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Detection of *Legionella pneumophila* bacteria from water sources in Palembang City, Indonesia

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Abstract. *Kautsar R, Fauziyah S, Aquaresta F, Widya AM, Fajar NS, Damayanti M, Sucipto TH. 2024. Detection of* Legionella pneumophila *bacteria from water sources in Palembang City, Indonesia. Biodiversitas 25: 1499-1504. Legionella* is a pathogenic bacterial genus that causes legionellosis. This bacteria is a gram-negative bacteria which is often found in warm and humid aquatic environments. *Legionella* spp. infection occurs due to inhalation of aerosols contaminated with *Legionella* spp. Until now, legionellosis is still a crucial public health problem in the world. Therefore, it is necessary to take preventive measures to detect the presence of *Legionella* spp. as early as possible. This study aimed to detect the presence of *Legionella pneumophila* from well water samples and drinking water sources in Palembang City using the Nested PCR method. The primers used in this study are specific primers that amplify the mip gene. The PCR amplification results showed that 2 of the 22 samples were positively contaminated with *L. pneumophila*. These positive samples came from well water and drinking water samples. Based on physical parameters, all samples are still safe for consumption because they have a normal temperature, are odorless and clear. Meanwhile, based on chemical parameters, the pH of all samples is still safe for consumption because it is still below the maximum threshold.

Keywords: Drinking water supply system, *Legionella pneumophila*, legionellosis, mip gene, nested PCR

INTRODUCTION

Legionella bacteria are pathogenic bacteria that cause legionellosis, a condition of chronic pneumonia (Zacharias et al. 2023). It is a Gram-negative rod bacteria or coccobacilli which can be found in warm and humid aquatic environments, as well as in various water sources such as water reservoirs, air conditioning systems (cooling towers), swimming pools, hospital shelters, offices and hotels, and housing (Moehario et al. 2019). *Legionella* is the only genus in the *Legionellaceae* family which consists of more than 50 species and 70 serogroups (Arslan-Aydogdu and Kimiran 2018).

Legionella spp. first isolated and described in 1947 as a "rickettsia-like" organism (Iliadi et al. 2022). In 1977, the genus *Legionella* was first recognized when *Legionella pneumophila* was identified as the causative agent of a major pneumonia outbreak that occurred in Philadelphia. Since then, many other *Legionella* species have been identified sequentially (Yang et al. 2023). To date, more than 50 species and 70 serogroups of *Legionella* spp. are known which belongs to the Legionellaceae family (Arslan-Aydogd and Kimiran 2018), but not all species cause Legionellosis (Yang et al. 2023). *Legionella* is assumed to survive and reproduce in water in three different forms; (i) generally, they exploit their ability to live in free-living protozoa, (ii) they often colonize the biofilms that line the inner surfaces of artificial water systems, (iii) although less frequently, *Legionella* can survive as planktonic forms in the water phase (Zacharias et al. 2023).

Legionella spp. infection occurs due to inhalation of aerosols or contaminated water droplets (Llewellyn et al. 2017). However, not all *Legionella* spp. can be an agent of legionellosis. About 70% of legionellosis cases are caused by *Legionella pneumophila* serogroup 1, another 20-30% are caused by other serogroups, and only 5-10% are caused by infection with non-pneumophila *Legionella* species, such as *L. micdadei* (60%), *L. bozemanii* (15%), *L. dumoffii* (10%), *L. longbeachae* (5%), and the remaining 10% are *L. feeleii*, *L. gormanii*, *L. jordanis*, *L. oakridgensis*, *L. wadsworthii* (Moehario et al. 2019). A study of 140 sporadic cases of community-acquired pneumonia in Japan from December 2006 to March 2019 showed that the most frequently isolated species was *L. pneumophila* (90.7%) (Yang et al. 2023).

In the United States, *Legionella* is the main cause of death due to waterborne plague and has increased almost 4-fold in the period 2000-2014 (Llewellyn et al. 2017). A case of severe pneumonia was reported in the city of San Miguel de Tucumán, Argentina in early September 2022. This case resulted in four deaths, three of whom were health workers. Laboratory test results stated that *Legionella* spp. from four clinical specimens from patients who died. *Legionella* spp., especially *L. pneumophila*, serogroup 1, is generally recognized as a cause of respiratory disease outbreaks but is underdiagnosed.

In Indonesia, only three cases of *Legionella* spp. were reported in several areas, such as Bali, Jakarta and Tangerang. The low number of case reports is believed to be due to a lack of capacity to diagnose or identify *Legionella* spp., including *L. pneumophila* serogroup characteristics from clinical specimens (Rahmawaty et al. 2022). The results of research conducted by Aksono et al. (2017) stated that *L. pneumophila* bacteria were detected in swimming pool water in the city of Surabaya. Other research also states that *L. pneumophila* was found in water samples from several hospitals in Jakarta (Moehari et al. 2019).

The presence of *Legionella* spp. will potentially cause public health problems. Therefore, it is necessary to take preventive measures to detect the presence of *Legionella* spp. as early as possible. Palembang City is one of the densely populated cities in Indonesia. The large population in this city will of course have an impact on the high need for clean water for daily activities, including consumption activities. According to WHO (2022), clean water suitable for consumption is water that does not pose significant health risks if consumed over a long period of time. On this basis, this research aims to detect the presence of *L. pneumophila* in several water sources in Palembang City, Indonesia.

MATERIALS AND METHODS

Sampling locations

The map creation was carried out using the QGIS Desktop 3.32.3 application. The location of the sample coordinates was taken from the Google Maps page at www.google.com/maps.

Procedures

Sampling collection

Water samples were taken from 22 water source locations in the Palembang City area, South Sumatra in November 2021 through purposive sampling method. Total 50 mL of each water source was taken and stored in a sterile bottle. To neutralize, 50% Fetal Bovine Serum (FBS) and 0.5 M Tris-HCl were added to the bottle to stop bacterial activity and then put into a cool box.

Preparation of DNA extraction

The genomic DNA extraction was carried out the next day. Each 50 mL water sample was stored in a sterile bottle and homogenized. Once homogeneous, the sample was filtered using Whatman™ No. 1 diameter 125 mm filter papers with Cat No 1001 125. A total of 500 µL samples were taken for the extraction process. DNA extraction of *Legionella* spp. performed using a DNA extraction kit (QIAamp® DNA mini kit Qiagen, Germany) following the instructions provided. The extracted DNA was stored at -20°C until use. DNA isolation involves the process of destroying cells (lysis), separating DNA from other impurity components (extraction), purification (purification) to ensure the absence of other impurity components in pure DNA isolates, as well as deposition (precipitation) of DNA molecules (Gupta 2019).

Nested Polymerase Chain Reaction

The amplification process was carried out using the Nested PCR method. In the first amplification, the PCR reagent was added to an Eppendorf tube including 12.5 µL GoTaq® Green Master Mix (USA); 0.5 µL distilled water; 1 µL forward primer (10 pmol/µL); 1 µL reverse primer (10 pmol/µL) mip gene (Table 1); and 5 µL template DNA. The PCR reagent mixture is inserted into the PCR thermocycler with the following temperature program; Initial denaturation 95°C for 5 minutes, DNA denaturation 95°C for 1 minute, annealing 55°C for 1 minute, extension 72°C for 1 minute, followed by post-extension at 72°C for 10 minutes which was repeated for 35 cycles.

The second amplification used PCR product from the first amplification in the amount of 2.5 µL which was added with 12.5 µL GoTaq® Green Master Mix (USA), 7.5 µL distilled water, 1 µL forward primer (10 pmol/µL), and 1 µL reverse primer (10 pmol/µL) mip gene (Table 1). The mixture is then put into a PCR thermocycler with the same temperature settings as the first amplification.

PCR amplification results were analyzed using 2% agarose gel electrophoresis with an electrophoresis apparatus. The concentration of agarose gel is related to the viscosity of the gel or the size of the gel pores which influences the speed of molecular migration (Fahlevi et al. 2018). The speed of movement of molecules in an electric field is also influenced by the amount of electric voltage used (Harahap 2018). Electrophoresis was carried out with a constant voltage of 100 volts for 30 minutes. The gel electrophoresis results were observed using Gel Documentation. A sample is declared positive for *L. pneumophila* if there is a mip gene band with a band length of 403 bp.

Table 1. Primers used in research

Targeted gene	Sequences primer	Length
Legionella	F1:5'-GCTACAGACAAGGATAAGTTG-3'	649 bp
pneumophila	R1: 5'-GTTTTGTATGACTTTAATTCA-3'	
(mip gene)	F2: 5'-CATGCAAGACGCTATGAGTG-3'	403 bp
	R2: 5'-CAAGTTGATCCAGCTGGCAT-3'	

Note: F1, R1: Forward and Reverse primers in the first amplification; F2, R2: Forward and Reverse primers in the second amplification

RESULTS AND DISCUSSION

Sampling locations

Legionella pneumophila is often found in freshwater environments, such as lakes, rivers, and drinking water supply systems (DWSS) (Lesnik et al. 2016). The samples used in this research were samples of well water and water sources managed by the regional drinking water company (PDAM) at 22 points in Palembang city, South Sulawesi, Indonesia (Figure 1). PDAM is a company that provides and distributes clean water in Indonesia (Tambunan 2014).

Nested PCR results

The nested PCR method is used to detect *L. pneumophila*. The results of the nested PCR test are presented in Table 2.

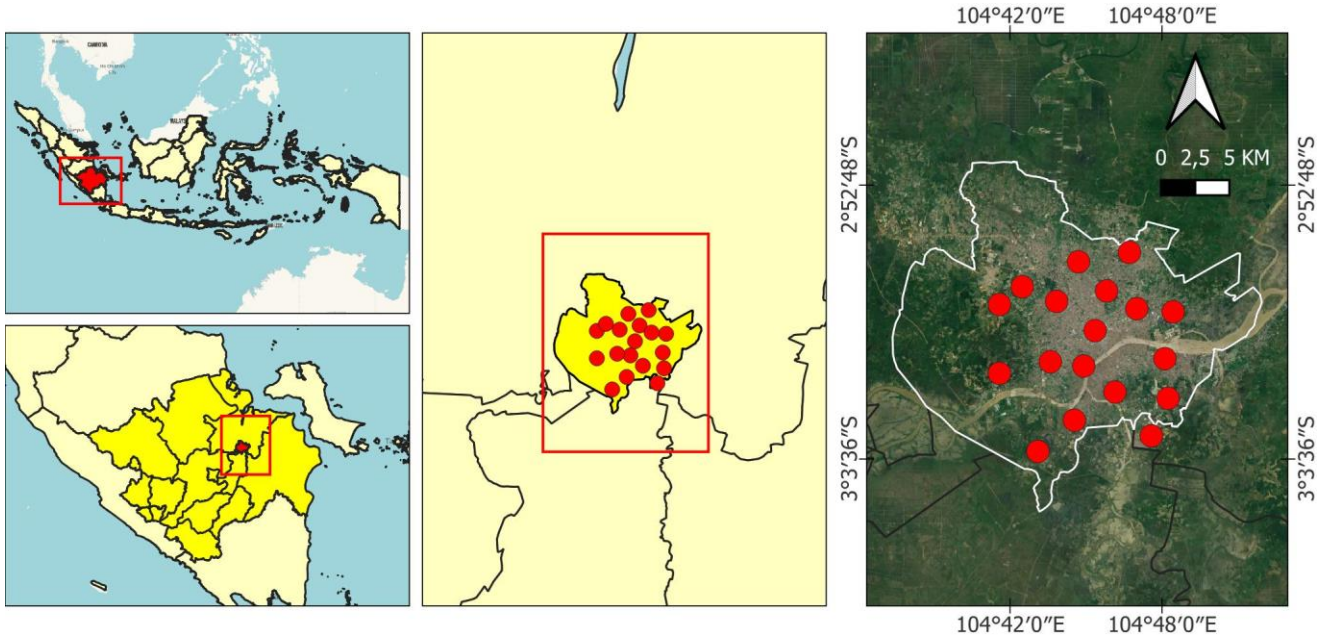

Figure 1. Map of sampling location points in Palembang City, South Sumatra, Indonesia

Table 2. Results of molecular screening of *Legionella pneumophila* and water quality test

Sample code	Sample type	Water condition				PCR result
		Smell	Clarity	pH	Temperature	
L1	DWSS	Odorless	Clear	5	25	Negative
L2	DWSS	Odorless	Clear	5	27	Positive
L3	DWSS	Odorless	Clear	6	24	Negative
L4	DWSS	Odorless	Clear	6	26	Negative
L5	Well Water	Odorless	Clear	7	25	Negative
L6	DWSS	Odorless	Clear	6	26	Negative
L7	Well Water	Odorless	Clear	6	26	Negative
L8	DWSS	Odorless	Clear	6	27	Negative
L9	Well Water	Odorless	Clear	5	24	Negative
L10	DWSS	Odorless	Clear	5	25	Negative
L11	DWSS	Odorless	Clear	7	27	Negative
L12	Well Water	Odorless	Clear	5	25	Negative
L13	Well Water	Odorless	Clear	6	24	Negative
L14	Well Water	Odorless	Clear	5	27	Negative
L15	Well Water	Odorless	Clear	6	26	Negative
L16	DWSS	Odorless	Clear	6	25	Negative
L17	Well Water	Odorless	Clear	6	27	Negative
L18	Well Water	Odorless	Clear	6	26	Negative
L19	Well Water	Odorless	Clear	6	26	Positive
L20	Well Water	Odorless	Clear	5	26	Negative
L21	Well Water	Odorless	Clear	6	25	Negative
L22	Well Water	Odorless	Clear	6	24	Negative

Discussion

Water is an important component that supports human life, including consumption activities. Drinking water is a natural resource that is very necessary for human survival. As a consumable, the levels and presence of physicochemical and biological parameters are expected to be within recommended limits. In this case, the water will not pose a health threat to its users. Microorganisms such as bacteria, fungi, protozoa, viruses, etc. Naturally grow in water and cause disease, especially when associated with contaminated and untreated sources. Every source of drinking water must be free from microorganisms and other dangerous substances (Ezugwu et al. 2022).

Water contaminated by pathogenic microorganisms will result in water-borne diseases. Waterborne diseases are a serious threat due to their ability to infect large numbers of people in a short period (Toplitsch et al. 2018). On the other hand, the need for clean water will continue to increase along with the increase in human population (Manetu and Karanja 2021). This significantly highlights the need for rapid detection and quantification of bacteria in environmental water samples. One of the waterborne diseases is the transmission of *L. pneumophila*. *Legionella* is a species of bacteria that causes pneumonia and legionellosis (WHO 2022).

Pneumonia cases occur in various provinces in Indonesia, including South Sumatra Province. Palembang is one of the densely populated metropolitan cities in South Sumatra (Wazir 2018). Palembang city consists of 18 sub-districts (Rohimawati and Ardillah 2021). In 2022, pneumonia cases in Palembang will reach 5,284 cases among toddlers. This figure has increased from the previous year, namely 5,035 cases in 2021 (South Sumatra Provincial Health Service 2023).

Detection of *L. pneumophila* was carried out molecularly, using the Nested PCR method. Nested PCR is an amplification method that comes from modifying the conventional PCR method with two PCR processes (Brun et al. 2020). The Nested PCR method has a sensitivity one hundred times better than the conventional PCR method (Salih et al. 2020). This PCR-based method uses primer sequences from specific gene for *L. pneumophila* (Borthong et al. 2018).

In this study, the target gene that was amplified was the MIP gene. The mip gene is a marker gene used to detect *L. pneumophila* using a PCR-based test (Borthong et al. 2018). The Macrophage Infectivity Potentiator (MIP) gene is a virulence factor known to contribute to the survival of *L. pneumophila* in host cells (Shen et al. 2022). The MIP gene functions to encode a 24-kDa protein virulence factor that facilitates the entry of *L. pneumophila* into amoebae and macrophages, and has considerable sequence variability between *Legionella* spp. (Ebrahimi et al. 2022). This gene is well conserved in *L. pneumophila* so it is suitable for use as a target gene in detecting the presence of *L. pneumophila* using PCR (Ahmadrajabi et al. 2016).

Based on the PCR amplification results that were obtained, it was discovered that there were 2 samples containing *L. pneumophila* out of 22 samples that were examined carefully. Determining a positive sample (Table 2) is based on the appearance of a DNA band resulting from PCR at a length of 403 bp (Figure 2) (Aksono et al. 2017). These two positive samples came from well water and drinking water managed by PDAM. Interestingly, both of them come from the same sub-district.

Well water is one of the natural habitats of *L. pneumophila*. According to Mapili et al. (2019), private wells are generally more susceptible to microorganism contamination. *Legionella* was detected in 9-8% of private well in Indian Reservation. In Poland, 9-28% of private wells was positive *L. pneumophila*. This can happen because private wells generally do not implement a regular disinfection system. Detection of microbes in well water samples is generally limited to the total number of coliforms and *Escherichia coli*. On the other hand, detection of *L. pneumophila* bacteria in well water is very rare.

In contrast, research conducted by Hapsari et al. (2023) shows different results. Detection of *L. pneumophila* in well water samples in Magetan Regency, Indonesia showed zero results. There are several reasons why the results were null in this study. In aquatic habitats, amoebas act as hosts for *Legionella* to reproduce. Thus, when *Legionella* leave the intracellular environment, they experience stress caused by changes in diet, temperature, pH, salinity, and oxygen. In addition, this study used non-sterile sample bottles which resulted in the failure of *Legionella* culture from environmental samples.

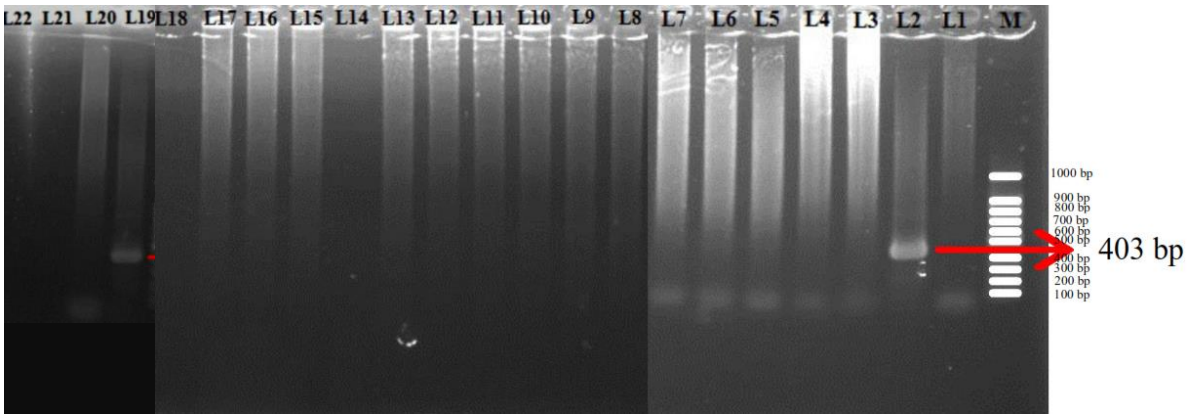

Figure 2. Visualization of PCR result

Apart from wells, *L. pneumophila* was also found in drinking water managed by PDAM. *L. pneumophila* infections are commonly associated with drinking water in building plumbing systems, cooling towers, hot tubs, and drinking water supply systems. However, drinking water supply companies usually do not monitor this important pathogen (LeChevallier 2019). According to Lesnik et al. (2016), several species of *Legionella* spp. known to be able to adapt well to low nutrient conditions in water, so it can adapt to conditions in drinking water. In drinking water supply systems (DWSS), *L. pneumophila* is able to survive planktonically growing in biofilms and infecting and replicating in protozoa. Biofilm helps bacteria to obtain nutrients and protects them from excessive temperature and pH, chemicals, and predators (Arslan-Aydogdu and Kimiran 2018).

In this research, physical and chemical water quality tests were also carried out in the form of odor, clarity, temperature and pH. Based on physical tests, all samples have odorless and clear characteristics with temperature variations ranging from 24 to 27°C. Chemically, the pH of all samples is still within safe limits ranging from 5-7.

Temperature is an important factor in supporting the growth of *Legionella* spp. in natural environments and artificial water systems. *Legionella* spp. is a type of mesophilic bacteria that can grow in the temperature range of 12 to 42°C (Lesnik et al. 2016). Positive samples in this study had temperature characteristics of 27 and 26°C. This temperature is the optimal condition to support the growth of *L. pneumophila*. Uniquely, this bacterium is also able to survive for a long time in damp places at relatively high temperatures, even in the presence of disinfectants such as chlorine. This bacterium has thermotolerant characteristics that can live in environments up to 50°C. This ability means that *L. pneumophila* can also survive in warm environments (Zhang and Lu 2021). Apart from that, environmental chemical factors such as pH also play a role in supporting the growth of *L. pneumophila*. This bacterium can live in waters in the pH range 5-8.5 (Talapko et al. 2022).

Not only found in waters, research conducted by Montagna (2016) states that *Legionella* spp. Water samples taken from several health facilities in Italy were also found. Other research also explains that *L. pneumophila* is found in air conditioners. Research conducted by Elsanousi and Elsanousi (2017) stated that 222 samples out of 525 total samples taken from air conditioners in several places in Khartoum State were positive for *L. pneumophila*. These positive samples are most often found in hospitals, office buildings, schools, homes and universities. This is of course very dangerous for health because it can cause pneumonia.

Until now, pneumonia is still a global public health problem. Pneumonia is an acute infection that attacks lung tissue caused by bacteria, fungi and viruses. Nearly 30% of pneumonia cases in developing countries, including Indonesia, occur in children under five years of age, around 10-20 cases per 100 children per year. Pneumonia causes more than 5 million deaths per year in children under five in developing countries (Junaidi et al. 2021). According to data from the Ministry of Health of the Republic of Indonesia (2023), pneumonia cases in toddlers tend to fluctuate in the last 10 years. Pneumonia is the second largest cause of death in the post-neonatal phase (babies aged 29 days-11 months) with a death rate of 15.3% in 2022. Therefore, preventive measures are needed to reduce pneumonia cases by carrying out early detection in water sources that are prone to contamination by *Legionella* spp. This research can be used as a reference by the Health Service and related institutions in carrying out early detection of the presence of *Legionella* spp. In this way, the number of pneumonia cases can be reduced so that it does not cause a high death rate.

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