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Article

Morphological Characteristics and Differentiation of *Acinetobacter baumannii* Colony on Selective Medium CHROMagar *Acinetobacter*

Edy Wiranto[1], Ariyani Kiranasari[2*], Lia Kusumawati[3,4], Mutiara[4], Tryna Tania[4]

[1]Master's Programme in Biomedical Sciences, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia
[2]Department of Clinical Microbiology, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia
[3]Department of Microbiology, Faculty of Medicine, Universitas Sumatera Utara, North Sumatra, Medan, Indonesia
[4]Murni Teguh Memorial Hospital, North Sumatra, Medan, Indonesia

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Abstract. *Acinetobacter* is an opportunistic pathogen that causes nosocomial infections and is one of the pathogens with the highest prevalence in the world. In addition to its prevalence, the number of isolates that are resistant to antibiotics is increasing every year worldwide. Identification of these bacteria in patients is generally carried out using various methods however, each method has drawbacks such as a long time, low accuracy, and requires high costs. To overcome this, CHROMagar *Acinetobacter* is used and is expected to be one of the solutions for identifying *Acinetobacter* in patient samples due to its selectivity and accuracy. However, in this research, several non-*Acinetobacter* bacteria are able to grow in this media, making identification of *A. baumannii* using this media remain a challenge. Using phenotypic tests, 5 isolates were successfully separated from 32 *A. baumannii* isolates in this study (13,51%), where these 5 isolates showed the same characteristics as *A. baumannii* phenotypically on CHROMagar *Acinetobacter* results but differ in biochemical tests. Therefore, false-positive results will be obtained based solely on CHROMagar *Acinetobacter* results. In sum, the use of CHROMagar *Acinetobacter* must be followed by other conventional tests to increase the accuracy of *Acinetobacter* identification in specimens.

Corresponding Author :
Ariyani Kiranasari
Department of Clinical Microbiology, Faculty of Medicine,
Universitas Indonesia, Jakarta, Indonesia
Email : gahariyani@gmail.com

1. Introduction

Acinetobacter belongs to the Moraxellaceae family which comes from the Greek word *a-kinetos-bacter* which means rod-shaped and non-motile bacteria. However, this bacterium is motile due to the twitching motility mechanism [1]. This bacterium also has the characteristics of a gram-negative cocci-bacillus, obligate aerobes, positive catalase, and negative oxidase [2]. These bacteria are ubiquitous, which means that they are abundant in the environment so they are easily found in soil, air, animals, or other surfaces. This bacterium can grow well in standard culture media such as Nutrient Agar (NA) or other media [3-5].

In recent years, this genus of bacteria has received worldwide attention and has become a target of research urgency due to several factors, including its relatively high prevalence worldwide and its resistance mechanisms. One of the most frequently encountered mechanisms and a major problem worldwide is the mechanism of increasing intrinsic resistance to existing antibiotics and resistance to some of the latest antibiotics [6-7]. Because of its ubiquitous characteristics, this bacterium is one of the highest contributors to mortality in clinical installations that cause nosocomial infections [8]. Clinical manifestations of *Acinetobacter* spp. also vary, ranging from asymptomatic to fatal such as sepsis, pneumonia, and meningitis [9]. Therefore, initial detection of this bacterial infection in patients cannot be enforced without identification process [4], [10].

Identification of *Acinetobacter* bacteria was carried out using bacterial culture process using a differential medium. However, this process is inaccurate considering that *Acinetobacter* colonies have the same morphology as other bacteria, making it difficult to differentiate [11]. Another way that can be done is to use biochemical tests, either conventionally (using artificial media) or manufactured (such as Vitek-2). Although testing using manufacturing techniques is considered more accurate than conventional processes, the identification process is still difficult because the biochemical properties of this bacterium are similar to other bacteria, so there is no definitive test for the identification [12]. The other most accurate methods of assistance are using molecular approaches such as Polymerase Chain Reaction (PCR) [13-14] or by using Whole-Genome Sequencing (WGS) [15-16]. However, both require longer time and costs more [12]. Based on this, it is necessary to develop new methods or media for the assistance process so that the assistance process can be carried out as quickly and efficiently as possible.

One of the selective media that can be used for the isolation process of *Acinetobacter* bacteria is CHROMagar *Acinetobacter* media (Cat. No. AC092; CHROMagar, Paris, France) where this media utilizing a chromogenic substrate which will be broken down enzymatically to produce certain colors. Thus, the target bacteria show certain colored colonies after being incubated in this medium for 18-24 hours so that they can be observed and identified easily. In addition, this media also contains compounds that can inhibit the growth of non-target bacteria thereby reducing the occurrence of other bacterial contamination [17-18]. This study aims to show the morphological characteristics of *Acinetobacter* spp. and other bacteria that can grow on CHROMagar medium *Acinetobacter*.

2. Materials and Methods

The number of bacterial samples used was 37 collection isolates from the Microbiology Laboratory of the Hospital. Murni Teguh Memorial Hospital Medan, North Sumatra. The isolate is suspected to be *Acinetobacter* spp. based on colony morphology and culture results grown on CHROMagar

Acinetobacter media. The preparation of CHROMagar *Acinetobacter* media was carried out according to the manufacturer's instructions by dissolving 32.8 grams of base media powder (containing 15 grams of agar, 12 grams of peptone and yeast extract, 4 grams of salts and 1.8 of chromogenic mixture) in 1L of distilled water.

Supplements containing growth factors were added to the mixture in the ratio of 4 mL of supplement to 1 L of base media mixture. The mixture is heated in a water bath at a temperature of no more than 100°C with periodic stirring until it is homogeneous. The media is cooled to a warm temperature and then aseptically poured into sterile petri dishes. Bacterial isolates were inoculated on the media by streak method using loops

Bacteria inoculated on CHROMagar *Acinetobacter* were characterized based on their morphology such as color, shape, edges, and elevation of the colony on the media. Bacteria that do not show a morphology compatible with *Acinetobacter* spp. on CHROMagar media was tested further by culturing it in MacConkey Agar media and Gram staining as well as a series of biochemical tests such as the oxidase test, catalase test, motility test, and sugar fermentation test which include glucose, sucrose, lactose, mannitol, and maltose.

Preparation of MacConkey media Agar No. 3 (Cat. No. 100181; Oxoid) was carried out by dissolving 51.5 g of media powder in 1L of distilled water according to the manufacturer's instructions. The mixture was heated on a heater until homogeneous and sterilized using autoclave at 121°C for 15 minutes. The oxidase test was carried out by inoculating bacterial colonies on the Bactident® Oxidase strip (Cat. No. 100181; Sigma-Aldrich) where a positive result was indicated by a change in the color of the inoculated strip to blue. 3% H_2O_2 is dripped on the surface of the object glass and inoculated with the isolate will produce air bubbles when the bacteria produce catalase enzymes to break down H_2O_2. Bacterial motility was tested by inoculating the bacteria on SIM (Sulfur-Indole Motility) media using a straight loop perpendicular to the center of the media where a positive result was indicated by the presence of faint traces on the media. The ability of sugar fermentation in bacterial isolates was indicated by a change in color to yellow due to changes in the pH of the media containing simple sugars and pH indicators such as bromocresol purple.

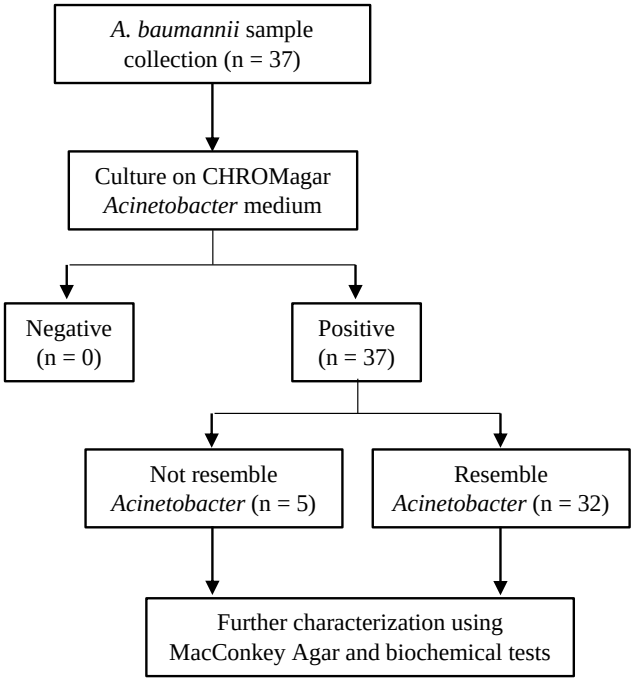

Figure 1. Flowchart of research

3. Results and Discussion

A total of 37 bacterial isolates are suspected of being *Acinetobacter* spp. obtained using CHROMagar *Acinetobacter* medium. A positive result was indicated by the growth of bright red colonies with a circular shape, flat edges, and convex elevation although with varying sizes after 18-24 hours of incubation. Oxidase test is performed on all isolates as confirmation.

Figure 2. Colonies of isolates of *Acinetobacter* spp. On CHROMagar *Acinetobacter* media

After the oxidase test, 32 out of 37 isolates gave negative results. These results indicate that the bacteria are *Acinetobacter* spp. while 5 bacterial isolates gave positive oxidase results. In addition to the oxidase test, another supporting test was carried out on these 5 bacterial isolates with the results that can be seen in Table 1.

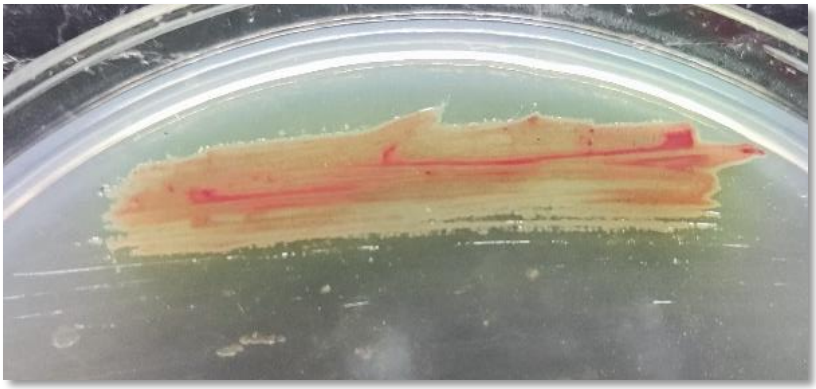

Figure 3. Pigments and colony morphology of oxidase-positive bacterial isolates on CHROMagar *Acinetobacter* media

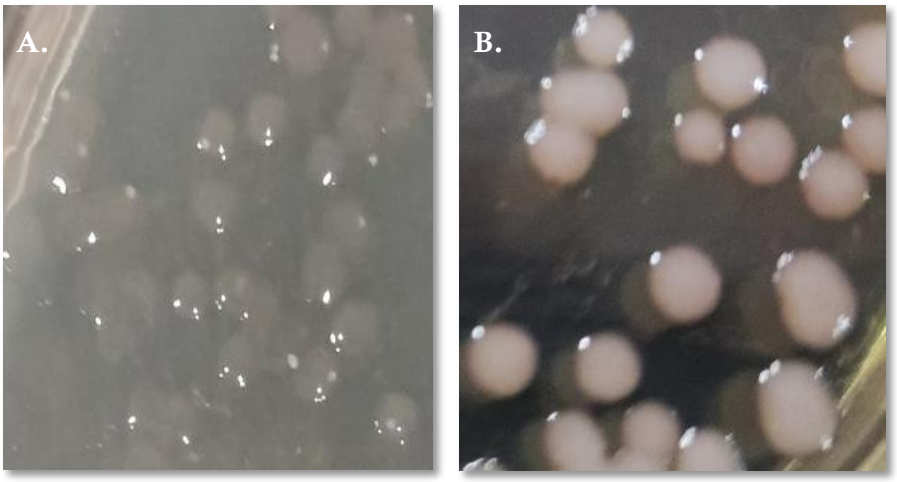

Figure 4. Colonies of bacterial isolates (A.) positive oxidase bacteria on MacConkey Agar media, (B.) *Acinetobacter* spp.

Table 1. Biochemical tests on all bacterial isolates.

Test	Results	
	(+) oxidase isolates	*A. baumannii* isolates
Gram staining	Gram (-), rod	Gram (-), rod
Motility	Positive	Negative
Catalase	Positive	Positive
Oxidase	Positive	Negative
Sugar fermentation		
• Glucose	Negative	Positive
• Sucrose	Negative	Negative
• Lactose	Negative	Positive
• Mannitol	Negative	Negative
• Maltose	Negative	Negative

Acinetobacter spp. is one of the pathogenic bacteria that cause infection in humans with a prevalence that varies throughout the world. Some species of this genus are reported to have a higher prevalence than other species, but the species that generally becomes a major problem in medical installations is *A. baumannii* [5], [12]. Due to its persistence, *A. baumannii* is one of the pathogenic bacteria with the highest prevalence among other pathogenic bacteria where the *Acinetobacter* genus can grow only with simple nutrient [3]. This bacterial infection is exacerbated considering that most of these bacteria are found to be resistant to antibiotics in patients so the treatment process becomes more difficult [4-6].

Identification of *Acinetobacter* spp. generally carried out not only by utilizing a differential media but also by utilizing an automatic system such as the Vitek-2. Identification using differential media alone is not effective because the morphology and characteristics are almost the same as other bacteria [12]. Therefore, several biochemical tests need to be carried out, although this alone cannot provide an accurate identification. Among several biochemical tests, the oxidase test is the most important test. Differentiation of *Acinetobacter* from other bacterial genera of the same family (Moraxellaceae) can be carried out by the oxidase test considering that only this genus of bacteria is oxidase negative [3]. The findings of biochemical testing on 32 isolates with negative oxidase results are consistent with

the features of *A. baumannii isolates*, as shown in Table 1. However, other tests for example particular sugar fermentation, may provide variable results even though all the isolates are of the same species [19-20]. Therefore, further identification techniques like genetic and serological techniques should be used in addition to biochemical testing, which cannot be regarded as the only reference for identifying this bacteria. These inconsistent results may exacerbate the difficulty of identifying this bacteria in patients, resulting in a laborious and ineffective therapeutic and infection control procedure.

Not only for the process of growing bacteria, CRHOMagar is also used for the purpose of identifying bacteria that grow on the media [21-22]. The use of this media can be applied in hospitals, considering the high level of sensitivity and specificity when compared with other conventional techniques [23-24]. However, in the case of CHROMagar *Acinetobacter* used in this research, some bacteria are known to grow on this medium, even though the media is selective [17]. When grown on CHROMagar *Acinetobacter* media, colonies of *Acinetobacter* spp. will show red colonies as shown in Figure 1.

In this study, 5 isolates with positive oxidase test results were able to grow with colony resembling *Acinetobacter* spp. and produce a blue-yellow pigment around the colony. In addition, the bacteria show colony characteristics that are different from *Acinetobacter* spp. when grown on MacConkey Agar media, where the bacterial colonies are clear. Together with the results of biochemical tests, these characteristics are consistent with the characteristics of the genus *Pseudomonas* which generally also produces soluble pigments in media such as pyocyanin and pyoverdine [3], [25]. The growth of *Pseudomonas* in this media is thought to be caused by the characteristics of *Pseudomonas* themselves, namely being able to utilize a variety of carbon sources, and is also a pathogen commonly found in hospital patients [26-27]. Nonetheless, further identification and testing need to be carried out on these isolates to obtain definitive information.

Besides CHROMagar *Acinetobacter*, MacConkey Agar is often used for the initial identification process of *Acinetobacter* spp [11]. MacConkey agar is frequently used in hospitals to differentiate bacteria in patients based on its ability to ferment lactose. Most pathogenic bacteria (for example: *Enterobacterales*) are capable of fermenting lactose while others pathogen do not have this ability, and therefore can simplify the identification process based on that trait [28]. However, it should be noted that the results of the culture process of these bacteria on MacConkey Agar also can show varying results. When grown on MacConkey Agar, *Acinetobacter* colonies can show the results of the absence of fermentation which is indicated by the yellow color of media. However, after 48 hours or more, the media and bacterial colonies slowly turned red, showing that *Acinetobacter* are a slow-fermenter bacterium [18], [29].

On the other hand, other bacteria that are also part of the Non-Fermenting Gram Negative Bacteria (NFGNB) group such as *Acinetobacter* are the genus *Pseudomonas*, which in MacConkey Agar media this genus has the characteristics of having clear colonies with yellow surrounding media. *P. aeruginosa* does not have the ability to ferment lactose and therefore, the media will remain yellow [25], [30]. Because of this, MacConkey agar also cannot be used as a sole definitive identification method for *A. baumannii*, even though its use is very common in hospitals.

4. Conclusion

CHROMagar *Acinetobacter* is a selective and differential medium that can be used to culture bacteria of the genus *Acinetobacter*. The process of identifying *Acinetobacter* bacteria in specimens can be done quickly through the morphological characteristics of the colonies that grow on the media. Nonetheless, the accuracy of the identification process can be improved by methods such as Gram stain where *Acinetobacter* spp. has the characteristic coccus-bacilli shape while other bacteria such as *Pseudomonas* which also grows on CHROMagar *Acinetobacter* media have the characteristic of a rod shape. Other tests are also necessary, such as oxidase and catalase tests considering that some non-*Acinetobacter* bacteria can still grow on CHROMagar *Acinetobacter* media.

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