Role Selective and Nonselective Media for Isolation of Burkholderia Species from Patients with Suspected Melioidosis

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Abstract: Melioidosis is an infectious disease caused by the Burkholderia bacteria species, especially *Burkholderia pseudomallei* and *Burkholderia cepacia*, disease is endemic in Southeast Asia and Northern Australia. As a tropical country like Indonesia, this is a very serious global threat. This study aims to compare selective and non-selective media in diagnosing Melioidosis based on the results of culture of clinical specimens of patients with suspected Melioidosis. The results showed that as many as 112 (100%) of suspected clinical samples of bacterial melioidosis were grown on nonselective media, Mac-Conkey agar and 110 (98.2%) bacteria growing on Columbia agar agar medium. While on Ashdown's Selective Agar (ASA) there is bacterial growth of 10 samples (8.9%.). Where 7 of 10 samples are is Burkholderia species (*Burkhoderia cepacia* 5 and *Burkholderia pseudomallei* 2). The results of this study indicate that Ashdown's media plays an important role in selecting Burkholderia species as the cause of Melioidosis.

1 INTRODUCTION

The Burkholderia genus is made up of a variety of species, Gram-negative bacilli, saprophytes in soil and water reservoirs, endemic to tropical and subtropical regions such as Southeast Asia and Northern Australia. Some species of the Burkholderia genus are widely used in biotechnology, bioremediation, biocontrol and agricultural industries (Estrada-De et.al, 2001). Three known species from this genus as an etiologic agent and cause fatal diseases in humans and animals are *Burkholderia pseudomallei*, *Burkholderia mallei*, and *Burkholderia cepacia*[2]. The disease that caused by these three species are known as melioidosis. Melioidosis is an infectious disease that has a complex spectrum such as local skin lesions, sub acute pneumonia, abscess on infected organ, musculoskeletal infections, and fulminant pneumonia (Cheng et.al, 2005). *Burkholderia pseudomallei* has been known for causing severe sepsis that leading to mortality of the patient (Currie et.al, 2010).

This situation is a serious global threat, especially for a tropical country, such as Indonesia. The clinical diagnosis of melioidosis remains difficult since the disease has no pathognomonic signs and symptoms (Wiersinga et.al, 2006). Current standard diagnostics are routine culture on non-selective media such as blood agar and selective gram-negative Mac-Conkey. However, both media are not selective for Burkholderia species, so Burkholderia bacterial colonies are difficult to distinguish from other bacterial colonies. Therefore, selective media is needed to overcome difficulties of the diagnosis. Selective media such as Ashdown's Selective Agar (ASA) as a standard to establish laboratory diagnosis of melioidosis. This study focused on the role of selective and nonselective media for culture clinical samples collected from patients suspected with Melioidosis.

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2 METHOD

A descriptive study, comparing the role of selective and nonselective media in growing Burkholderia species bacteria. Samples were collected by using total sampling method between January and June 2018, based on inclusion and exclusion criteria. Identification of bacterial colonies was phenotypically tested by using Vitek 2 Compact.

2.1 Specimen Collection

samples were collected from all clinical specimens that sent to Universitas Sumatera Utara Hospital Microbiology Laboratory from patients suspected with melioidosis based on the physician diagnosis on the Clinical Microbiology laboratory request form. Several types of specimens collected in the form were throat swab, blood, urine, pus, sputum and respiratory secretions.

2.2 Non-selective Media Columbia Agar and Mac-Conkey Agar

Columbia agar media preparation are made by dissolving 3.8 gram of Columbia into 100 mL of sterile aquadest and sterilized by using autoclave at 121^{o}C for 15 minutes. After sterilization, put solution at room temperature until medium temperature reaches 40-45^{o}C, 5% blood sheep was added and homogenized, then poured on sterile petridish. For Mac-Conkey agar media, 5.15 gram are dissolved into 100 mL of sterile aquadest and autoclaved. After sterilization process, put the solution at room temperature until medium temperature reaches 50^{o}C and finally poured on sterile petridish.

2.3 Selective Media Ashdown's Selective Agar (ASA)

Selective media preparation by weighing the composition of media consisting of Tryptone soya broth 10 gram, agar bacterial 15 gram, 40 ml glycerol, 5 mL of 0.1% crystal violet, 5 mL of 1% neutral red and 950 mL of distilled water, all of the material were dissolved into an Erlenmeyer and sterillized by using an autoclave at 121^{o}C for 15 minutes. Put the solution at room temperature until the temperature reaches 50^{o}C, gentamycin was added with concentration 4mg/liter and homogenized, then the media is poured on sterile petridish.

2.4 Bacterial Culture

Bacterial culture was done on by using selective ASA medium, as well as on routine media, Columbia Blood Agar and Mac-Conkey Agar. Bacterial culture was incubated at 35°C for 24-48 hours. Microscopic and macroscopic identification were done for every grown colonies.

2.5 Identification

The identification was started with macroscopic observation of bacterial colonies by performing a morphological selection of the suspected *Burkholderia* species bacteria. Followed by microscopic observation with Gram staining. The suspected *Burkholderia* species underwent identification stage by using the Vitek 2 Compact. Phenotypically, this tool can identify *Burkholderia* species bacteria using GN card and simultaneously performing antibiotic susceptibility test by using AST GN card.

2.6 Data Analysis

Data of the comparison between selective and non-selective media in growing Burkholderia species was analysed. All results were presented in the tabulation and percentage.

3 RESULTS AND DISCUSSIONS

Based on the results of culture of clinical specimens from patients suspected with Melioidosis on routine or non-selective media (Columbia Agar and Mac-Conkey Agar) for 24 hours found the growing bacteria *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas putida*, *Pseudomonas aeruginosa, Pseudomonas stutzeri*, *Pseudomonas fluorescens, Serratia marcescens* and *Acinetobacter baumannii*. While Burkholderia colony species were not seen yet, the incubation time on nonselective media therefore was extended to 48 hours. Based on observed colonies of Burkholderia species after 48 hours, it was seen in streaks thus continued to subculture and identification stage. However, in both media colonies of Burkhokderia species bacteria were not typical, making it a little difficult to do the selection.

Columbia or blood agar was used as a nonselective medium to evaluate the vitality of the

strain. Almost all bacteria can grow on this medium so there will be a competition for growth between species of bacteria (Edler, et.al, 2017). Whereas Mac-Conkey was more selective towards Gram-negative bacteria, but the growth of Burkholderia species requires accuracy and a longer incubation time (> 48 hours) to ensure the presence or absence of Burkholderia species colonies.

In contrast to Ashdown's agar selective media, at 24 hours the bacteria had grown and shown a distinctive colony morphology such as round, convex, absorbed little of the red pigment, and wavy surfaces. Based on the identification results using the GN identification card Vitek 2 Compact, colonies that grow on Ashdown's selective media were *Burkholderia pseudomallei* and *Burkholderia cepacia*. While other colonies that grew on this selective media were *Pseudomonas putida* and *Pseudomonas stutzeri*. The results of the growth comparison on selective and nonselective media can be seen in table 1.

Table 1: Growth of clinical strains at 48 hours on selective and nonselective media

Bacterial growth	Nonselective		Selective
	CA	MCA	ASA
Escherichia coli	+	+	-
Klebsiella pneumoniae	+	+	-
Pseudomonas putida	+	+	+
Pseudomonas aeruginosa	+	+	-
Pseudomonas stutzeri	+	+	+
Pseudomonas fluorescens	+	+	+
Burkholderia pseudomallei	+	+	+
Burkholderia cepacia	+	+	+
Serratia marcescens	+	+	-
Acinetobacter baumannii	+	+	-

The results of culture from 112 specimens of patients suspected with Melioidosis showed bacterial growth in Columbia Agar Blood medium which was 110 (9.8%) and in Mac-Conkey media. While on Ashdown's selective media, bacterial growth was seen in 10 samples, consisted of *Burkholderia pseudomallei* 2 (1.8%), *Burkholderia cepacia* 5 (4.4%), *Pseudomonas stutzeri* 2 (1.8%) and *Pseudomonas putida* 1 (0.9%). All bacterial growth from all clinical samples of patients suspected with Melioidosis were presented in Table 2.

Table 2: Other microorganisms growth seen on media

Bacterial species	No. (%) growth of bacterial isolates in each media		
	CA (n=112)	MCA (n=112)	ASA (n=112)
Escherichia coli	8(7.1)	8(7.1)	0(0)
K. pneumoniae	28(25)	28(25)	0(0)
P. putida	1(0.9)	1(0.9)	1(0.9)
P. aeruginosa	54(48)	54(48)	0(0)
P. stutzeri	2(1.8)	2(1.8)	2(1.8)
P. fluorescens	3(2.6)	3(2.6)	0(0)
B.pseudomallei	1(0.9)	1(0.9)	2(1.8)
B.cepacia	1(0.9)	3(2.6)	5(4.4)
S. marcescens	2(1.8)	2(1.8)	0(0)
A. baumannii	10(8.9)	10(8.9)	0(0)
Total :	110(98.2)	112(100)	10(8.9)

Based on the results of selective and nonselective media comparison, it can be seen that Ashdown's selective media has a higher selection rate for the growth of Burkholderia. This findings indicated the use of media has of great significance role in establishing diagnosis of Melioidosis. Although the media is very selective, Pseudomonas growth was also seen in this study. It is possible that Pseudomonas-type bacteria were found to be resistant to Gentamycin so that it grew in selective Ashdown's Media agar. So the composition of the media needs to be modified to the ASA medium.

Modification of ASA media media was needed as *Burkholderia pseudomallei* selective agar (BPSA), this medium was designed to improve recovery from strains that easier to be inhibited by *Burkholderia pseudomallei*, *Burkholderia cepacia*, and *Pseudomonas aeruginosa*, used to determine the selectivity and sensitivity of BPSA. The purpose of BPSA was to inhibit the growth of *Pseudomonas aeruginosa* making the identification of Burkholderia species easier to characterize because of the typical morphology of the colony Howard, et.al, 2005).

The sensivity was equal between ASA and BPSA based on their sensitivity ratio, although BPSA selectivity was found to be lower than ASA medium. Culture from 86 of 155 clinical specimens showed growth in at least one selective medium (range, 1 to 4 positive samples per patient) (Peacock et.al, 2005). BPSA has advantages in showing typical morphology such as crinkled and undulating colonies, while in ASA media it was seen to be smooth surface and convex colonies. Both media did not show any significant difference overall (Howard, et.al, 2005). ASA media supported the growth of

Burkholderia species including *Burkholderia mallei* and suitable for screening during situation when *Burkholderia pseudomallei* and/or *Burkholderia mallei* were suspected (Peacock et.al, 2005).

ASA media has an important role to support the diagnosis of melioidosis at the University Hospital of North Sumatra. This study findings demonstrated that to use only the current routine or nonselective media as the standard diagnostic tools of culture was definitely not enough to establish the diagnosis of melioidosis from clinical samples. The use and application of ASA as standard culture media in clinical microbiology laboratory routine services to support and establish the challenging diagnosis of melioidiosis from clinical samples collected from patients suspected with melioidosis was imperative.

4 CONCLUSIONS

This study showed that the use of ASA media was necessary especially in clinical microbiology laboratory settings and has high selectivity rate for Burkholderia. In this study, 7 of 10 types of bacteria that had successfully grown on ASA media were found to be Burkholderia species (5 of *Burkholderia cepacia* and 2 of *Burkholderia pseudomallei*).

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