memerlukan waktu yang lama untuk mendeteksi bakteri *Legionella*. Oleh karena itu, dalam penelitian ini dikembangkan uji PCR duplex (dPCR) untuk deteksi *Legionella* sp. dan *L. pneumophila* secara simultan pada sampel air tower. Metode kultur digunakan sebagai baku emas.

**Metode:** Dilakukan optimasi metode dPCR untuk mendapatkan teknik uji yang memiliki sensitivitas dan spesifisitas tinggi. Metode kemudian diuji pada 9 sampel air tower yang diperoleh dari 9 gedung di Jakarta. Untuk metode kultur, bakteri ditumbuhkan pada media selektif 'growth factor supplemented-buffered charcoal yeast extract' (BCYE).

**Hasil:** Dari 9 sampel yang diuji dengan dPCR, 6 menunjukkan positif *Legionella* sp., 1 positif *L. pneumophila*, dan 2 menunjukkan hasil uji negatif. Untuk sampel yang sama, metode kultur menunjukkan hasil uji negatif.

**Kesimpulan:** Uji dPCR adalah uji yang sangat sensitif dibandingkan dengan metode kultur, dan uji dPCR ini dapat digunakan untuk pemeriksaan rutin *Legionella* sp. dan *L. pneumophila* pada sampel air dari 'tower'. (*Med J Indones* 2010; 19:223-7)

### Abstract

**Aim:** Since culture method is time-consuming and has low sensitivity, we developed a duplex PCR (dPCR) assay for the detection of *Legionella* sp. and *L. pneumophila* in cooling tower samples. We used culture method as a gold standard.

**Methods:** Optimization of dPCR method was performed to obtain an assay with high sensitivity and specificity. The optimized method was used to detect *Legionella* sp. dan *L. pneumophila* in 9 samples obtained from 9 buildings in Jakarta. For culture method, the bacteria were grown or isolated on selective growth factor supplemented-buffered charcoal yeast extract (BCYE) media.

**Results:** Of 9 samples tested by dPCR assay, 6 were positive for *Legionella* species, 1 was positive for *L. pneumophila*, and 2 showed negative results. For the same samples, no *Legionella* sp. was detected by the culture method.

**Conclusion:** dPCR assay was much more sensitive than the culture method and was potentially used as a rapid, specific and sensitive test for routine detection of *Legionella* sp. dan for *L. pneumophila* in water samples. (*Med J Indones* 2010; 19:223-7)

**Key words:** BCYE media, mip gene, 16S-rRNA gene

*Legionella* sp. are the etiological agents that cause both legionnaires' disease and pontiac fever. The outbreak of legionnaires' disease was first reported in the United States at the American Legion Convention, Philadelphia.<sup>1</sup> Fifty-two species and 72 serogroups of *Legionella* have been identified.<sup>2</sup> Twenty species have been associated with fatal pneumonia (Legionnaires' disease) and a non-pneumonic self-limiting flu-like illness (pontiac fever).<sup>3</sup> Among *Legionella* sp., *L. pneumophila* is a predominant cause (90%) of all reported cases of legionellosis in the United States.<sup>4</sup>

*Legionella* sp. are ubiquitous in water environments and able to survive in extreme conditions.<sup>5</sup> Human

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infection occurs through inhalation of *Legionella* sp containing aerosols.<sup>6</sup> Potential sources of *Legionella* include *Legionella*-contaminated water in cooling towers and air conditioners, hot tubs, showerhead water, and public fountains.<sup>7, 8</sup> Among those sources, the cooling towers have been involved in some community outbreaks of legionellosis.<sup>9, 10</sup> Previously, Koide *et al.* reported that of 27 cooling tower water samples, 25 were positive for *Legionella* sp., and 14 of these contained *L. pneumophila*.<sup>11</sup>

In our laboratory, culture method is a routine examination for detection and isolation, whereas latex agglutination method was used for identification of *L. pneumophila* from

environmental samples. However, the methods have limitations i.e. time-consuming, high cost, and low sensitivity.<sup>12, 13</sup> A PCR-based detection, an attractive and sensitive technique, has been suggested as a way to overcome problems of the culture method.<sup>4, 12, 14</sup> For the reason, we developed and evaluated the duplex PCR (dPCR) assay for simultaneous detection of *Legionella sp.* and *L. pneumophila* in cooling tower water samples, and compared the sensitivity of the dPCR assay with the sensitivity of the culture method as a gold standard.

## METHODS

### Bacterial isolates

Bacterial isolates used in this study were *L. pneumophila* ATCC 33152, and wild strains of *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae*, *Enterobacter aerogenes*, *Streptococcus viridans*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Acinetobacter anitratus*, *Moraxella catharralis* and *Haemophilus influenzae*.

### Cooling tower water samples

Nine cooling-tower water samples were obtained from 9 buildings in Jakarta, Indonesia from November 2007 to June 2008. Four hundred milliliter of sample volume was collected in a sterile glass bottle from the basin of each cooling tower. Samples were filtered by sterile membrane Millipore 0.2 µm in class II biological safety cabinet. The filter was removed aseptically and placed into a 10 ml sterile physiological saline containing 15 ml-sterile tubes. The suspension was vortexed for 1 minute, then aliquoted into 1 ml. For culture, one aliquot (1 ml) was treated with HCl 0.2 N to achieve pH 2 and incubated at room temperature for 15 minutes, and 200 µl was inoculated onto selective growth factor supplemented-buffered charcoal yeast extract (BCYE) agar. For duplex PCR assay, another aliquot (1 ml) was centrifuged at 7500 rpm for 5 min. The supernatant was discarded and the pellet was used for DNA extraction.

### DNA extraction

The bacterial genomic DNA was extracted by DNA extraction kit (*QIAamp DNA mini kit* Qiagen) following the manufacturer's instructions. The extracts were suspended in a final elution volume of 40 µl. The DNA containing final elute was stored at -20°C until used. Seven micro liter of elute was used as template for duplex PCR assay.

### Primers

The primers used in this study were previously reported by Templeton *et al.*<sup>15</sup> A primer pair, forward (AGGCTAATCTTAAAGCGCC) and reverse (CCTG GCTCAGATTGAACG), was specific for 16S rRNA gene of *Legionella sp.* with a 212-bp PCR product. Another primer pair, forward (TGGTGACTGCAGCTGTTATG) and reverse (CATTGCTTCCGGATTAACAT), was specific for *mip* gene of *L. pneumophila* with a 124-bp PCR product.

### Duplex PCR assay

The assay was performed in a 25 µl of reaction mixture with the following compositions: 1x HotStar buffer, 1 mM MgCl<sub>2</sub>, 0.1 µM of each primer for 16S rRNA, 0.2 µM of each primer for *mip* gene, 0.025 U HostStart Taq DNA polymerase (Qiagen), and 7 µl of DNA template. Products were amplified using the following conditions (AB Applied Biosystems GeneAmp PCR system 2004): 95°C for 15 minutes, then 40 cycles of 94°C for 30 seconds, 59°C for 45 seconds, and 72°C for 30 seconds followed by one cycle of 72°C for 7 minutes. The amplification product was run on 9% polyacrylamide gel and DNA bands on gel was detected by Gel Doc XR with ultraviolet transilluminator (Bio-Rad).

### Sensitivity

*L. pneumophila* ATCC 33152 were used to establish the sensitivity of the duplex PCR assay. The bacteria was isolated on BCYE media and incubated at incubator (5-7% CO<sub>2</sub>) at 35° C for 48-72 h. Time needed for culture was 14 days.

Effects of different water samples on sensitivity of the dPCR assay were tested by a simulation technique using sterile 0.9% NaCl, sterile distilled water, and non-sterile tap water as diluents. For this purpose, the bacteria were suspended into sterile physiological saline until a cell density of 10<sup>8</sup> cells per ml. The suspension was then two-folded diluted. Ten milliliter of each dilution was added into sterile 0.9% NaCl, distilled water, and non-sterile tap water with final volume of 400 ml. The mixture was immediately processed as mentioned above for the preparation of cooling tower water samples.

### Specificity

Specificity of duplex PCR assay was evaluated towards *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae*, *Enterobacter aerogenes*, *Streptococcus viridans*, *Klebsiella*

*pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Acinetobacter anitratus*, *Moraxella catharralis* and *Haemophilus influenza*.

## RESULTS

### Sensitivity

In this study, the positive result of dPCR assay was defined as the existence of a 212-bp DNA band detected on acrylamide gel for the *Legionella sp.* and both 212 bp and 124 bp for *L. pneumophila* (Figure 1).

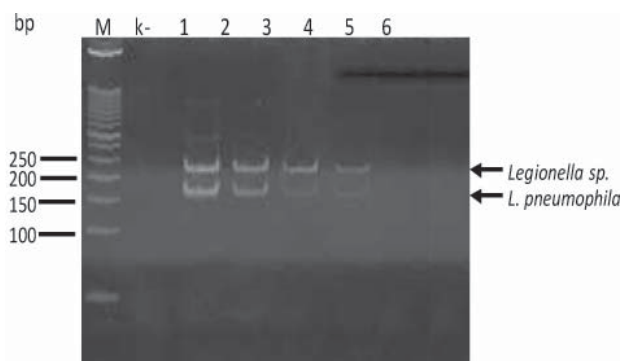


Figure 1. Detection of *L. pneumophila* genome with different concentrations by dPCR assay. M: DNA ladder. Line 1-6: 3.5 ng/ $\mu$ l, 0.35 ng/ $\mu$ l, 35 pg/ $\mu$ l, 3.5 pg/ $\mu$ l, 1.75 pg/ $\mu$ l, and 0.87 pg/ $\mu$ l respectively. k-: negative control. bp: base pairs.

Further dPCR assay on different water samples showed that the assay had same sensitivity ( $3.2 \times 10^1$  CFU/ 400 ml) for 0.9% NaCl and distilled water samples. The sensitivity decreased up to  $6.2 \times 10^1$  CFU/ 400 ml if the assay was tested for non-sterile tap water samples. The result indicated that non-sterile water decreased the sensitivity of dPCR assay. In addition, minimal detection of *Legionella sp.* and *L. pneumophila* DNA by dPCR assay was 3.5 pg/ $\mu$ l in 0.9% NaCl (Figure 1).

### Specificity

The dPCR assay, which was developed in this study, showed negative results or no cross-reactivity with *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae*, *Enterobacter aerogenes*, *Streptococcus viridans*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Acinetobacter anitratus*, *Moraxella catharralis*, and *Haemophilus influenza*.

### Detection of *Legionella sp.* and *L. pneumophila* in cooling tower water

Of 9 cooling tower water samples tested by dPCR assay, 6 were positive for *Legionella sp.*, 1 was positive for *L. pneumophila*, and 2 was negative for either *Legionella sp.* or *L. pneumophila* (Table 1; Figure 2). For culture method, all tested samples showed negative results (Table 1). Thus, the results showed that the dPCR assay was much more sensitive than the culture method.

Table 1. Results of the culture method and the dPCR assay for cooling tower water samples

Cooling tower	Culture method	dPCR assay
1	-	-
2	-	(+) <i>L. pneumophila</i>
3	-	(+) <i>Legionella sp.</i>
4	-	(+) <i>Legionella sp.</i>
5	-	(+) <i>Legionella sp.</i>
6	-	-
7	-	(+) <i>Legionella sp.</i>
8	-	(+) <i>Legionella sp.</i>
9	-	(+) <i>Legionella sp.</i>

(-): negative; (+): positive

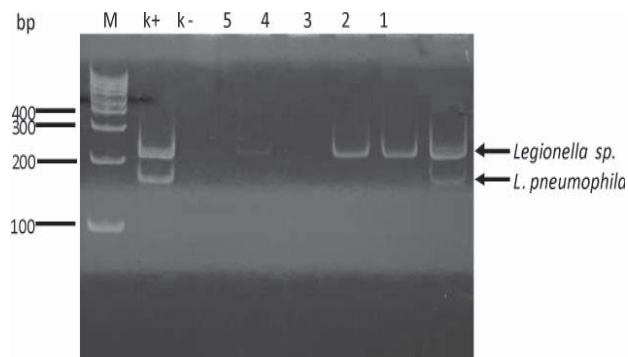


Figure 2. Results of duplex PCR assays. M: DNA ladder. Line 1-5: five examples of tested samples. k-: negative control. k+: positive control. bp: base pairs.

## DISCUSSION

Because of the potential for any cooling tower to harbor, amplify, and disseminate *Legionella sp.*, survey and monitoring of the bacteria are needed. The presence of *L. pneumophila* in certain environment may be used as a predictive risk factor for legionellosis. In this study, *Legionella sp.* could not be recovered by culture method from the tested samples. The results possibly due to the rapid loss of cultivable *L. pneumophila* and

its metabolic activity in cooling tower water samples. The factor that caused this phenomenon was not clear; however, some trace minerals have been known to play an important role in bacterial metabolism. Another study showed that *L. pneumophila* could not be cultured from cooling towers that were suspected as infection sources particularly when biocide have been used as disinfectant.<sup>16</sup> Moreover, lower levels of certain minerals such as iron, zinc, and potassium were important factors in survival and growth of *L. pneumophila*; in contrast, higher levels of minerals were toxic.<sup>15</sup> The bacterial populations that are released to an environment frequently face stresses due to limitation and changes in nutrient availability (temperature, salinity, oxygen and pH), and to adapt to such a stressful environment, bacteria often enter a viable but non-cultivable (VBNB) state.<sup>17</sup>

In contrast to culture method, the dPCR assay developed in this study provided an effective way to detect *Legionella sp.* and *L. pneumophila* simultaneously for environmental water samples. *Legionella sp.* were much more frequently detected than *L. pneumophila* (Table 1). Other works also reported the same results.<sup>11, 13</sup>

Comparing the dPCR to the culture assays showed that the dPCR assay was much more sensitive than the culture method (Table 1). There was one possible reason why the dPCR assay could detect *Legionella sp.* or *L. pneumophila* while the culture method could not; the PCR technique based on DNA amplification could detect any specific DNA without considering viable or cultivable bacteria as discussed above. Thus, result of the PCR assay is an indicator of the presence of a particular bacteria. This is a main advantage of the PCR technique that can be applied for monitoring of the presence of *Legionella* cells in environments especially in water supply of certain buildings.

Dealing with sensitivity, type of water sample influenced the sensitivity of the dPCR assay. The sensitivity of the method was the same when the assay was tested for sterile 0.9% NaCl or sterile distilled water, but it decreased twofold for non-sterile tap water samples. This result indicated that the water samples from environments could contain particular substances capable of inhibiting the PCR reaction. This result is different with that reported by Declerck *et al*<sup>18</sup> in which they found no PCR inhibitor in the tap water samples. This difference might be due to the different water sources that are possibly influenced by difference in certain region from where the water was supplied. In the future investigations, eradicating PCR inhibitors without performing distillation process is needed.

The major disadvantage of PCR assay developed in this study is its inability to evaluate the bacterial viability due to the persistence of DNA in cells or environment after cell death. Therefore, the assay results in an overestimation of the risk of infection because of false-positive results. As having been reported that the real risk from *Legionella* is determined by the living fraction of the total *Legionella* population, only living or viable *Legionella* cells can replicate in pulmonary macrophages that lead to severe pneumonia.<sup>4, 19</sup> Thus, further research is needed to develop a PCR assay that is able to discriminate between living and dead cells in order to measure *Legionella* infection risks and to prevent legionellosis.

In conclusion, the dPCR assay could be used as a rapid, specific and sensitive test for simultaneous detection of *Legionella sp.* and *Legionella pneumophila*. The assay was much more sensitive than the conventional culture method. However, two main works, eradication of PCR inhibitors and differentiation of viable from non-viable *Legionella* cells should be conducted in the future in order to achieve the surveillance and monitoring programs of legionellosis appropriately.

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