

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

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Abstrak

Latar belakang: *Legionella pneumophila* (*L. pneumophila*) telah banyak diketahui sebagai penyebab legionellosis; habitat nya di berbagai sumber air, lingkungan lembab dan hangat. Metode kultur menggunakan media spesifik masih menjadi baku emas untuk identifikasi *L. pneumophila*. Penelitian ini bertujuan membandingkan recovery rate *L. pneumophila* ATCC®33823 pada medium spesifik BCYE, dan medium selektif BMPA.

Metode: Dilakukan dilusi serial pada suspensi 0,5 McFarland *L. pneumophila* ATCC®33823; 100 ul dari tiap tingkat dilusi diinokulasi pada medium Buffered Charcoal Yeast Extract (BCYE) dan medium BMPA (medium BCYE yang ditambahkan suplemen BMPA- α), secara duplo. Konsentrasi suspensi dihitung menggunakan metode Angka Lempeng Total (ALT) dari Standar Nasional Indonesia No. 01-2332.3-2006. Persentase recovery rate dihitung dan dianalisis secara statistik menggunakan SPSS versi 23,0.

Hasil: Jumlah koloni *L. pneumophila* yang tumbuh pada medium BMPA jauh lebih tinggi daripada yang tumbuh di medium BCYE; konsentrasi tertinggi yang diperoleh adalah pada medium BMPA sebesar $1,45 \times 10^7$ CFU/ml. Persentase recovery rate pada medium BMPA adalah 96,67%, dan 60,67% pada medium BCYE.

Kesimpulan: Recovery rate medium BMPA untuk pertumbuhan koloni *L. pneumophila* ATCC®33823 jauh lebih tinggi daripada media BCYE, karena itu medium BMPA dapat direkomendasikan untuk kultivasi *L. pneumophila*, khususnya pada program surveilans berbagai sumber air dengan biaya lebih terjangkau. (*Health Science Journal of Indonesia* 2020;11(1):32-7)

Kata kunci: *Legionella pneumophila*, medium spesifik, BCYE, BMPA, recovery rate

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 for the cultivation of Legionella, 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. 1.45×10^7 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.^{1,2} The incidence of legionellosis in the United State has increased around 4.5 times since 2000, meanwhile cases requiring hospitalisation exceeded average frequency.^{3, 4} *L. pneumophila* are widespread in freshwaters such as lakes, rivers, and groundwater. These bacteria gain entry to the man-made water reservoir^{5, 6} and were detected by Bryne et. al in Pittsburgh (70%) and Paris (60%)⁶ and Al-Matawah Q et. al (2015) in Kuwait which was dominated by *L. pneumophila* serogroup 7.⁷ In Indonesia, *L. pneumophila* was found in the swimming pool sample in Surabaya and cooling water samples in Jakarta.^{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.¹⁰ Regarding these reports, it assumes that *Legionella* commonly colonize man-made water system, leading to transmission of disease via aerosol.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.^{14, 15}

The cultivation method remains as the gold standard in *L. pneumophila* detection.^{16,17} Since the concentration of this bacteria in the building water system was very low and could not be detected by routine sampling,¹⁸ Charcoal Yeast Extract (CYE) agar supplemented with Buffered Charcoal Yeast Extract (BCYE), a supplement containing L-cysteine, is used as specific medium.^{20, 21} Addition of selective supplements consist of antimicrobials could promote *L. pneumophila* growth and reduce the competing bacteria and fungi.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®33823 on CYE supplemented with BCYE and BCYE-BMPA supplements.

METHODS

This study was descriptive-analytic research, conducted in the Microbiology Laboratory of Department 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™ *L. pneumophila* ATCC 33823 (Thermo Scientific™) 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% CO₂ 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 10⁻¹ 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 as above until 10⁻⁸ dilution. A total of eight tubes represented for dilution of 10⁻¹-10⁻⁸ 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 01-2332.3-2006.²⁵

Inoculation was carried out on media as followed: *Legionella* CYE agar base medium (CM0655 Oxoid™) was added with *Legionella* BCYE Growth Supplement (SR0110 Oxoid™), referred as BCYE medium, and BCYE medium plus *Legionella* BMPA-α selective supplement (SR0111 Oxoid™) consists of Cefamandole, Polymyxin B, and Anisomycin, referred as BMPA medium. Both media were tested for fertility by inoculating Culti-Loops™ *Staphylococcus epidermidis* ATCC®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. pneumophila* serogroup identification; Microgen® Legionella Agglutination Kits M45CE was used. The agglutination is expected to occur for the *L. pneumophila* 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:

$$N = \frac{\Sigma C}{[(1 \times n_1) + (0,1 \times n_2)] \times (d)}$$

N : Number of product colonies, expressed in colonies per ml or colonies per g

Σ C : Number of colonies in all plates counted

n₁ : Number of plates in the first dilution calculated

n₂ : 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.

Table 1. Colonies number of *L. pneumophila* ATCC 33823 and the concentration yielded from the cultivation on BCYE medium

Tube	Dilution	Colonies number			Concentration (CFU/ml)
		I	II	Mean	
I	10 ⁻¹	TNTC*	TNTC*	TNTC* TNTC*	TNTC* TNTC*
II	10 ⁻²	TNTC*	TNTC*	540	0.54 x 10 ⁷
III	10 ⁻³	510	570	90.5	0.91 x 10 ⁷
IV	10 ⁻⁴	95	86	5	0.50 x 10 ⁷
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		

*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 0.91×10^7 CFU/ml and 1.45×10^7 CFU/ml respectively. Further, on the BMPA plates,

colonies growth was observed up to tube VI, and resulted to a concentration of 1.00×10^7 CFU/ml, while it only showed colonies up to dilution 10^{-5} (tube V), which produced a concentration 0.50×10^7 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	II	Mean	
I	10^{-1}	TNTC*	TNTC*	TNTC*	TNTC*
II	10^{-2}	TNTC*	TNTC*	TNTC*	TNTC*
III	10^{-3}	840	990	915	0.92×10^7
IV	10^{-4}	150	140	145	1.45×10^7
V	10^{-5}	5	14	9.5	0.95×10^7
VI	10^{-6}	1	1	1	1.00×10^7
VII	10^{-7}	0	0	0	0
VIII	10^{-8}	0	0	0	0

*Too Numerous Too Count

The recovery rate of BCYE and BMPA medium was calculated by dividing the concentration obtained, i.e. from tube IV of the BCYE and BMPA by the initial concentration of the bacterial suspension used, i.e. 0.5 McFarland (1.5×10^7 CFU/ml) and multiplied by 100; thus showed 60.67% and 96.67% respectively, where the BMPA medium had a much higher recovery rate than BCYE medium. Colonies grew from tube IV were chosen referring to SNI number 01-2332.3.-2006 in which showed colonies range from 25-250 colonies. The distribution of data obtained using Shapiro Wilk test showed normal distribution. Comparison of the concentration from each medium was carried out from tube III-V as shown in Figure 1, and was statistically analysed using t-independent test, the result showed p-value of 0.102 ($P > 0.05$). Both medium showed similar growth capability for *L. pneumophila*, nevertheless, the BMPA medium had much higher recovery rate than the BCYE.

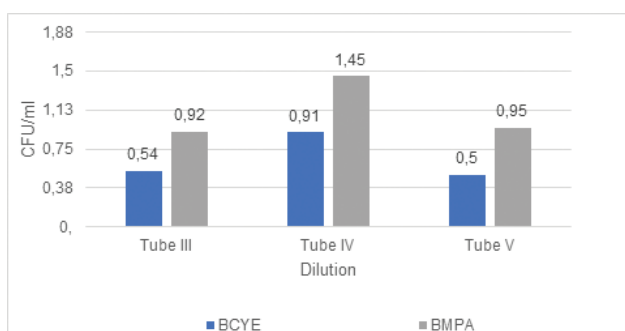


Figure 1. The concentration (CFU/ml) of tube III, IV, and V plated on BCYE and BMPA media

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.²⁸ 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.²⁹ 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*.²⁷ Fliermans et al. (1981)³¹ 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.³² 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.

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