

COMPARISON OF MICROBIOLOGICAL EXAMINATION BY TEST TUBE AND CONGO RED AGAR METHODS TO DETECT BIOFILM PRODUCTION ON CLINICAL ISOLATES

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ABSTRAK

Biofilm pada perangkat medis dapat menyebabkan penyakit dan kematian yang signifikan dan memberi pengaruh besar pada penularan penyakit di antara pasien dan penyedia layanan kesehatan dan berpotensi meningkatkan biaya perawatan pasien. Dengan mengetahui adanya biofilm pada pasien, seseorang dapat membedakan pengelolaan pengobatan untuk pasien tertentu dari pasien tanpa biofilm pada alat kesehatan mereka. Tujuan penelitian ini adalah untuk mendapatkan metode diagnostik untuk mendeteksi pembentukan biofilm pada isolat dari alat kesehatan menggunakan metode sederhana yang mudah dilakukan dan bisa diterapkan di laboratorium mikrobiologi terbatas sumber daya. 36 spesimen yang diperoleh dari IV Line, CVC, kateter urin dan ETT ditanam pada agar Muller Hinton dan dilanjutkan dengan 3 metode, yaitu metode Test Tube, metode Congo Red Agar dan metode Microtiter Plate Assay. Hasil penelitian ini menunjukkan Uji Tabung (nephelometer), Test Tube (visual) dan Congo Red Agar memiliki sensitivitas yang sama, yaitu 100%, namun memiliki spesifisitas yang lebih tinggi dibandingkan metode Test Tube (visual) dan metode Congo Red Agar dalam mendeteksi produksi biofilm pada isolat dari alat medis yang telah terpasang ke tubuh pasien. Pembentukan biofilm di dalam perangkat tergantung pada faktor, yaitu inang, alat dan mikroorganisme itu sendiri. (FMI 2018;54:22-28)

Kata kunci: Biofilm; perangkat; metode; sensitivitas; spesifisitas

ABSTRACT

Biofilm on medical devices can cause significant diseases and deaths and give a large effect on disease transmission among patients and health providers and potentially increase the cost of patient treatment. By knowing the presence of biofilm on a patient, one can differentiate the treatment management for that particular patient from the patients without biofilm on their medical device. The purpose of this study was to obtain diagnostic method to detect biofilm formation on isolates from the medical devices by simple method that is easy to do and can be applied in resource-limited microbiology laboratory. 36 specimens obtained from IV Line, CVC, urinary catheter and ETT were grown on Muller Hinton agar and continued with 3 methods, i.e., Test Tube method, Congo Red Agar method and Microtiter Plate Assay method. Results of this study showed Test Tube (nephelometer), Test Tube (visual) and Congo Red Agar in order to have the same sensitivity of 100% but has higher specificity compared to Test Tube method (visual) and Congo Red Agar method in detecting biofilm production on isolates from medical devices that had been plugged into patients body. The biofilm formation inside devices depends on factors, i.e., host, device and the microorganism itself. (FMI 2018;54:22-28)

Keywords: Biofilms; device; method; sensitivity; specificity

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INTRODUCTION

Biofilm is a community of microorganisms which attached on solid surface, either living or nonliving surface. Microorganisms in biofilm can be prokaryotic or eukaryotic, and can live in either one of two forms, sessile or planktonic. Sessile form is a result of attachment and usually expands into multispecies biofilm with unique characteristics, in many ways similar to hydrated polymer. Planktonic form is free-flowing microorganism (Mahon et al 2011).

Biofilm on the surface of medical device can cause significant diseases and deaths and give great impact in

infectious disease transmission among patients and health care workers (Donlan 2008). Particularly in patients with infection, one of solutions in patient management, i.e. by detecting biofilm on medical device in patients body.

Microbiological examination of biofilm detection by Microtiter Plate Assay Method/Tissue Culture Plate Method is the gold standard to detect presence of biofilm on medical device in patients body. Optical Density (OD) of attached bacteria which had been stained by crystal violet was assessed by micro ELISA autoreader (mode PR 601, quklinger S) on 630 nm (OD 630 nm).

The value of OD based on index of bacterial attachment on the surface and biofilm formation.

Other than Microtiter Plate Assay Method/Tissue Culture Plate Method, which are quantitative methods, there are also qualitative methods, i.e. Test Tube Method and Congo Red Agar Method. In Test Tube examination, biofilm formation is positive when a blue layer is seen on the wall and bottom of tube, and negative when no blue color is seen. Retained blue color from crystal violet staining indicate slime attachment of biofilm on the wall and bottom of tube. In Congo Red Agar method, positive result is shown by black colony and dry crystal consistency. Pink colony appears if there is no presence of biofilm. Congo Red acts as pH indicator. Color changes into blue in pH 3,0 and red in pH 5,2. Color of colonies turns into black after isolates on Congo Red Agar were incubated. When incubated, sucrose in Congo Red medium breaks into glucose and fructose. During metabolism, bacteria also produce glucose. Glucose is acidic. Indicator in Congo Red bound with glucose from bacteria metabolism and sucrose degradation, resulting in pH change into acidic. With the high acidification, the color were no longer blue but turned into black (Mathur et al 2006, Yoon et al 2007, Hassan et al 2011, O'Toole 2011, Abidi et al 2013, Deka 2014, Vasanthi et al 2013, Lotfi et al 2014).

The purpose of this study was to detect biofilm formation in isolates from medical device using diagnostic method that is easy to do and applicable in simple clinical microbiology laboratory. This research specifically analyzes accuracy of microbiology examination by Test Tube method, Congo Red Agar and Microtiter Plate Assay as gold standard in biofilm formation detection in isolates from medical device.

MATERIALS AND METHODS

Samples of medical devices had been removed from patients according to standard procedure of the medical device length of use in intensive care unit Dr. Soetomo Hospital, Surabaya. The location of this study was intensive care unit of Dr. Soetomo Hospital, Surabaya. Culture and detection of biofilm-forming bacteria were done in clinical microbiology laboratory of Dr. Soetomo Hospital, Surabaya. Optical Density measurement by ELISA reader was done in Institute of Tropical Disease Universitas Airlangga, Surabaya. This research was done in June – July 2016. Devices that had been collected were cut in 5 -7 cm. Every part of medical devices lumen was flushed by 2 ml of BHI and incubated for 24-48 hours in 10 ml BHI. Primary isolation was done on MH agar. Identification was done by Gram staining of colony from primary isolation.

Biofilm formation of isolates were detected by three methods to reveal phenotype of each isolate: Test Tube found by Cristensen GD, Congo Red Agar Method found by Freeman, and Microtiter Plate Assay Method found by Cristensen GD.

These three methods have been standardized by ATCC 35984 *Staphylococcus epidermidis* which is known as biofilm producer and ATCC 12228 *Staphylococcus epidermidis* as non-slime biofilms producer. As control *Staphylococcus aureus* ATCC 35556, *Pseudomonas aeruginosa* ATCC 27853, and *Escherichia coli* ATCC 35218.

Test Tube (TT)

Examination by Test Tube in this study was divided into visual and nephelometer. Test Tube method with nephelometer up to now has not been published in journal or research articles so that researchers do not have any guideline to refer. However, by using nephelometer, accurate value of biofilm density can be obtained.

Test Tube Visual

A loopful of bacteria colony from primary isolation that has been incubated for 24 hours in 37°C was added into TSB Media with 1% glucose as much as 10 ml. Then the tube that had been filled with broth was washed by phosphat buffer saline (pH 7.3) and dried. Dried tube was then fixated by passing it through flame three times and stained with crystal violet (0.1%). Remove crystal violet and wash tube with ionized water. Tube was then dried in reversed position and observed for biofilm formation. Biofilm formation is positive when a blue layer is seen on wall and bottom of the tube. To avoid subjectivity bias, examination was done by minimal two observers. In this study, the examination was done by three observers and repeated three times to obtain maximal result.

Test Tube Nephelometer

A loopful of bacteria colony from primary isolation that has been incubated for 24 hours in 37°C was added into TSB Media with 1% glucose as much as 10 ml. Then the tube that had been filled with broth was washed with phosphat buffer saline (pH 7.3) and dried. Dried tube was then fixated by passing it through flame three times and then stained with crystal violet (0.1%). Remove crystal violet and wash tube with ionized water. Dry tube in reversed position. Interpretation of the result was done with nephelometer, i.e., positive when the value = 0.36 and negative when the value <0.36.



Fig. 1. Test Tube with positive result (visual (+), nephelometer (+)).

Congo Red Agar (CRA) method

Congo Red Agar media consists of 37 g/L BHI broth, 10 g/L agar base, 50 g/L sucrose, 1 L water and 0.8 g/L Congo Red indicator. Congo Red indicator was prepared as concentrated liquid apart of other media constituents, and autoclaved in 121°C for 15 minutes and then added into cooled agar in 55°C. Isolate was then inoculated and incubated for 24 hours in 37°C. Positive result was shown by black colored colonies. Negative result was shown by pink color on colonies.

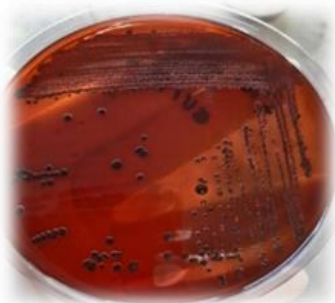


Fig. 2. Congo Red Agar with positive result.



Fig. 3. Congo Red Agar with negative result.

Microtiter Plate Assay (MPA) method

Isolate was put into TSB media that had been added with 1% glucose (TSBglu) and incubated for 24 hours in 37°C and then diluted 1:100 with new medium. Every sterile polystyrene well (out of 96 wells) was filled with 0.2 ml of diluted culture and only broth was used as control for sterility test and non-specific binding of media. Culture was incubated for 24 hours in 37°C. After incubation, plate was gently tapped and then washed four times with 0.2 ml PBS in pH 7.2 to remove planktonic bacteria. Biofilms made of microorganisms that was attached on plate (sessile) binded with sodium acetate (2%) which stained by crystal violet (0.1% w/v). Stain was washed with ionized water and plate was dried. Cells that were attached usually form biofilms and wells were stained with crystal violet. They were then washed with PBS once and fixated with ethanol for 15 minutes. OD of bacteria was attached on wells and stained by crystal violet which was determined by micro ELISA autoreader (mode PR 601, quklinger S) on wave of 630 nm (OD 630 nm). This process was repeated three times to obtain optimal result. Biofilm formation is interpreted as negative if $0 \leq 0.2$, moderate if $0.2 - 0.4$, and positive if ≥ 0.4 . In this study, the moderate results were categorized as positive.

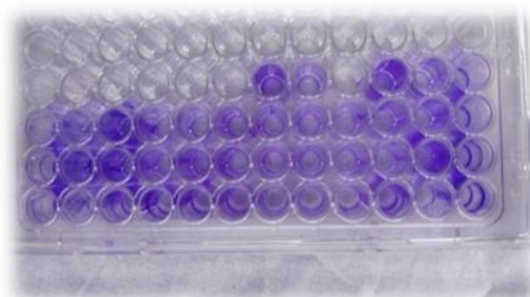


Fig. 4. Microtiter Plate Assay after staining with crystal violet.

RESULTS

Device specimens that had been collected were 86 devices. However, out of 86 devices, only 36 specimens that grow bacteria can be continued with Test Tube method, Congo Red Agar method, and Microtiter Plate Assay method. Based on device distributions, devices most common obtained from Intensive Observation Room (IOR) and Intensive Care Unit (ICU) were 25 intravenous catheters (69.4%), 7 urinary catheters (19.4%), 2 central venous catheters (5.6%) and 2 endotracheal tubes (5.6%). The distribution of devices with bacterial growth is shown in Fig. 5.

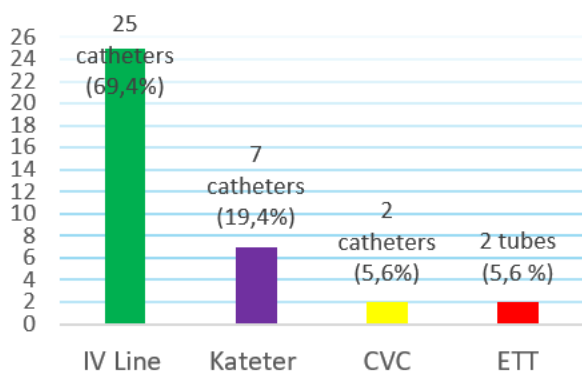


Fig. 5. Distribution of devices with bacterial growth.

Based on distribution on Fig. 6, the device specimens obtained were IOR 24 devices (66.7%) and ICU 12 devices (33.3%). Distribution of units where the device specimens were obtained is shown in figure 6.

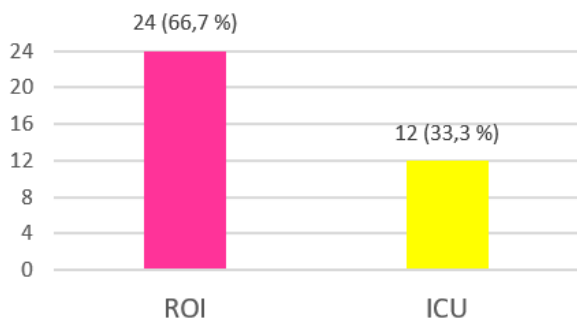


Fig. 6. Distribution of units where the device specimens with bacterial growth were obtained.

Distribution of positive and negative results of all methods is shown in Table 1 and results of cross tabulation is shown in Table 2.

Table 1. Distribution of positive and negative results of Test Tube method (visual) and Test Tube method (nephelometer), Congo Red Agar method, and Microtiter Plate Assay method

	Positive	Negative
Congo Red	25 (69.4%)	11 (30.6%)
Test Tube (Visual)	32 (88.9%)	4 (11.15%)
Test Tube (Nephelometer)	22 (61.1%)	14 (38.9%)
Microtiter Plate Assay	15 (41.7%)	21 (58.3%)

DISCUSSION

Medical devices play important part in nosocomial infections, especially in patients on intensive care. Infections related to medical devices can be trouble-

some in clinical practice and ends with economical problems. Bacteria colonization on medical devices can result in infection and malfunction of the devices.

Table 2. Comparison of sensitivity, specificity, accuracy of all methods

	Sensitivity	Specificity	Accuracy
Congo Red Agar to Microtiter Plate Assay	100%	52.3%	72.3%
Test Tube (Visual) to Microtiter Plate Assay	100%	19.4%	52.8%
Test Tube (Nephelometer) to Microtiter Plate Assay	100%	66.6%	80.6%
Test Tube (Visual) to Congo Red Agar	84%	0%	58.3%
Test Tube (Nephelometer) to Congo Red Agar	72%	63.6%	59.4%
Test Tube (Visual) to Test Tube (Nephelometer)	100%	28.57%	72.2%

The core of pathogenesis of device-associated infection is interaction between bacteria, device that is used and host factors. Bacteria use different adhesins to colonize medical devices. Initially, rapid bacteria attachment is facilitated by non-specific factors such as surface tension, hydrophobic and electrostatic condition and the presence of specific adhesin.

Device factor increases bacteria virulence. Data analysis for bacterial attachment on surface holds five principles, i.e. (1) Different bacteria can attach in different manners on devices of same material, (2) Same bacteria can attach differently on devices of different materials, (3) Same bacteria can attach differently on devices of same material under different condition, including where the devices are put (hydrophobic or hydrophilic media), electrical current type (dynamic or static), and temperature, (4) In vitro inhibition of bacteria colonization on device cannot predict effectivity of in vivo inhibition, (5) Clinical profit of surface modification approach can be different in one bacteria from another (Darouiche 2001).

There are two host factors, i.e. (1) Host factors that cause bacteria attachment on device, (2) Host factors that can support or hinder bacteria presence on device surfaces. IV line and CVC are intravascular catheters

that are used to instill parenteral liquid, drugs, and nutrition, and blood products; to monitor haemodynamic status, and to assist haemodialysis (Mermel et al 2001). It means that intravascular devices harbor more risk of device-related infection compared to other medical devices (Klevins et al 2005). Microorganisms can colonize both catheter's external and internal (luminal) surfaces (Raad et al 1993).

Biofilm forms in three days following catheter insertion (Anaissie et al 1995). Raad et al reported in 1993 that catheters indwelling less than 10 days have more colonization compared to external surface of long-term catheters (over 30 days), but biofilm formation is more dominating inside catheter lumen.

Microorganisms colonizing catheters can be originated from skin of insertion area and migrating along catheter surface, or originated from the catheter, related to transmission of organisms from healthcare personnel into catheter lumen (Elliot et al 1997, Raad et al 1997).

Microorganisms can spread hematogenous from infected device to other places through patient's catheter (Anaissie et al 1995). Platelets, plasma and protein such as fibronectin, fibrin and laminin will be absorbed to catheter surface after the catheter inserted into blood vessel (Raad et al 1997) and these materials will transform surface characteristics and cause microbial attachment (Rupp et al 1999, Murga et al 2001).

UTI contributes 25%-40% of nosocomial infection and become the most common infection (Maki and Tambyah 2001, Foxman 2003 Kalsi et al 2003, Bagshaw & Laupland 2006, Wagenlehner & Naber 2006). There are many interventions to be done based on evidence-based practice guidelines to prevent VAP, the same goes to ETT management. Microaspiration and biofilm formation are two important mechanisms that play important roles in colonization on tracheal bronchial tree and the development of VAP. Microaspiration happens when microorganisms migrate from distal part of secretion above ETT cuff, and biofilm grows from secretion on ETT surface and migrate along ETT polymer cuff and entire ETT surface, which facilitate bacteria transfer to the normally sterile bronchial tree (Fernandez et al 2012).

Congo Red Agar method in this study had some pros and cons. The pros were: easy to do, one time incubation, media Congo Red Agar could be produced in many number a batch so that we did not have to make new media every time we isolated bacteria, only little amount of Congo Red indicator was needed, i.e. 0.8 mg/L, for the making of media, black color of colonies for biofilm identification could be observed easily, those

colonies could also be used for antibiotic susceptibility tests. The cons of Congo Red Agar methods were: Congo Red indicator was not on sale in Indonesia, so that it took much time and cost, and it needs pure subculture.

Test Tube examination in this study was done in two ways: visual and nephelometer. Up to dated Test Tube method with nephelometer has not been published on journals or research articles so that we do not have any source to refer to. By using nephelometer, accurate values for biofilm density can be obtained.

The pros of visual Test Tube were: faster, simple and easy to do, and only take one time incubation. The con of visual Test Tube was that it needed minimal 3 observers to decide positive or negative results. The pros of Test Tube nephelometer were: fast, simple and easy to do, can give accurate value of biofilm density, do not require a many observers, and just one time incubation. The con of Test Tube nephelometer was that it needed nephelometer.

Microtiter Plate Assay (MPA) method is a quantitative test introduced by Christensen et al (1985), which is a good gold standard to detect biofilms. Microtiter Plate Assay method is a quantitative method that can be used to detect biofilms and be recommended as general screening method to detect bacteria that produce biofilm in diagnostic laboratory (Hassan et al 2011). In this study, results of Microtiter Plate Assay method have lower sensitivity and specificity value compared to Test Tube Nephelometer because of many factors, such as short time period and only few devices used as samples.

In this study, Test Tube Visual gave more positive results, although it is presumed that Test Tube Visual has lower value compared to Test Tube Nephelometer. However, positive and negative results was based on subjectivity of observers. It was because there was no standardized control of color to compare positive or negative results visually. The result of unavailability of control is false positive results, which then cause higher value of Test Tube Visual than those of Test Tube Nephelometer. Results of this research suggest Test Tube Visual as initial screening to detect biofilm formation on medical devices but not as standardized microbiological examination to decide presence of biofilms.

Microbiological examination to detect biofilm production from clinical isolates by Test Tube method, particularly by the use of nephelometer, because this method is easy to do, and applicable in Microbiology laboratory of Dr. Soetomo Surabaya Hospital.

CONCLUSION

Test Tube nephelometer, Test Tube visual, and Congo Red Agar have the same sensitivity, which is 100%. However, the specificity of Test Tube nephelometer is higher than Test Tube visual and Congo Red Agar in determining biofilm production of isolates from medical device that had been taken out from patients body. Biofilm formation in device depends on three factors: host, device, and the bacteria. Based on this study, further studies with larger and more representative samples needs to be done upon each method of examination. Studies focusing on effectivity of each method to detect biofilms on different medical device specimens and also needs to be conducted. Besides, it is also interesting to raise a topic about the effect of different materials that the devices made of to biofilm formation on the devices to further give input and advice clinicians in using devices with specific materials, especially in IOR and ICU, and the need of further investigation by phenotypic and molecular techniques of each bacteria on factors that contribute to biofilm formation in medical devices.

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