DIFFERENCES OF LEGIONELLA PNEUMOPHILA SEROGROUPS DISTRIBUTION IN WELL WATER, TAP WATER, ICE CUBES, HOSPITAL AND HOTEL WATER IN EAST JAVA

Eduardus Bimo Aksono^{1,2,+,*}, Kadek Rachmawati^{2,+,}, Retno Bijanti^{2,+,}

¹ Institute of Tropical Disease, Universitas Airlangga. Surabaya-Indonesia. 60115.

² Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya-Indonesia. 60115.
eduardus-b-a-h@fkh.unair.ac.id; kadekrachmawati@yahoo.co.id; bijantiretno@yahoo.co.id

⁺ These authors have quality contributed to this work

^{*} Correspondence : eduardus-b-a-h@fkh.unair.ac.id; Tel +62-31-5992445

Abstract. *Background* Legionella pneumophila is one of the causes of legionellosis. Water environments serve as the natural habitat and the main sources of Legionella pneumophila. *Objectives* The aims of this study was to understand the differences of Legionella pneumophila serogroups distribution in well water, tap water, ice cubes, hospital and hotel water in East Java-Indonesia. *Methods* a total of 60 water samples in east java-Indonesia; from well water (n=25), tap water (n=5), ice cubes (n=5), water from the hospital (n=16), and hotel water (n=9) were detected using polymerase chain reaction with mip gene spesific primers and then it was analyzed by phylogenetic tree. *Results* For the 60 water samples collected in East Java, 12% of the samples (7/60) were positively contaminated by L. pneumophila. In details, there was 8% of the well water samples (2/25), 2% of the tap water samples (1/5), 2% of the ice cubes samples (1/5), 0% of the hospital water samples (0/16) and 33.33% of the hotel water samples (3/9). The phylogenetic tree showed that Legionella pneumophila contaminating well water isolate 1 from Surabaya and tap water isolate from Sidoarjo was closer to L.pneumophila serogroup 2, 4, 6, 8, 10, 12, isolates from Brazil, China, Spain and Australia. L.pneumophila contaminating the ice cubes from Sidoarjo was closer to serogroup 1, 2, 4, 7, 8, 11, 13, 14, while the bacteria contaminating well water isolate 2 from Sidoarjo as well as water in hotel of Surabaya (hotel water isolate 1, 2 and 3) classified into their own group. *Conclusion* There is a difference in the distribution of L. pneumophila serogroups between well water, tap water, ice cubes, and hotels.

Keywords: L.pneumophila, well water, tap water, ice cubes, hospital water, hotel water

1. Introduction

Legionella spp. are gram-negative, found everywhere in nature, and spread aerogenically until 300 m radius. These bacteria infect the lungs and cause symptoms of pneumonia and they can be a life-threatening after inhalation of contaminated small droplets of water in the air from various water sources (1). These bacteria grow well in any environments; 56% in domestic hot water system, 44% in cooling water system and the ideal condition is 6.9 pH level, 35°C-37°C, with availability of ferric oxide as nutrients in the water. The bacteria are prevalent in any buildings with cooling and hot water system such as in offices

building and hotels. That is why the illness caused by the bacteria is also called “sick building syndrome”.

The water environments are the main sources of *Legionella* and in their natural habitat, they are found in biofilms (2) and they can multiply actively in this complex structure (3) and spread through the flow of water (4). There are not any reports on person-to person transmission of *Legionella*, so they are considered as pathogens from the environments (5). Distribution of *Legionella* species and serogroups were reported to be based on the type of water, such as in ground water and drinking water, while *Legionella pneumophila* is often found in hot water system where the bacteria may multiply well. Different species distribution of *Legionella* may bring significant impact to the public health because Legionnaires disease is caused by *L.pneumophila* and the most infectious is serogroup 1 (6;7).

There are various methods developed for *Legionella* detection. One of them is gold-standard culture-based detection of *Legionella* in the laboratory. This method has 50-90% sensitivity. However, *Legionella* are slow-pace growing bacteria and they require selective medium so their colony only appear after 3 to 4 days. Recently, a new method has been developed to detect *Legionella* in water samples. PCR method is used to address the limitation of culture method. PCR method is faster, more thorough and easier to do for bigger samples (8;9;10). Therefore, the objective of the research is to know the differences of *Legionella pneumophila* serogroups distribution in well water, tap water, ice cubes, hospital and hotel water in East Java-Indonesia.

2. Material and Methods

2.1. Water Samples

Samples collected from the environments in East Java were 60 samples; from well water (25 samples), tap water (5 samples), ice cubes (5 samples), water from the hospital (16 samples), and hotel water (9 samples). Each 200 ml sample of water collected in a sterile vial was then filtered using a Millipore membrane 0.22 μm . Millipore membrane was taken and then put in a 50 ml conical tube and rinsed with 1 ml PBS vortex for 10 minutes. 1 ml was taken and transferred into eppendorf tubes. Then, it was centrifuged at 13,000 rpm for 3 minutes. The supernatant was discarded and pellet was ready to be used for DNA extraction (11).

2.2. Polymerase Chain Reaction

Legionella pneumophila DNA was extracted using a DNA extraction kit (QIAamp®DNA mini kit Qiagen) following the manufacturer's instructions. The final volume of 50 mL of extraction obtained was used as template DNA. DNA contained in the final elution was stored at -20°C for use later. The process of amplification in this study consisted of two rounds. In the first round of amplification, PCR reagent mixture was inserted in eppendorf tubes consisting of 12.5 µl GoTaq®Green Master Mix, 0.5 µl of distilled water, 1 µl for forward primer (F1: 5'-GCTACAGACAAGGATAAGTTG-3') and 1 µl of reverse primer (R1: 5'-GTTTTGTATGACTTTAATTCA-3') of the mip gene and 5 µl of template DNA (Bernander et al, 1997). PCR reagent mixture was then put in a thermocycler with the following temperature program: pre denaturation 95°C for 5 minutes, then DNA denaturation 95°C for 30 seconds; annealing 55°C for 30 seconds, extension 72°C for 1 min, followed by post-extension at 72°C for 10 minutes and was repeated as many as 30 cycles. Amplification in the second round was as follows: using the results of the first round reagent mixture and then adding 12.5 µl GoTaq®Green Master Mix, 5 µl of distilled water, 1 µl for forward primer (F2:5'-CATGCAAGACGCTATGAGTG-3') and 1 µl of reverse primer (R2:5-'CAAGTTGATCCAGCTGGCAT-3') and 0.5 µl of template DNA. PCR reagent mix was then fed back into the thermocycler with the same temperature program as the first round (Bernander et al, 1997). The result of PCR amplification that had been done before was then analyzed by electrophoresis using a 2% agarose gel. Each amplification product was lowered into 2% agarose gel well submerged in a tank containing TBE buffer. DNA marker was also incorporated into agarose gel well to determine the size of the PCR product DNA. Electrophoresis was run at a constant voltage of 100 volts for 30 minutes. Electrophoresis was stopped and then the gel was lifted and observed with transilluminator-UV. Samples tested positive for *Legionella pneumophila* were determined from the presence of mip gene with a 403 bp band length (11).

2.3. Phylogenetic Tree Analysis

PCR products obtained were purified in accordance with the procedure of QIAGEN kit. After purification, labelling and sequencing were then performed using the ABI Prism 310. Phylogenetic analysis using software Genetix Mac Ver. 10.0 against *Legionella* from environmental water samples in East Java was compared with *Legionella pneumophila serogroups* sequence data that has been reported in GenBank (11).

3. Results

Table 1. Results of Legionella bacteria detection in water samples from East Java by PCR

Samples Origin	Results		Species
	Negative	Positive	
Well water	23/25	2/25	<i>L. pneumophila</i>
Tap water	4/5	1/5	<i>L. pneumophila</i>
Ice cubes	4/5	1/5	<i>L. pneumophila</i>
Hospital water	16/16	0/16	<i>L. pneumophila</i>
Hotel water	6/9	3/9	<i>L. pneumophila</i>
TOTAL	53/60	7/60	<i>L. pneumophila</i>

Table 1 and figure 1 showed that using PCR method, from the 60 water samples collected in East Java, 12% of the samples (7/60) were positively contaminated by *L. pneumophila*. In details, there was 8% of the well water samples (2/25), 2% of the tap water samples (1/5), 2% of the ice cubes samples (1/5), 0% of the hospital water samples (0/16) and 33.33% of the hotel water samples (3/9).

Figure 1. Results of PCR *Legionella pneumophila* in water samples from East Java by electrophoresis gel in 2% (403 bp). M (marker); K+ (positive Control); K- (negative control); 1 (positive from tap water); 3 (positive from ice cubes)

Figure 2. Results of phylogenetic analysis of *Legionella pneumophila* from water samples in the environments of East Java

Results of Phylogenetic analysis in Figure 2 showed that *Legionella pneumophila* contaminating well water isolate 1 from Surabaya and tap water isolate from Sidoarjo was closer to *L.pneumophila* serogroup 2, 4, 6, 8, 10, 12, isolates from Brazil, China, Spain and Australia. *L.pneumophila* contaminating the ice cubes from Sidoarjo was closer to serogroup 1, 2, 4, 7, 8, 11, 13, 14, while the bacteria contaminating well water isolate 2 from Sidoarjo as well as water in hotel of Surabaya (hotel water isolate 1, 2 and 3) classified into their own group.

4. Discussion

All of the water samples were collected from some regions in East Java such as Kediri, Sidoarjo dan Surabaya. From the 60 samples, 25 samples were collected from well water, 5 samples were from tap water, 5 samples were from ice cubes, 16 samples were from hospital water, and 9 samples from hotel water. The result showed that 33.33% of the hotel water samples were contaminated by *L. Pneumophila*. There was 8% contamination found from well water samples, 2% contamination found from tap water samples and 2% contamination found from ice cubes. Eventhough the *Legionella* bacteria contamination was quite high, it was only

found a little in quantity ($>1,000$ CFU/liter) (12) and it is not dangerous in the water, including in tap water (11). However, they may multiply fast in inundated water. The infection is transmitted through water spray or aerosol containing *Legionella* microbes. The infected person will feel ill after five or six days from the infection. Our knowledge on *Legionella* infection in small family housing is lesser than the risk of infection in big building. In small family housing, the infection found reached 6-30% (13). Therefore, to prevent contamination of *L.pneumophila*, chlorine is added about >2 mg/liter of water in family housing or housing complex (14;15).

Species and serogroups analysis result based on sequence profile acquired from the PCR with mip (macrophage inhibitor potentiator) gene specific primer was compared to the isolates from GenBank of Brazil, China, Spain and Australia. It showed that isolates distribution of the water was different from the region. It can be seen from the 7 positive samples from sources with contamination risk. *L. Pneumophila* contaminating the well water, tap water and ice cubes from Kediri, Sidoarjo and Surabaya is close to the classified serogroups reported to GenBank (serogroups 1-14). However, *L. Pneumophila* found in well water from Surabaya and hotel water have different serogroup cluster.

In this research, *Legionella pneumophila* serogroup distribution from the natural water (such as well water) is different from artificial water source (such as water tank and drinking water system in hotels). These results are in line with the previous research conducted in Japan and Canada (16,17), they may correlated with water source characteristic differences (such as, temperature and pH level). There are various characteristics of water sources, while cooling tower and drinking water system tend to have similar characteristics due to same water treatments. *Legionella* has high prevalence, fast intracellular growth, and diverse genetics in hot water source (18). In this study, isolate of *L. pneumophila* from hot water tank in hotels, has higher genetic diversity, different from natural water and drinking water system. This result may be related to amoebae host which adapts in different environment. It has been reported that the growth of *L. pneumophila* in amoebae host depends on the bacterial genetic backgrounds (19,20). Hot water isolate with unique sequence adapts with the amoebae, and may be transmitted to humans.

5. Conclusion

The research showed that there is differences of *Legionella pneumophila* serogroups distribution isolated from the public facility such as well water, tap water, ice cubes and samples collected from hotel facility. Moreover, our findings showed some unique profile

sequences from *L.pneumophila* serogroup 1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 12, 13, 14 is the common type serogroups found in East Java, Indonesia except serogroups 5. Routine water monitoring for *Legionella pneumophila* is expected to reduce bacterial contamination in water system. Addition of chlorine compound is also one way to reduce water contamination. Further researches on the correlation of *Legionella* environmental and clinical isolates are needed.

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