Comparison of BCYE and BMPA media on recovery rate of *Legionella pneumophila*

DOI: dx.doi.org/10.22435/hsji.v11i1.3127

Lucky H. Moehario¹, Enty Tjoa¹, Mutiara J. Taslim², and Yohanna Angelina¹

¹Department of Microbiology, School of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia
²Undergraduate Program, School of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia

Corresponding author: Lucky H Moehario
Email: lhmoehario@gmail.com, lucky.hartati@atmajaya.ac.id

Received: December 18, 2019; Revised: April 23, 2020; Accepted: May 29, 2020.

**Abstract**

**Background:** *Legionella pneumophila* (*L. pneumophila*) has been known as the etiology of legionellosis; they live in aquatic environment, warm and moist. Culture method using specific medium remains as the gold standard in the identification of *L. pneumophila*. This study aimed to compare the recovery rate of *L. pneumophila* ATCC® 33823 on the specific medium BCYE, and BMPA, the selective medium.

**Methods:** Suspension of *L. pneumophila* ATCC® 33823 of 0.5 McFarland was diluted to 10 fold serial dilution; 100 ul of each dilution was inoculated on Buffered Charcoal Yeast Extract (BCYE) medium, and BMPA (BCYE supplemented with BMPA-α) in duplicate manner. The concentration was calculated using Total Plate Count standard as of Indonesian Nasional Standard number 01-2332.3-2006. The percentage of recovery rate was calculated, and the statistical analysis was performed using SPSS version 23.0.

**Results:** Numbers of colonies of *L. pneumophila* grew on BMPA was much higher than on BCYE medium; the highest concentration was yielded on BMPA medium i.e. 1.45x10⁷ CFU/ml. The recovery rates were 96.67% and 60.67% on BMPA medium and BCYE subsequently.

**Conclusion:** The recovery rate of the BMPA medium on the colony growth of *L. pneumophila* ATCC®33823 was markedly higher than the BCYE, therefore BMPA medium can be suggested to be used in the cultivation of *L. pneumophila* especially in the routine surveillance program for water sources with less cost. *(Health Science Journal of Indonesia 2020;11(1):32-7)*

**Keywords:** *Legionella pneumophila*, specific medium, BCYE , BMPA, recovery rate
Legionella pneumophila (L. pneumophila) causes legionellosis with pneumonia as one of the most common clinical manifestations.\textsuperscript{1, 2} The incidence of legionellosis in the United States has increased about 4.5 times since 2000, meanwhile cases requiring hospitalisation exceeded average frequency.\textsuperscript{3, 4} L. pneumophila are widespread in freshwaters such as lakes, rivers, and groundwater. These bacteria gain entry to the man-made water reservoir\textsuperscript{5, 6} and were detected by Bryne et al in Pittsburgh (70%) and Paris (60%)\textsuperscript{7} and Al-Matawah Q et al (2015) in Kuwait which was dominated by L. pneumophila serogroup 7.\textsuperscript{7} In Indonesia, L. pneumophila was found in the swimming pool sample in Surabaya and cooling water samples in Jakarta.\textsuperscript{8, 9} Cases of legionellosis in Indonesia were reported in Bali, Karawaci Tangerang and other cities; a survey conducted in 2001 showed that the cases were related to transmission of bacteria from cooling towers.\textsuperscript{10} Regarding these reports, it assumes that Legionella commonly colonize man-made water system, leading to transmission of disease via aerosol.\textsuperscript{11–13} However, legionellosis cases are still underreported, therefore surveillance of L. pneumophila is necessary for a long-term approach in eradicating infection. Ministry of Health Republic of Indonesia (2019) has released a regulation no.7/2019 on routine surveillance that should be performed in the water system, especially in cooling towers.\textsuperscript{14, 15}

The cultivation method remains as the gold standard in L. pneumophila detection.\textsuperscript{16, 17} Since the concentration of this bacteria in the building water system was very low and could not be detected by routine sampling,\textsuperscript{18} Charcoal Yeast Extract (CYE) agar supplemented with Buffered Charcoal Yeast Extract (BCYE), a supplement containing L-cysteine, is used as specific medium.\textsuperscript{19} Addition of selective supplements consist of antimicrobials could promote L. pneumophila growth and reduce the competing bacteria and fungi.\textsuperscript{22–24} Since the use of lots of media for identification of Legionella is not cost effective, we aimed to evaluate the recovery rate of L. pneumophila ATCC\textsuperscript{\textregistered}33823 on CYE supplemented with BCYE and BCYE-BMPA supplements.

**METHODS**

This study was descriptive-analytic research, conducted in the Microbiology Laboratory of Department of Microbiology School of Medicine and Health Sciences, Atma Jaya Catholic University Indonesia, from August 2019 to September 2019; this had passed the ethical evaluation by the School of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia No. 10/08/KEP-FKUAJ/2019.

**Bacterial Suspension**

Culti-Loops\textsuperscript{\textregistered} L. pneumophila ATCC 33823 (Thermo Scientific\textsuperscript{TM}) was streak directly according to manual instruction on CYE agar supplemented with BCYE supplement, followed by incubation at 37°C in the presence of 5% CO\textsubscript{2} for 3 to 7 days. Observation of the growth of colonies was conducted after 72 hours incubation and regularly every 48 hours to find the growth of expected colonies. Colonies with Legionella morphology characteristics were harvested in 5.0 ml of sterile Phosphate Buffered Saline (PBS) and adjusted to 0.5 McFarland turbidity standard.

**Cultivation and Enumeration**

Dilution of 10\textsuperscript{-1} was made by transferring 1 ml of 0.5 McF bacterial suspension to 9 ml of diluent (PBS) in the first tube (Tube I) and homogenised by vortex. Every dilution was prepared from drawing 1 ml aliquot from previous dilution. The method was performed in the same manner past above until 10\textsuperscript{-8} dilution. A total of eight tubes represented for dilution of 10\textsuperscript{-1}-10\textsuperscript{-8} was obtained, assigned as tube I-VIII. Every suspension must be mixed well using vortex prior to drawing an aliquot for each subsequent serial dilution. The concentration of viable bacteria from each tube was subjected to enumeration by Total Plate Count (TPC) standard from Indonesian Nasional Standard (SNI) number 01-2332.3-2006.\textsuperscript{25}

Inoculation was carried out on media as followed: Legionella CYE agar base medium (CM0655 Oxoid\textsuperscript{TM}) was added with Legionella BCYE Growth Supplement (SR0110 Oxoid\textsuperscript{TM}), referred as BCYE medium, and BCYE medium plus Legionella BMPA-α selective supplement (SR0111 Oxoid\textsuperscript{TM}) consists of Cefamandole, Polymyxin B, and Anisomycin, referred as BMPA medium. Both media were tested for fertility by inoculating Culti-Loops\textsuperscript{TM} Staphylococcus epidermidis ATCC\textsuperscript{\textregistered}12228 as recommended by the manufacturer. The expected result was absence of the growth of Staphylococcus epidermidis on BMPA medium, while the growth was observed on BCYE medium. A total of 100 ul aliquots from each dilution was plated onto each media in duplicate manner by spread plate techniques. The inoculated culture media were incubated and inspected in the same condition as above. Viable colonies were counted using colony counter and the concentration (CFU/ml) of sample tested was calculated.

**Identification of morphology characteristics and agglutination test**

Characteristics of Legionella colonies identified as greyish-white shiny colonies with the typical ground
glass appearance, Gram-negative rods, oxidase-positive, catalase-positive. Latex agglutination test was performed for *L. pneumohila* serogroup identification; Microgen® Legionella Agglutination Kits M45CE was used. The agglutination is expected to occur for the *L. pneumohila* ATCC®33823 using reagent test 2-15. This strain of *L. pneumophila* is identified by the manufacturer as serogroup 7.

**Concentration and recovery rate measurement**

The concentration was measured using the TPC formula, based on Indonesian SNI No. 01-2332.3-2006, shown below:

\[ N = \frac{\sum C}{[(1 \times n_1) + (0.1 \times n_2)]x(d)} \]

- **N**: Number of product colonies, expressed in colonies per ml or colonies per g
- **\(\Sigma C\)**: Number of colonies in all plates counted
- **\(n_1\)**: Number of plates in the first dilution calculated
- **\(n_2\)**: Number of plates in the second dilution calculated
- **\(d\)**: First dilution calculated

The calculation then multiplied by 10 due to the volume used was 0.1 ml.

**Recovery rate**

\[ \frac{N1}{N0} \times 100\% \]

- **N1**: Number of colony forming units per ml (CFU/ml) obtained
- **N0**: Number of colony forming units per ml (CFU/ml) as equal to 0.5 McFarlands (1.5 x 10⁷ CFU/ml)

The percentage of recovery rate was calculated by dividing the concentration obtained by the initial concentration and multiplied by 100.²⁶,²⁷

**Statistical analysis**

Statistical analysis was carried out using a t-independent test to compare the concentration (CFU/ml) on BCYE and BMPA media using SPSS software version 23.0, 2015 with \(P < 0.05\) was significance, and 95% confidence interval. Normality of the data was tested using Shapiro Wilk test. If the value is greater than 0.05, it shows a normal distribution. Mann Whitney U test was used instead if the distribution was not normal.

**RESULTS**

The identification of the colonies was conducted on day 5 of incubation since at this time of incubation colony morphology has shown its best. The bacteria colonies were identified by morphologies characteristics, Gram staining, biochemical and agglutination test as mention in the method above. The results on all plates by a 100 ul diluted bacterial suspension from tube I to tube VIII showed colonies with characteristics of *L. pneumophila*; agglutination occurred when tested with Microgen® Legionella Agglutination Kits M45CE, which confirmed of the presence of *L. pneumophila* serogroup 2-15. The growth of expected colonies of *L. pneumophila* was observed on BCYE plates inoculated by the bacterial suspension from tube I to tube V, while on BMPA plates, colonies growth was observed up to tube VI, which was 10 fold more diluted than tube V. Numbers of colonies on each plate were counted, and the concentration of the bacterial suspension in each tube was calculated using the TPC formula (Indonesian SNI No. 01-2332.3-2006). The results were as shown in Table 1 and 2.

| Tube | Dilution | I  | II  | Mean | Concentration (CFU/ml) |
|------|----------|----|-----|------|------------------------|
| I    | 10⁻¹     | TNTC* | TNTC* | TNTC* | TNTC* | 0.54 x 10⁷ |
| II   | 10⁻²     | TNTC* | TNTC* | 540  | 0.91 x 10⁷ |
| III  | 10⁻³     | 510  | 570  | 90.5 | 0.50 x 10⁷ |
| IV   | 10⁻⁴     | 95   | 86   | 5    | 0         |
| V    | 10⁻⁵     | 2    | 8    | 0    | 0         |
| VI   | 10⁻⁶     | 0    | 0    | 0    | 0         |
| VII  | 10⁻⁷     | 0    | 0    | 0    | 0         |
| VIII | 10⁻⁸     | 0    | 0    | 0    | 0         |

*Too Numerous Too Count*
The highest concentration obtained on BCYE and BMPA media was of those that were inoculated by bacterial suspension of tube IV which gave a concentration of $0.91 \times 10^7$ CFU/ml and $1.45 \times 10^7$ CFU/ml respectively. Further, on the BMPA plates, colonies growth was observed up to tube VI, and resulted to a concentration of $1.00 \times 10^7$ CFU/ml, while it only showed colonies up to dilution $10^{-5}$ (tube V), which produced a concentration $0.50 \times 10^7$ CFU/ml on BCYE medium.

Table 2. Colonies number of L. pneumophila ATCC 33823 and the concentration yielded from the cultivation on BMPA medium

| Tube | Dilution | Colonies number | Concentration (CFU/ml) |
|------|----------|-----------------|------------------------|
| I    | $10^{-1}$ | TNTC*           | TNTC*                  |
| II   | $10^{-2}$ | TNTC*           | TNTC*                  |
| III  | $10^{-3}$ | 840             | 990                    |
| IV   | $10^{-4}$ | 150             | 140                    |
| V    | $10^{-5}$ | 5               | 14                     |
| VI   | $10^{-6}$ | 1               | 1                      |
| VII  | $10^{-7}$ | 0               | 0                      |
| VIII | $10^{-8}$ | 0               | 0                      |

*Too Numerous Too Count

This figure showed the concentration (CFU/ml) obtained from three different dilutions of bacterial suspension inoculated on BCYE and BMPA agar. The CFU/ml showed up from the cultivation on BMPA agar was higher than on BCYE. Moreover, the highest concentration was obtained from the suspension dilution of $10^{-4}$ cultivated on BMPA medium.

**DISCUSSION**

The present study used different medium formulations for the recovery of L. pneumophila strain. We compared the recovery rate and concentration on BCYE and BMPA medium and showed the BMPA medium had better performance than BCYE. A study by Descours et. al (2014) showed selective media supplemented with antibiotic and anti fungi yielded higher isolation rates than BCYE medium. On the contrary, however, statistical analysis demonstrated no significant difference in L. pneumophila growth on BCYE and BMPA media ($P=0.102$, t-independent test).

The ability of a medium for the isolation of L. pneumophila varies depend on the sample types and medium composition. Edelstein (1981) isolated L. pneumophila from the contaminated water specimen and showed a significant difference of mean viable counts on BCYE and BMPA media. BCYE medium is specific but not selective for the isolation of L. pneumophila due to absence of antibiotics component to inhibit contaminants. Our study, however, we used sterile Phosphate Buffered Saline (PBS) that has
been seeded with *L. pneumophila*, of which none of the contaminants were present. Thus, it could be assumed that there would be no significant difference in growth on BCYE and BMPA media using PBS seeded with *L. pneumophila*.

Pharmacopeia recommends a recovery rate of 50%-200%, whereas the recovery rate of *L. pneumophila* on BCYE (60.67%) and BMPA (96.67%) media were within the range.³⁰ Recovery rates could vary depending on the type of water sample.³¹ Boulanger and Edelstein stated that the results obtained could be different between seeded water samples and actual water specimens, further the presence of other flora in water specimens could decrease the recovery of *L. pneumophila*.³² Fliermans et al. (1981)³³ found the recovery rate of Legionella from seeded water samples was consistently around 80%, whereas the BMPA medium in the present study showed higher i.e. 96.67%. Our earlier study of water resources from tap water, water reservoir, condensed water from split air conditioning (AC), and hot water obtained from two private hospitals in Jakarta showed a better growth of *L. pneumophila* on BMPA.³⁴ Edelstein (1981)²⁹ recommended a laboratory with limited funds could use BMPA medium which might show lower yield instead of using BCYE medium with a higher risk of contamination. The present study is in agreement with Edelstein that the use of BMPA medium increases both selectivity and sensitivity of *L. pneumophila*.

In conclusion, the present study demonstrated the recovery rate of *L. pneumophila* was markedly higher on the BMPA medium than BCYE. Therefore, the BMPA medium can be suggested to be used for cultivation of *L. pneumophila*, especially in the routine water sources surveillance program with less cost.

**Conflict of interest**

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

**REFERENCES**

1. CDC [Internet]. About legionnaires disease and pontiac fever. 2019. [Cited 2020 May 25]. Available from: https://www.cdc.gov/legionella/about/index.html.

2. Farver CF. Bacterial Diseases. Pulmonary Pathology [Internet]. 2nd ed. Philadelphia, PA: Elsevier; 2018. [Cited 2018 Sep 16] p 163–200. Available from: https://www.clinicalkey.com/#/content/book/3-s2.0_B97803233930890000108?scrollTo=%23hl0001580.

3. Yackley JK. <em>Notes from the Field</em>: Legionellosis outbreak associated with a hotel aquatics facility - Tennessee, 2017. [Cited 2019 Dec 6]. MMWR Morb Mortal Wkly Rep [Internet], 2018; 67. Available from: https://www.cdc.gov/mmwr/volumes/67/wr/mm6702a5.html.

4. Edelstein PH. Legionnaires’ disease, pontiac fever, and related illnesses. In: Feigin and Cherry’s Textbook of Pediatric Infectious Diseases [Internet], 8th ed. Philadelphia, PA: Elsevier; 2019. [Cited 2018 Sep 18] p 1228-1235. e4. Available from: https://www.clinicalkey.com/#/content/book/3-s2.0-B9780323376921001374?scrollTo=%23hl0000638.

5. Rubin LG. Legionella Species. Principles and Practice of Pediatric Infectious Diseases [Internet]. 5th ed. Philadelphia, PA: Elsevier; 2018 [Cited 2018 Sep 16]; p. 948-952.e1. Available from: https://www.clinicalkey.com/#/content/book/3-s2.0-B9780323240181400178?scrollTo=%23hl0000336.

6. Byrne BG, McColl S, McElmurry SP, Kilgore PE, Sobekk J, Sadler R, et al. Prevalence of infection-competent serogroup 6 Legionella pneumophila within premise plumbing in Southeast Michigan. mBio. 2018 Mar 7; 9(1): e00016-18.

7. Al-Matawah Q, Al-Zenki S, Al-Azmi A, Al-Waalan T, Al-Salameen F, Hejji AB. Legionella detection and subgrouping in water air-conditioning cooling tower systems in Kuwait. Environ Sci Pollut Res Int. 2015 Jul; 22(13): 10235–41.

8. Aksono EB, Farahdiba AA, Hestianah EP. Legionella pneumophila bacteria detected in swimming pool water of Surabaya by using nested Polymerase Chain Reaction. J Vet. 2017; 18(2): 221–5.

9. Yasmon A, Yusmaniaria Y, Anis A, Bela B. Simultaneous detection of Legionella species and Legionella pneumophila by duplex PCR (dPCR) assay in cooling tower water samples from Jakarta, Indonesia. Med J Indones. 2010 Nov 1; 19(4): 223–7.

10. Ministry of Health Republic of Indonesia. Minister of Health Decree No. 1538/Menkes/SK/1/2003 about Standards of Legionella Specimen Management. Jakarta : Ministry of Health of Indonesia. 2003.

11. Sikora A, Wojtowicz-Bobin M, Kozioł-Montewka M, Magrys A, Gladysz I. Prevalence of Legionella pneumophila in water distribution systems in hospitals and public buildings of the Lublin region of eastern Poland [Internet]. [Cited 2020 May 25]. Available from: http://www.aatem.pl/Prevalence-of-Legionella-pneumophila-in-water-distribution-systems-in-hospitals and,72258,0,2.html doi.org/10.5604/12321966.1152064.

12. D’Alessandro D, Fabiani M, Cerquetani F, Orsi G. Trend of legionella colonization in hospital water supply. Ann Ig Med Prev E Comunità. 2015 Jun 5; 27: 460–6.

13. Prussin AJ, Schwake DO, Marr LC. Ten questions concerning the aerosolization and transmission of Legionella in the built environment [Internet]. [Cited
14. Ministry of Health Republic of Indonesia. Minister of Health Decree No.7_Th_2019 ttg Kesehatan Lingkungan_Rumah_Sakit.pdf [Internet]. [Cited 2019 Oct 29]. Available from: http://aspak.yankes.kemkes.go.id/beranda/wpcontent/uploads/downloads/2019/03/PMK_No__7_Th_2019_ttg_Kesehatan_Lingkungan_Rumah_Sakit.pdf.

15. Agarwal S, Abell V, File TM. Nosocomial (Health Care–Associated) legionnaire’s disease. Infect Dis Clin North Am. 2017 Mar; 31(1): 95–133.

16. Mercante JW, Winchell JM. Current and emerging Legionella diagnostics for laboratory and outbreak investigations. Clin Microbiol Rev. 2015 Jan; 28(1): 95–133.

17. Kotrbancová M, Špaleková M, Fulová M, Trnková K, Perželová J. Legionellosis and its diagnosis. Epidemiol Mikrobiol Imunol Cas Spolecnosti Epidemiol Mikrobiol Ceske Lek Spolecnosti JE Purkyne [Internet]. 2017; 66(3): 133–9. [Cited 2020 May 25]. Available from: https://pubmed.ncbi.nlm.nih.gov/28948808/.

18. Giglio O, Diella G, Trerotoli P, Consomni M, Palermo R, Tesauro M, et al. Legionella detection in water networks as per ISO 11731:2017: can different filter pore sizes and direct placement on culture media influence laboratory results? Int J Environ Res Public Health. 2020 Mar 20; 17: 1-8.

19. CDC [Internet]. Monitoring your building water for Legionella. 2019. [Cited 2020 May 25]. Available from: https://www.cdc.gov/legionella/wmp/monitor-water.html.

20. Whelen C. Legionella and Bordetella. Textbook of diagnostic microbiology. 6th ed. Philadelphia: Saunders; 2018. p. 401-7.

21. Caroll KC, Brooks GF, Jawetz E, Melnick JL, Adelberg EA (ed). Bacteriology. Jawetz, Melnick, & Adelberg’s medical microbiology. 28th ed. New York: McGraw Hill Medical; 2019. p. 315–8.

22. Pierre DM, Baron J, Yu VL, Stout JE. Diagnostic testing for Legionnaires’ disease. Ann of Clin Microbiol and Antimicrob [Internet]. 2017 Aug 29 [Cited 2020 May 24]; 16 (59) : 1-4. Available from: https://annclinmicrob.biomedcentral.com/articles/10.1186/s12941-017-0229-6.

23. Veenendaal HR, Brouwer-Hanzens AJ, van der Kooij D. Incubation of premise plumbing water samples on Buffered Charcoal Yeast Extract at elevated temperature and pH selects for Legionella pneumophila. Water Res. 2017 15; 123: 439–47.

24. Engl ards unit NISPH, st 61 Colindale Avenue London NW9 5EQ Email, st. UK Standards for Microbiology Investigation: ID 18 Identification of Legionella species - GOV.UK. Public Health Eng. (03): 09–13. Public Health England. (2015).

25. Badan Standardisasi Nasional. Cara uji mikrobiologi-bagian 3: penentuan angka lempeng total (ALT) pada produk perikanan. Standar Nasional Indonesia. Jakarta; 2006. Indonesian.

26. Roelofsen E, Leeuwen MV, Meijer-Severs GJ, Wilkinson MHF, Degener JE. Evaluation of the effects of storage in two different swab fabrics and under three different transport conditions on recovery of aerobic and anaerobic bacteria. Journal of Clinical Microbiology [Internet]. 1999 [Cited 2019 October 30]; 37(9): 3041-3. Available from: https://jcm.asm.org/content/37/9/3041.

27. Catherine AB, Edelstein PH. Precision and accuracy of recovery of Legionella pneumophila from seeded tap water by filtration and centrifugation. Applied and Environmental Microbiological [Internet]. 1995 [Cited 2019 November 21]; 61(5):1805–9. Available from: https://aem.asm.org/content/aem/61/5/1805.full.pdf.

28. Descours G, Cassier P, Forey F, Ginevra C, Etienne J, Lina G, et al. Evaluation of BMPA, MWY, GVPC and BCYE media for the isolation of Legionella species from respiratory samples. J Microbiol Methods [Internet]. 2014 Mar 1; 98: 119–21. Available from: 10.1016/j.mimet.2014.01.001.

29. Edelstein PH. Improved semiselective medium for isolation of Legionella pneumophila from contaminated clinical and environmental specimens. J Clin Microbiol [Internet]. 1981 Sep [Cited 2018 August 16];14(3):298–303. Available from: https://jcm.asm.org/content/jcm/14/3/298.full.pdf.

30. Council of Europe. Microbial examination of non-sterile products: microbial enumeration tests. Pharmacopoeia. 2018.

31. Fliermans CB, Cherry WB, Orrison H, Smith SJ, Tison DL, Pope DH. Ecological niche of Legionella pneumophila. Applied and Environmental Microbiology. 1981;41(1):9-16.

32. Moehario LH, Robertus T, Grace Y, Tjoa E. Screening of Legionella pneumophila from water sources in the hospitals in Jakarta. Health Science Journal of Indonesia [Internet]. 2019 [Cited 2018 August 15]; 10(1):21–6. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC243633/pdf/aem00194-0029.pdf.