Screening of *Legionella pneumophila* from water sources in the hospitals in Jakarta

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Abstrak

Latar belakang: *Pneumonia akibat bakteri Legionella masih menjadi masalah di berbagai tempat di dunia; menjadi penyebab 2-15 % dari pneumonia yang perlu di rawat di Rumah Sakit. Kasus legionellosis di Indonesia dilaporkan terjadi di Bali pada tahun 1996 dan di Tangerang tahun 1999. Keberadaan Legionella di fasilitas Pelayanan Kesehatan berpotensi sebagai penyebab infeksi nosokomial. Bakteri Legionella hidup di lingkungan perairan hangat dan lembab, juga ditemukan diberbagai sumber air seperti, sumber air sistem pendingin ruangan, kolam renang, tempat penampungan air di rumah sakit, perkantoran, hotel, dan perumahan sehingga turut berkontribusi dalam terjadinya community acquired dan pneumonia nosokomial.*

Metode: *Penelitian ini merupakan penelitian deskriptif yang bertujuan untuk penapisan keberadaan Legionella pneumophila diberbagai sumber dan penampungan air di Rumah Sakit (RS) di Jakarta dengan menggunakan medium Legionella Charcoal Yeast Extract (CYE) dan dengan berbagai suplemen. Tujuh belas sampel air yang berasal dari berbagai sumber air di dua RS yang berlokasi diJakarta Utara dan Barat telah diteliti.*

Hasil: *Dua puluh satu koloni yang ditemukan memiliki karakterisitik L. pneumophila dari semua varian medium, namun pada tes agglutinasi latex tidak memberikan reaksi positif.*

Kesimpulan: *L. pneumophila tidak ditemukan diberbagai sumber air dari dua RS ini. Penggunaan metode yang lebih sensitif dan spesifik perlu dilakukan untuk memastikan ditemukannya L. pneumophila.* ***(Health Science Journal of Indonesia 2019;10(1):21-6)***

Kata kunci: *Legionellosis, Legionella pneumophila, medium BCYE*

Abstract

Background: Pneumonia due to Legionella bacteria is still a problem in various places in the world, causes 2-15% of pneumonia that need hospitalization. In Indonesia, legionellosis cases have been reported in Bali in 1996 and Tangerang in 1999. The existence of Legionella in healthcare facilities is potential to cause nosocomial infections. Legionella bacteria live in warm and humid waters, and are also commonly found in various water sources, such as water cooling systems, swimming pools, water reservoirs in hospitals, offices, hotels and housing. These bacteria contribute to the occurrence of community-acquired and nosocomial pneumonia.

Methods: This study was a descriptive research, and aimed to screen water sources and reservoirs in the hospitals in Jakarta for the existence of *Legionella pneumophilla* using Legionella Charcoal Yeast Extract (CYE) medium with various supplements. A total of 17 water samples from 2 hospitals located in West and North Jakarta have been examined.

Results: The results showed a total of 21 colonies with characteristics as of *L. pneumophila* were obtained from those water samples, however, none showed positive results in the latex agglutination test.

Conclusion: *L. pneumophila* was not found thus far in the water sources in these two hospitals. A more sensitive and specific approaches might be used to enable the findings of *L. pneumophila.* ***(Health Science Journal of Indonesia 2019;10(1):21-6)***

Keywords: Legionellosis, *Legionella pneumophila,* BCYE medium

Legionella is a rod or cocobacilli, aerobic, fastidious Gram-negative bacteria. These bacteria live in water, attach to biofilms and multiply intrinsically in amoeba.[1] Legionella habitats are warm and humid aquatic environments, and commonly found in various water sources such as water reservoirs, air conditioning systems (cooling tower)[2], swimming pool[3], hospital shelters[4,5,6], offices and hotels[6,7], and housing. *Legionella pneumophila* contributes to the occurrence of Community Acquired Pneumonia (CAP) and Health Care Associated Pneumonia (HAP).[6,8,9]

The incidence of Legionnaire disease globally remains uncertain. Legionellosis occurs when humans breathe aerosols or aspirate water containing *L. pneumophila* serogroup 1 and 6, and/or some other Legionella species; WHO in 2007 stated that legionellosis is a collection of various respiratory diseases caused by infection with *L. pneumophila* and several bacteria in the family Legionellaceae.[2] Seventy percents of cases of legionellosis are caused by *L. pneumophila* sero group 1, 20-30% of cases caused by other serogroups, and only 5-10% due to infection with other non-pneumophilla Legionella species such as *L. micdadei* (60%), *L. bozemanii* (15%), *L. dumoffii* (10%), *L. longbeachae* (5%), and the remaining 10% are *L. gormanii*, *L. wadsworthii, L. jordan*, *L. feeleii*, *L. oakridgensis*.[1,2,3,6]

ELDSNet, 2012 reported that in Europe, the cases of Legionnaire disease were from 1 to 30 cases per million cases and it was estimated that unreported cases were as much as 20 times.[8] The study by Phin et al, 2014 showed that the largest contributor to the case of Legionnaire disease was men and the world population was above 50 years old.[4] In the United States, there has been an increase in the crude incidence of Legionnaire disease from 2000 to 2009 which was associated with increased temperature and humidity.[4] In Iran, Yaslianifard et al, 2012 stated that the mortality rate from hospital-acquired Legionellosis was 80%, especially in patients with immunosuppression who were not treated with antibiotics. Visca et al, 1999[9] reported 30% of HAP was caused by Legionella in which *Legionella pneumophila* (*L. pneumophila*) serogroup 6 was found in a hospital heated-water systems. In Indonesia, legionellosis has been reported in Bali in 1996 and Tangerang in 1999.[10] Yasmon et al., 2010 using duplex-PCR method found Legionella sp. in 6 samples, and 1 positive sample of *L. pneumophilla* from a total of 9 samples from water reservoirs in offices towers in Jakarta.[6] Also, in Surabaya, Aksono et al., 2017, found *L. pneumophila* in 1 out of 10 samples of swimming pool water.[3]

Feeley et al, 197811 and Feeley J. C. et al, 197912 developed a medium specifically for Legionella i.e. Buffered Charcoal Yeast Extract (BCYE), which then was further modified by Edelstein, 198113 and Edelstein, 1982[14] and was used as a selective medium for Legionella species. BCYE contains amino acid L-cysteine which is specifically needed by Legionella bacteria for its growth when cultured from environmental samples and clinical specimens; supplements containing antibiotics i.e. Cefamandole, Polymyxin and Anisomycin are also needed to inhibit many other bacteria found in water and specimens originating from the human body so that *L. pneumophila* can thrive. Various studies on Legionella have been carried out, however, information whether water sources in health facilities (hospitals) in Jakarta has been contaminated by *L. pneumophila* is not available thus far. This study aimed to screen the presence of *L. pneumophila* in various water sources and reservoirs in hospitals in Jakarta using BCYE variants medium.

METHODS

Study design

This research was a descriptive study with a cross-sectional design.

Place, population and time of study

The study was carried out in the Microbiology Laboratory of Department Microbiology Faculty of Medicine and Health Sciences Atma Jaya Catholic University of Indonesia from March 2018 to October 2018 (Research contract: No. 0419/III/ LPPM.10.01/ 04/2018), ethical clearance No. 21/12/ KEP-FKUAJ/2018. Two private hospitals located in North and West Jakarta Jakarta participated in this study. Water samples of each 200 mL were collected aseptically from several sources i.e. central air conditioning, storage water, tap water and hot water (pipe systems were either galvanised iron, PVC and polyethylene), and were put into sterile bottles and stored in boxes at room temperature for only a short time or less than an hour.[14,15] Samples were immediately sent to the Microbiology laboratory for processing. Samplings were conducted once on each sources.

The collection of environmental samples is in accordance to the regulation from Indonesia Health Ministry No.1538/MENKES/SK/XI/2003 concerning Standard Management of Legionella Specimens.

Bacterial Cultivation and Identification

Water samples, each of 10 ml, were centrifuged at 5500 rpm for 15 minutes, supernatant was discarded, and the sediment was pipetted on to selective media and streaked using Koch method. Incubation was carried out at 37^0C for 3-10 days in anaerobic jar contained 5%-10% CO_2.[15] Bacterial colonies were observed on day 3, 7 and 10. Morphology characteristics of the colony of *L. pneumophila* are as follow: gray in colour, shiny, convex, circular, smooth edge, and darker at center with 3-4 mm diameter.

Four variants of selective medium were used as follow:

Variant 1: Charcoal Yeast Extract (CYE) agar base medium (Oxoid™) contained activated charcoal, yeast extract with addition of BCYE (Oxoid™) supplements containing potassium hydroxide, ferric pyrophosphate, cysteine and ketoglutarate.

Variant 2: CYE agar base medium with addition of BCYE supplement that did not contain cysteine (Oxoid™)

Variant 3: The same as variant 1, plus supplement BMPA-α (Oxoid™) which contains antibiotics cefamadole, polymyxcin and anisomycin[14]

Variant 4: The same as variant 2 with addition of BMPA-α supplement[14]

The suspected colonies were picked up and stained with Gram stain, and further tested for their properties of oxidase and catalase. The oxidase test was conducted using oxidase strip (Oxoid™), and the catalase test was carried using H_2O_2 3%; *L. pneumophila* showed variable results with oxidase test and positive with catalase test. Finally, latex agglutination test was performed using Legionella (M45) from Microgen®, in which individual *L. pneumophila* serogroups 1, 2-15 and non *L. pneumophila* latex and positive control were included in the kit separately. The test permits the identification of *L. pneumophila* serogroups 1, 2-15, and non *L. pneumophila* species. The principle of the method is that the latex particles are coated with purified polyvalent serum against *L. pneumophila* serogroups. The test is positive when suspension of *L. pneumophila* mixed with the reagent and show aggregation of latex particles. *L. pneumophila* ATCC 225493 is used as a positive control bacteria.

RESULTS

Cultivation of control bacteria, *L. pneumophila* ATCC 22549, was carried out using variant 3 and 4 media following the procedures mentioned above. On day 3, the control bacteria grew and appeared thriving in both media as shown in Figure 1. Morphology of the colonies grew in both variants were as described above, they showed the same morphology characteristics.

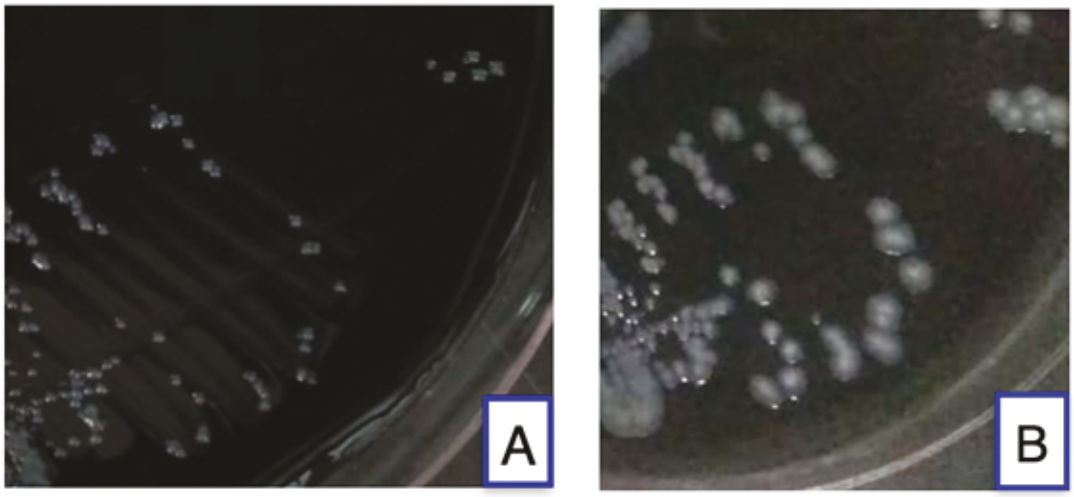

Figure 1. Colonies of L. pneumophila ATCC 225493 cultivated on CYE medium with addition of BCYE and BMPA-α supplements (variant 3) dan BCYE without cysteine and BMPA-α supplement (varian 4)
This figure showed colonies of *L. pneumophila* ATCC 225493 grew on variant 3 medium (A) and on variant 4 medium (B). These colonies showed similar morphology characteristics on both media.

A total of 17 water samples were collected from 17 different sources, in which 10 samples were obtained from the hospital in North Jakarta (A), and 7 samples from the one in West Jakarta (B) (see Table 1). Water samples were all cultivated in all four variants media as described in the Methods section. The cultivated media were taken out from the incubator on day 3, 7 and 10 for observation of the growth and the presence of colonies that were in accordance with the characteristics of Legionella. Eight out of 17 water samples showed bacterial colony morphology with characteristics as of *L. pneumophila* (Table 1). Sixty percents (6/10) water samples from hospital A showed suspected *L. pneumophila* colonies, while those from hospital B showed only *29% (*2/7). The growth of the colonies suspected as *L. pneumophila* in variant 1 medium was shown in Figure 2. All suspected colonies showed no diffferences in their morphology regardless the medium used. The colony that showed characteristics of *L. pneumophila* was then picked and Gram stained and tested for catalase and oxidase. The result of Gram staining of the suspected colony was shown in Figure 3.

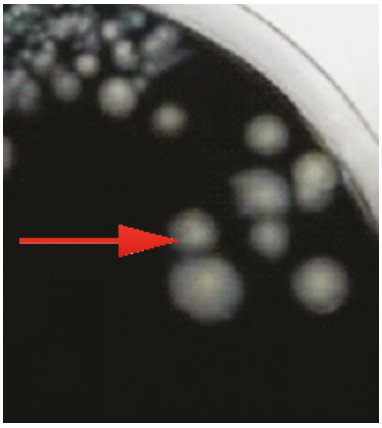

Figure 2. Appearance of bacterial colonies cultivated from water sample on CYE agar base medium contained BCYE supplement (variant 1 medium) Cultivation of water sample from water reservoir on variant 1 medium showed bacterial colonies with morphology characteristic in accordance of *L. pneumophila.*

A total of 21 colonies with morphology, catalase and oxidase characteristics in accordance to *L. pneumophila* were obtained (Table 1). Confirmation test using latex agglutination as described in the Method section was performed on these bacterial colonies and no agglutination showed up. None of the *L. pneumophila* serogroups 2-15 latex gave agglutination with all of the colonies tested.

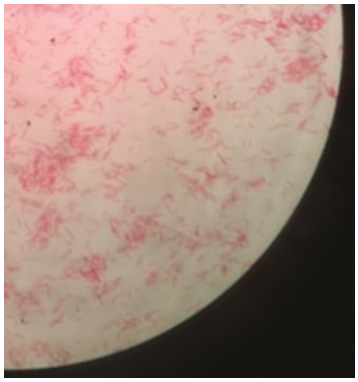

Figure 3. Gram stained of the bacteria colony with characteristic in accordance to L. pneumophila Gram staining of the suspected bacteria colony grew on variant 1 medium contained CYE (Oxoid™) agar base medium with addition of BCYE (Oxoid™) supplement showed rod bacilli Gram-negative.

DISCUSSION

Many studies reported that Legionella bacteria grew better in the present of L-cysteine amino acid.[11,12,13] On the contrary, however, this study showed that bacteria with characteristics such as *L. pneumophila* grew more on variant 2 medium in which supplement BCYE did not contain L-cysteine i.e. 5 water samples (sample number 3, 5, 8, 9 and 14), and only 2 water samples (no. 2 and 7) showed bacterial colonies suspected as *L. pneumophila* in variant 1 and 3 media which contained L-cysteine, which indicating that the growing colonies needed an amino acid L-cysteine. One water sample (no. 17) grew colonies suspected as *L. pneumophila* in variant 1 and 4 media, and seemed these bacteria could grow in either conditions i.e. with or without of L-cysteine.

Variant 3 medium contained L-cysteine and antibiotics, it was a highly selective medium and has been reported to be an enriched medium and could suppress the growth of other Gram negative bacteria found in water.[13,14] In this study, 5 water samples (no. 5, 7, 8, 9 and 14) showed colony growth with characteristics of *L. pneumophila* in variant 3 medium but also many other colonies that were not with the characteristics. Seemed many other bacteria also presence in the water sources that were not killed by the antibiotics in BMPA-α supplement. It was worth noted that 1 water sample i.e. no. 7 grew bacterial colonies only in variant 3 medium, indicating that the bacteria needed the present of amino acid L-cysteine and the growth was not inhibited by antibiotics. This colony was highly suited with the characteristics of *L. pneumophila.*

Table 1. Sources of water samples and the results of cultivation on Legionella CYE agar base medium with various supplements

No	Hospitals	Sources	Culture medium			
			Variant 1	Variant 2	Variant 3	Variant 4
1	A	Tap water (Staff room)	-	-	-	-
2		Water reservoir	+	-	-	-
3		Tap water (ICU)	+	+	-	+
4		AC (Pharmacy in operating theatre)	-	-	-	-
5		AC (Operating theatre 3)	-	+	+	+
6		AC (Operating theatre 2)	-	-	-	-
7		Tap Water (NICU)	-	-	+	-
8		AC (Operating theatre 1)	+	+	+	+
9		AC (Recovery room)	-	+	+	+
10		Tap water (Haemodialysis room)	-	-	-	-
11	B	Water reservoir (1st floor)	-	-	-	-
12		AC (ICU room 1)	-	-	-	-
13		AC (Operating theatre 1)	-	-	-	-
14		AHU (3rd floor)	+	+	+	+
15		AHU (4th floor)	-	-	-	-
16		Hot water (Operating theatre 3)	-	-	-	-
17		Water reservoir (2nd floor)	+	-	-	+

Note:
A: represented the hospital in North Jakarta; B represented the hospital in West Jakarta
+: Bacterial colonies showed morphology characteristics of *L. pneumophila*, catalase test positive, oxidase test variable
-: None showed characteristics of *L. pneumophila*
Variants 1, 2, 3 and 4: culture medium used as described in the Methods section
AC: Air conditioner; AHU: Air handling unit; ICU: Intensive care unit; NICU: Neonate intensive care unit.

In variant 4 medium, which did not contain L-cysteine but contained antibiotics, 6 water samples showed positive bacterial colonies in accordance with *L. pneumophila* characteristics; this variant medium showed the highest number of growth of suspected *L. pneumophila* colonies, though none of them showed positive reaction in the latex agglutination test.

Latex agglutination used in this study is specific for *L. pneumophila* serogroups 2-15 which are found in many water sources, but has a relatively low virulence compared to *L. pneumophila* serogroup 1. In this study none of the colonies with the characteristics of *L. pneumophila* showed agglutination, not even the one of colony from sample no.17 which was highly suspected. Serology tests such as latex slide agglutination are commonly used in the diagnostic laboratory because they are simple, rapid and widely available. However, the sensitivity and specificity of the agglutination test needs to be considered. Previous study reported the sensitivity of latex agglutination was 85.7% compared to 16S rRNA PCR-DNA methods, and also showed some cross reactivity.[16] Further, up to recently 42 Legionella species with 64 serogroups has been identified, in which *L. pneumophila* constituted 91.5% of the isolates. Serogroup 1 was the predominant (84.2%), and serogroups 2–13 (7.4%) accounted for the remaining serogroups.[17] CDC reported only less than 5% of cases of was caused by Legionella non-pneumophila species.[18]

Conventional methods somehow are more applicable to be used in the middle income country such as Indonesia since the practice does not require highly skilled personnels and expensive machineries. The use of specific medium with various supplements for the cultivation of Legionella in the present study showed some colonies with characteristics of *L. pneumophila.* Nonetheless, the confirmation test using latex agglutination was not successful to identify *L. pneumophila.* This might be due to the existence of many species of Legionella and serogroups of *L. pneumophila*. Therefore, more specific and sensitive methods such as PCR-DNA sequencing of 16S rRNA might be needed to enable the findings this microorganism.

In conclusion, screening of Legionella bacteria from many water sources collected from two private hospitals in Jakarta using four variants media specific for Legionella showed some growth of bacterial colonies with morphology, catalase and oxidase characteristics in accordance to *L. pneumophila.*

Nonetheless, the confirmatory test using latex agglutination was not successful to identify *L. pneumophila*. A more sensitive and specific approach such as 16S rRNA PCR and sequencing may be used in the up coming investigation to ensure the findings of *L. pneumophila*.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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