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## Original Article

### Screening of *Legionella pneumophila* from Well Water in Magetan Regency, Indonesia

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#### ABSTRACT

Legionellosis is a respiratory infection caused by *Legionella pneumophila*, a bacterium that can infect protozoa and human lung cells. The disease can be mild or severe, and sometimes fatal, especially in immunocompromised people. Some types of antibiotics that can be used to treat *Legionella* disease are macrolides such as rifampicin, azithromycin and clarithromycin, as well as fluoroquinolones such as levofloxacin and moxifloxacin. The aim was to investigate the distribution and prevalence of *Legionella* in well water. This study collected and tested water samples from a different well water in Magetan Regency, East Java, Indonesia using DNA extraction, two-step PCR methods, and visualization with 1.5% agarose gel in UV transluminator. The results were visualized with QGIS 3.28.6 and compared with other tests. The results showed that none of the eight water samples were contaminated with *L. pneumophila* compared with positive control (403 bp).

**Keywords:** *Legionella pneumophila*, well water, Magetan Regency, DNA extraction, and PCR.

**Highlights:** This Study used DNA extraction and two-step PCR methods to detect *Legionella* in water sources in Magetan Regency, East Java, Indonesia, and can provide useful information for public health authorities to prevent and control *Legionella* outbreaks and to improve the quality and safety of water sources in the area.

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## INTRODUCTION

The half of the 20th century saw widespread infection with a type of legionellosis caused by the bacterium *Legionella pneumophila*.<sup>1,2</sup> This type of bacteria has a morphology resembling the stem of  $\gamma$ -proteobacteria and can be found in freshwater habitats, moist soil, and compost material.<sup>3</sup> There are several grades of *Legionella* infection ranging from low-grade fever (Pontiac fever) to a potentially fatal form of pneumonia (Legionnaires' disease) that can affect anyone, particularly vulnerable hosts due to age, disease, immune suppression or other risk factors, such as smoking.<sup>4-7</sup>

*L. pneumophila* was the first bacteria described to multiply inside protozoan hosts, primarily aquatic amoeba, which had the idea that the bacteria's capacity to infect protozoa also allowed them to replicate inside human lung macrophages, a finding confirmed later through numerous studies.<sup>3</sup> Legionnaires' disease is an important but relatively uncommon respiratory infection that can cause great morbidity and mortality.<sup>8,9</sup> First recognized as a fatal cause of pneumonia more than three decades ago, little progress has been made in investigations, clinical and incident management, and public health responses to cases and outbreaks.<sup>10</sup> *Legionella* disease has spread to almost various countries around the world. In Indonesia, cases of this disease have been reported from 2010-2019 from foreign tourists traveling to Bali and West Java based on the discovery of cases from the country of origin. In addition, on May 30, 2023, the first 2 confirmed cases of Legionellosis (Indonesian citizen) have been reported in Bandung, West Java.<sup>11</sup>

Research conducted by Yasmon et al. from 9 cooling tower water samples taken from buildings in Jakarta, Indonesia from November 2007 to June 2008 tested with duplex PCR (dPCR) tests, 6 of them were positive for *Legionella sp.*, 1 positive for *L.*

*pneumophila*, and 2 negative for both.<sup>12</sup> Another study also examined *Legionella* bacteria where as many as 17 water samples were taken from 17 different sources, of which 10 samples were obtained from hospitals in North Jakarta, and 7 samples from West Jakarta. Water samples were all cultivated in all four media variants Culture media removed from the incubator on days 3, 7 and 10 to observe growth and presence of colonies corresponding to the characteristics of *L. pneumophila*. Eight of the 17 water samples showed colony morphology of bacteria with traits such as *L. pneumophila*. Sixty percent (6/10) of water samples from North Jakarta Hospital showed *L. pneumophila* colony suspects, while those from West Jakarta hospitals only showed 29% (2/7). Colony growth is suspected as *L. pneumophila* in media variant 1.<sup>13</sup>

The initial symptoms of Legionellosis disease are very similar to those of the flu (low-grade fever, headache, fatigue, joint and muscle pain, and loss of appetite). After a few days (1 or 2 days) more severe pneumonia symptoms may appear (high fever with a temperature of 39°C - 41°C, cough, dyspnea or difficulty breathing, chills, and chest pain). Gastrointestinal symptoms such as diarrhea and nausea may appear. In many cases severe pneumonia requires hospitalization and in some cases Legionellosis can lead to death. The probability of an immunocompromised person dying from Legionellosis is 40-80%. However, this figure can decrease by 5-30% if treatment or case management is carried out properly.<sup>14</sup>

Currently, treatment for *Legionella* disease usually involves administering antibiotics. Some types of antibiotics that can be used to treat *Legionella* disease are macrolides such as azithromycin and clarithromycin, as well as fluoroquinolones such as levofloxacin and moxifloxacin. Rifampicin may also be used to treat *Legionella's* disease. Fluoroquinolones should be used for 7-14 days, among macrolides azithromycin may be preferred

for 5-10 days. A potentially useful drug is also doxycycline (in the form of uncomplicated immunocompetent patients).<sup>15,16</sup>

*Legionella* detection can be done in several ways, such as traditional culture on buffered charcoal yeast extract agar, bronchoscopy to see signs of legionella bacteria in the lungs, thoracentesis to see signs of bacterial infection outside the lungs, and CT scan to get a picture of the condition of the lungs. For this reason, it is important to detect the spread of these bacteria in water samples in various sampling sites because bacterial detection is an important step for handling the spread of a disease because by detecting the location and characteristics of the bacteria spread, we can anticipate the source of infection and immediately plan further steps.<sup>17,18</sup>

An assay used for detecting *L. pneumophila* bacteria is two-step PCR. In two different tubes, for reverse transcription and amplification, have been prepared. That is why this variation is known as two-step PCR. Notably, both reactions have different conditions and materials are used. The first reaction uses reverse transcriptase enzymes, dNTPs, reaction buffers, oligo-primers (dT), and random primers to construct cDNA. After completing the reaction, a stock of cDNA is obtained. The stock of cDNA can be stored at adequate conditions or it can be used directly for gene expression studies. The second step is using cDNA as a template for measuring mRNA. In the second reaction, along with all PCR reagents, Taq DNA polymerase completes the reaction and is quantified using dyes or probes. At this stage, no reverse transcriptase reaction is required. The second reaction quantifies cDNA (formed from the first step). One-step PCR does have an easier and more efficient procedure, but Two-step RT-PCR switches quickly from RNA to more stable cDNA without knowing exactly which genes will be targeted downstream. It can choose various targets after seeing the initial qPCR results or

even store the cDNA samples indefinitely, and revisit them later to look for gene expression that might not have been thought of at first. One of the biggest advantages of the two-step method is that it can store reverse cDNA transcribed from RNA. In two-step PCR, three sets of primers are used, one set of oligo primers (dT), a set of random primers, and sequence-specific primers to subsequently have high specificity compared to the one-step method. This method performs very well for a small number of samples, but the use of additional steps makes it more susceptible to reaction failure and contamination.<sup>19</sup>

Various countries have recommended strategies to prevent the growth of *L. pneumophila*. Important differences can be seen in the way they measure hazardous concentrations, use water sampling frequency and *L. pneumophila* alertness levels, and institute necessary measures. The recommended strategy depends on local regulations, although this problem occurs worldwide because *Legionella* naturally exists in water. Geographic differences are not significant enough to require adjusting infection control practices by region.<sup>20</sup> The hope is that this detection can find out how the characteristics of bacteria live and multiply so that they can further develop drugs and vaccines that can reduce the severe impact of *Legionella* infection.

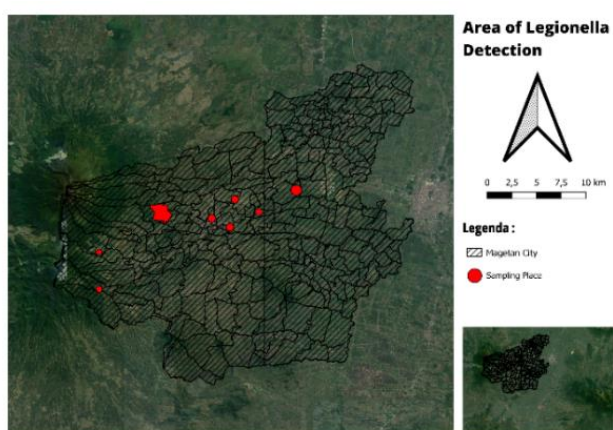
## MATERIALS AND METHODS

### Samples Collection

Water samples were collected in December 2021 from a variety of well that focus on the scope area of Magetan Regency, East Java, Indonesia. The location of the sample distribution was visualized with QGIS 3.28.6 by focusing on potential places for *Legionella* growth, namely tap water and also river water which is the main source of disease spread by *Legionella* bacteria in humans by obtaining 8 water samples from different places in Magetan Regency. Water

is transferred to a sterile container then tightly closed and taken to the laboratory for testing. To find out whether there are *Legionella* bacteria or not can be done by extracting bacterial DNA first which is then carried out PCR tests. This test can detect *Legionella* DNA in water samples or other environments. In addition, other tests such as culture tests and serology tests can be used to detect the presence of *Legionella*.<sup>21</sup>

**Figure 1.** Sample Collection Area for *Legionella* Detection



The research location was visualized with QGIS desktop ver 3.6.3 where water samples were collected at several points in Magetan Regency (Figure 1) with the following details in the Table 1.

**Table 1.** Location Details of Samples Collection

No.	Sampling location
1.	Singget Hamlet, Sukomoro District, Magetan Regency, East Java
2.	99CF+QR5, Jl. Raya Maospati, Kem, Sukomoro, Sukomoro District, Magetan Regency, East Java 63391 (Warung pecel pincuk marsel)
3.	Sumber Sawit Village, Sidorejo District, Magetan Regency, East Java
4.	Telaga sarangan, Plaosan District, Magetan Regency, East Java
5.	Jl. Mayjen Sukowati No.52, Bangunsari, Sukowinangun, Magetan District, Magetan Regency, East Java 63312

6. Warung Lesehan Suminar Jl. Panglima Sudirman No.2 Tambran, Magetan 63318 Indonesia
7. Jl. Sawo, Magetan Regency, East Java
8. Geni Langit Tourism, Genilangit, Poncol District, Magetan Regency

### DNA Extraction

DNA extraction using Zymo Kit™ by Zymo Research, USA, according to its label instructions. Preparing DNA / RNA Shield™ as much as 300 µL, PK Digestion Buffer 30 µL and Proteinase K 15 µL added to each water sample, then homogenized with vortex and incubated at 55°C for 5 hours to obtain maximum extraction results. The tissue sample dissolved in the liquid was then centrifuged at 15,000 g for 1 min. The supernatant (300 µl) is transferred to an DNase-free tube, added to an DNA lysis buffer of equal volume and vortexed. For DNA purification, the sample is placed on a yellow Spin-Away™ filter already attached to the collection tube and then centrifuged. The passing filter is transferred to a new collection tube, to which ethanol is added in a ratio of 1:1 to the sample volume and stirred. The mixture is then transferred to a green CG Zymo-Spin™ III column which is attached to a collection tube and centrifuged. Save the filtered sample, dispose of the solution on the collection tube and reinstall the collection tube. Then add 400 µL DNA Prep Buffer and centrifuge, store the filtered sample, dispose of the solution in the collection tube and reinstall the collection tube. The filtrate is discarded and the column is washed twice with 700 µl and 400 µl of DNA washing buffer. The column is then transferred to a DNase-free tube and centrifuged at maximum speed for 2 minutes to ensure the purity of the DNA sample. DNA samples were eluted with 100 µl of DNase/RNase-free water and centrifuged.

### Polymerase Chain Reaction

The analysis method uses the principle of thermal-based polymerization to

multiply DNA from bacteria contained in water samples so that it can be detected with gel documentation, first and second step PCR using GoTaq® DNA polymerase by Promega, USA, (contains taq DNA polymerase, dNTPs, MgCl<sub>2</sub>), *Legionella* primers (forward & reverse)<sup>22</sup>, nuclease free water, and RNA from samples. PCR was run with a pre-denaturation initial temperature of 95°C for 5 minutes and denaturation of 1 minute, annealing 55°C for 1 minute, extension of 72°C for 1 minute, final extension of 72°C for 10 minutes. After the second PCR product sample has been propagated then electrophoresis is performed to measure the bp length of the DNA.

### Electrophoresis

Prior to electrophoresis the sample was inserted into 1.5% agarose gel that had been perforated on one side. Electrophoresis is carried out by preparing 3 µl of 100 bp markers as a comparison or bp measuring target which was inserted into 1.5% agarose gel, then 3 µl of multiplied samples are also added to another hole, then put agarose gel into the electrophoresis instrument, then pour TAE buffer until the agarose gel is closed. Electrophoresis instruments allow DNA to move through these pores. The molecules in the sample will move towards the pole of the electrode opposite to its electric charge. Smaller, negatively charged molecules will move faster and farther away than larger, positively charged molecules. After running on electrophoresis with a power of 100 volt for 30 minutes then continue the staining stage (staining).

### Staining

The electrophoretic agarose gel is placed in a container filled with Ethidium bromide and shaken using a shaker for 30 minutes. The staining process is carried out for the staining process so that the sample molecules that have been separated can be seen.

### Gel Documentation

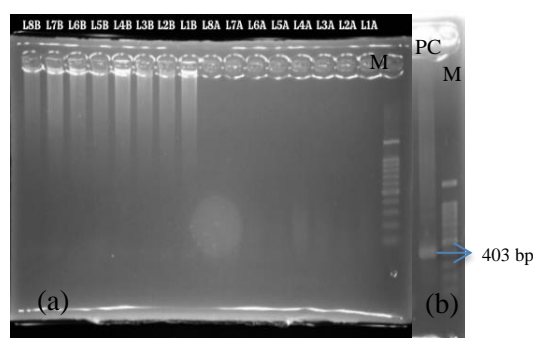
The agarose gel is then read using documentation gel with UV light radiation. The DNA bands on agarose gel appear according to their size, which can be measured by comparing DNA markers to known sizes. The readings are printed for analysis.

### Data Analysis

Readings with gel documentation were analyzed based on the molecular weight (bp) that appeared and then compared with the molecular weight (bp) of the target gene for *Legionella* bacteria, namely 403 bp. This is used to determine whether *Legionella* bacteria are present in the sample.

## RESULTS AND DISCUSSION

PCR is carried out in two stages where the first stage is carried out with the code L1A-L8A, then after PCR is complete, PCR is carried out with the same stage for the second time (L1B-L8B). The PCR results in electrophoresis are then visualized with gel documentation with the following results in the Figure 2.



**Figure 2.** (a) DNA Visualization of *Legionella pneumophila* from Magetan Regency (well water samples), (b) Positive Control (PC) of *Legionella pneumophila*, and DNA ladder (M) used 100 bp.

All of 8 water samples showed negative results for *L. pneumophila*. Warm pools and spas are the main routes of transmission of *Legionella*, which are optimal conditions for breeding as well as

containing nutrients for growth. The visualized in Figure 2a all amplified water not showing any bands. For positive control we can see at Figure 2b. Below which gave rise to the band at 403 bp that indicated contamination of *L. pneumophila* bacteria.

There are several factors that causes of bacterial detection to show negative results. Firstly, *Legionella* needs the amoeba's intracellular environment to reproduce. In aquatic habitats the amoeba itself acts as a host.<sup>22</sup> Thus, by the time *Legionella* bacteria leave the intracellular environment, they experience stress caused by changes in diet, pH, temperature, salinity, and oxygen. To adapt to the changing environment, *Legionella* enters a living state but cannot be cultured, meaning the bacteria are alive but not evolving.<sup>23,24</sup> When collecting water samples in Magetan, samples are stored using non-sterile bottles and long delivery to the laboratory, this causes a changes in pH, temperature, salinity, and oxygen so that *Legionella* bacteria in water samples die. This often leads to failure of *Legionella* cultures from environmental samples.<sup>25</sup> The suggestion for further research is to pay attention to storage of the sample to retain the bacteria before extraction.

Then according to the Indonesian Ministry of Health No: 416/MenKes/Per/IX/1990, these bacteria are increasingly active in water, especially in warm or hot temperature conditions. These bacteria can live at temperatures between 57 °C to 63 °C and thrive at temperatures of 30°C - 45°C. So that for storage of *Legionella* samples can maintain temperatures in the range of 30°C - 45°C. The results of this detection indicate the need for wider detection and attention to how to store samples from the point of collection to testing on a laboratory scale.<sup>26</sup>

Currently, steps that can be a recommendation to minimize the spread of *Legionella* disease is to pay attention to the source of water consumed and treat well

water that will be used first like cook water until cooked, namely at boiling temperatures (100°C) in order to ensure there are no bacteria contaminated in drinking water. In addition, maintenance and cleaning of air conditioning towers and condensers to prevent the development of *Legionella* at least 2 times a year cleaning and use of chlorine.

## STRENGTH AND LIMITATION

This research has advantages in supporting early detection of the spread of legionella bacteria which is the source of pneumonia, where early detection can be a benchmark for areas that must be of special concern for handling *Legionella* bacteria and characterizing the habitat of *Legionella* bacteria so as to reduce the risk of more severe spread, but this study has shortcomings where sampling from water samples is less widespread so that there are no bacteria which is detected from testing so that wider sampling is needed to determine the spread of the *Legionella* bacteria.

## CONCLUSIONS

Of the 8 samples taken from different places, all samples were negative for legionella bacteria.

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## ETHICAL CLEARANCE

This study does not require an ethical review.

## FUNDING

There is no funding for the research.

## CONFLICT OF INTEREST

All authors declare that there is no conflict of interest in writing this manuscript.

## AUTHOR CONTRIBUTION

Each author has contributed in the preparation of this study. Author THS, SF, and FA in this study is in charge of collecting of samples, NSF and AMW analyzing data, then NNH and MRHK writing article reviews. Meanwhile, EBAH is a principle investigator who provides study ideas and validates data.

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