EFFECT OF GLUCOSE INDUCTION ON BIOFILM DENSITY IN CLINICAL ISOLATE *Acinetobacter baumannii* PATIENTS IN INTENSIVE CARE UNIT OF DR. SOETOMO HOSPITAL, SURABAYA

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ABSTRACT

This study aimed to analyze the effect of glucose induction on the clinical isolate biofilm density of Acinetobacter baumannii. Thirteen clinical isolates of A. baumannii non biofilm forming were collected from non-DM patients who were treated at the ICU of Dr. Soetomo Hospital, Surabaya, was treated with the addition of 0.08% glucose, 0.15% glucose, 0.2% glucose, and 0.4% glucose in TSB growth media, followed by biofilm density examination with Tissue Culture Plate Method (TCPM) using 96 wells flatbottomed polyesterene tissue culture plate and read by autoreader ELISA with a wavelength of 630 nm (OD630). Biofilm density obtained was analyzed using ANOVA statistical analysis. The results of OD630 showed that the biofilm density increased significantly at the addition of 0.2% and 0.4% glucose. There was a significant increase in biofilm density at the addition of 0.2% and 0.4% glucose so that the management of blood sugar levels in ICU patients was needed before and when medical devices were installed.

Keywords: Biofilm density; glucose; A. baumannii

ABSTRAK

Penelitian ini bertujuan untuk menganalisis pengaruh induksi glukosa terhadap kepadatan biofilm isolat klinis Acinetobacter baumannii. Tiga belas isolat klinis A. baumannii non biofilm dikumpulkan dari pasien non-DM yang dirawat di ICU Rumah Sakit Dr. Soetomo, Surabaya, dirawat dengan penambahan glukosa 0,08%, glukosa 0,15%, glukosa 0,15%, dan 0,4% glukosa dalam media pertumbuhan TSB, diikuti dengan pemeriksaan kerapatan biofilm dengan Metode Kultur Jaringan (TCPM) menggunakan 96 sumur plat kultur jaringan poliester flatbottomed dan dibaca oleh ELISA autoreader dengan panjang gelombang 630 nm (OD630). Kepadatan biofilm yang diperoleh dianalisis menggunakan analisis statistik ANOVA. Hasil OD630 menunjukkan bahwa kepadatan biofilm meningkat secara signifikan pada penambahan 0,2% dan glukosa 0,4%. Ada peningkatan yang signifikan dalam kepadatan biofilm dengan penambahan 0,2% dan glukosa 0,4% sehingga pengelolaan kadar gula darah pada pasien ICU diperlukan sebelum dan ketika peralatan medis dipasang.

Kata kunci: Densitas biofilm; glukosa; A. baumannii

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INTRODUCTION

Acinetobacter baumannii is a non-fermenting environmental flora Gram negative coccobasilus with the ability to colonize the human body. Acinetobacter baumanni is grouped by the Infectious Diseases Society of America as one of the six most important multidrug-resistant (MDR) microorganisms found in hospital environments around the world (Talbot et al 2006). This bacterium is one of the pathogenic opportunistic bacteria which is responsible for 2-10% of the incidence of Gram negative bacterial infections in the hospital (Joly-Guillou, 2005).

Acinetobacter baumannii is currently recognized as a cause severe nosocomial infections, including skin and soft tissue infections, wound infections, urinary tract infections, and meningitis secondary (Bergogne-Bérézin & Towner 1996, Towner 2009; Roca et al 2012, McConnell et al 2013) and have the highest mortality rates in cases of ventilator-associated pneumonia and blood stream infection (Dijkshoom et al 2007) where VAP has a crude mortality rate of around 40-70% (Fagon et al 1996, Garnacho-Montero et al 2003) while BSI has a crude mortality rate of around 28-43% (Seifert et al 1995, Wisplinghoff et al 2004). The

incidence of *A. baumannii* infection is increased when associated with the length of time of hospitalization in hospital and in older patients (Wisplinghoff et al 1999).

Another thing that should be a concern of *A. baumannii* is the multi-drug resistance is caused by the intrinsic properties of its resistance against some classes of ß-lactam antibiotics and some other groups, and exacerbated by the misuse of broad-spectrum antibiotics. (Amyes & Young 1996). *A. baumannii* have the capacity to develop resistance mechanisms against many classes of ß-lactam antibiotics include a broad spectrum third generation cephalosporin, carboxy-penicillins (Joly-Guillou et al 1995) and the class of carbapenems (Mussi et al 2005). *A. baumannii* is able to produce many enzymes that activate aminoglycoside groups (Buisson et al 1990, Lambert et al 1990) and most strains are resistant to the fluoroquinolone class (Joly-Guillou et al 1995, Vila et al 1993).

A. baumannii has the ability to survive in a hospital environment because it is able to interact with a variety of surfaces of both abiotic objects such as furniture, linen and medical devices (Neely et al 1999, Neely 2000, Villegas & Hartstein 2003, Borer et al 2005) and biotics such as human epithelial cells or Candida albicans filaments (Lee et al 2006) and are able to form biofilms (Costerton et al 1999). There is a hypothesis that *A. baumannii* which survives in the hospital environment has primary virulence factors, namely multi-drug resistant properties and the ability to form biofilms. (Donlan & Costerton 2002, Gaddy & Actis 2009).

Biofilm formation often begins with colonization of bacteria on abiotic or biotic surfaces forming sessile microcolony and followed by the production of exogenous polysaccharide containing glycocalyx as a biofilm architectural structure. If environmental conditions do not support biofilms such as nutrient deficiencies or too many bacteria, the sessile organisms will freeze and become free-floating organisms, called planktonics, which will become pathogenic agents that cause infections associated with the installation of medical devices (Trautner & Darouiche 2004) Based on the latest public report conducted by the National Institutes of Health, more than 60% of all microbial infections are caused by biofilms and often in recurrent infections or chronic infections, including non-responsiveness to antibiotic therapy according to culture (Costerton et al 2003). Biofilms play an important role because they protect bacteria from antibiotic activity by changing the characteristics of bacterial cells and metabolic dormancy so as to cause resistance to antibiotics (Donlan 2002, Trautner & Darouiche 2004).

Glucose has a very important role for the survival of bacteria, besides acting directly as a carbon source in the metabolic process, glucose is capable of acting as a signaling molecule and influencing the expression of various genes, one of which is a role in the biofilm formation process. A study of *A. baumannii* showed an increase in Bap protein production involved in the sugar utilization process through Leloir pathway, where high glucose levels will stimulate the anabolic process of glucose to UDP-glucose thereby increasing extracellular polysaccharide production in *A. baumannii* biofilms (Shin et al 2009, Cabral et al 2011). This study aims to analyze the effect of glucose induction on the formation of *A. baumannii* clinical isolates biofilms which have been isolated from patients admitted to the ICU of Dr. Soetomo Hospital, Surabaya.

MATERIALS AND METHODS

The study sample was a non-biofilm forming *Acinetobacter baumannii* clinical isolate from non-DM patients in the Intensive Care Unit of Dr. Soetomo Hospital, Surabaya, from April to May 2018. The study was conducted at the Clinical Microbiology Laboratory of Dr. Surabaya Soetomo and biofilm density readings were carried out at ITD Airlangga University Surabaya. The ethics committee of the Dr. Soetomo Hospital, Surabaya, has approved this research as outlined in the ethics feasibility statement number 0213/KEPK/IV/2018.

Laboratory procedures

Clinical specimens from patients were inoculated on growth media and incubated for 24 hours. Identification with Gram staining taken from colonies in primary culture and continued identification using the BD Phoenix 100 system automatic method. Isolates were examined for detection of biofilms using Microtiter Plate Methode (MTPM) and the results were negative biofilm production. Isolates are stored in the -800C freezer until the glucose induction process is carried out.

Biofilm density assay using microtiter plate method (MTPM)

Each flat-bottomed microtiter plate well 96 polystyrene tissue culture-treated microtiter plate was filled with 180µl TSB and 20 µl of 0.5 McFarland bacterial suspension and as a negative control contained 180 µl TSB. To examine the effect of glucose induction on the biofilm density, we successively added glucose concentrations of 0.08%, 0.15%, 0.2% and 0.4% in TSB. Next the plate is closed and incubated at 350C for 24 hours. After incubation, the contents of the well were removed

and then washed with 300 μl phosphate buffer saline (pH 7.2) three times and dried in the inverted position.

Each well was filled with 150 μl methanol 100% for 20 minutes so that the biofilm produced by the bacteria will be fixed on the base and wall of the well. The microtiter plate was emptied by tapping and left in the inverted position then the biofilm was stained with crystal violet (0.1%) for 5 minutes. The remaining excess crystal violet is washed with deionized water and the plate is dried. Dried crystal violet on surface of each well diluted with 200 μl ethanol 100% for 30 minutes and continued for reading process.

Biofilm density which is bound to the base and wall of the microtiter plate well is read using ELISA reader with a wavelength of 630 nm, expressed as an Optical Density (OD630) value. These OD values are considered as indices of bacterial attachment to the surface of microtiter plate wells and forming biofilms. The interpretation of biofilm density is: 1. Negative if OD630 < 0.275; 2. Low if 0.275 < OD630 < 0.55; 3. Moderate if 0.55 < OD630 < 0.825; and 4. High if OD630 > 0.825. Data analysis was performed to assess the effect of glucose induction on the biofilm density by ANOVA method using statistical significance level (p value <0.05).

RESULTS

Thirteen clinical isolates of *A. baumannii* induced with concentration of glucose successively 0%, 0.08%, 0.15%, 0.2% and 0.4% respectively. Biofilm density checks were carried out using the iMark Bio Rad tool. Microplate Reader with a wavelength of 630 nm gives the average biofilm density results successively 0.126 + 0.062; 0.137 + 0.042; 0.272 + 0.124; 1,274 + 0,290; 3,407 + 0,269.

Statistical analysis of the effect of glucose induction on *A. baumannii* biofilm density using SPSS 17.0 with the same subject ANOVA method (p<0.05) showed a significant effect of glucose induction on biofilm density in clinical isolates of *A. baumannii* non-biofilm forming.

Table 1. Results of reading optical density with a wavelength of 630nm (OD630) samples of *A. baumannii* before and after the addition of glucose

Samples	No GLU	GLU 0.08%	GLU 0.15%	GLU 0.2%	GLU 0.4%
A. baumannii-001	0.132	0.119	0.177	0.870	3.269
A. baumannii-002	0.144	0.125	0.177	0.902	3.330
A. baumannii-003	0.058	0.112	0.480	1.412	3.455
A. baumannii-004	0.061	0.139	0.228	1.296	3.620
A. baumannii-005	0.075	0.133	0.211	1.206	3.536
A. baumannii-006	0.236	0.103	0.168	1.006	3.434
A. baumannii-007	0.199	0.096	0.224	1.385	3.387
A. baumannii-008	0.079	0.254	0.169	1.323	3.672
A. baumannii-009	0.148	0.166	0.423	1.519	3.675
A. baumannii-010	0.062	0.158	0.470	1.595	3.684
A. baumannii-011	0.216	0.140	0.394	1.870	3.416
A. baumannii-012	0.145	0.088	0.144	1.012	2.735
A. baumannii-013	0.085	0.145	0.268	1.173	3.076
Mean	0.126	0.137	0.272	1.274	3.407

Table 2. Descriptive statistic data

Glucose Induction	Mean	Std. Deviation	Total Samples
0%	0.126	0.062	13
0.08%	0.137	0.042	13
0.15%	0.272	0.124	13
0.20%	1.274	0.290	13
0.40%	3.407	0.269	13

Table 3. Double comparison data of influence of glucose concentration on biofilm density

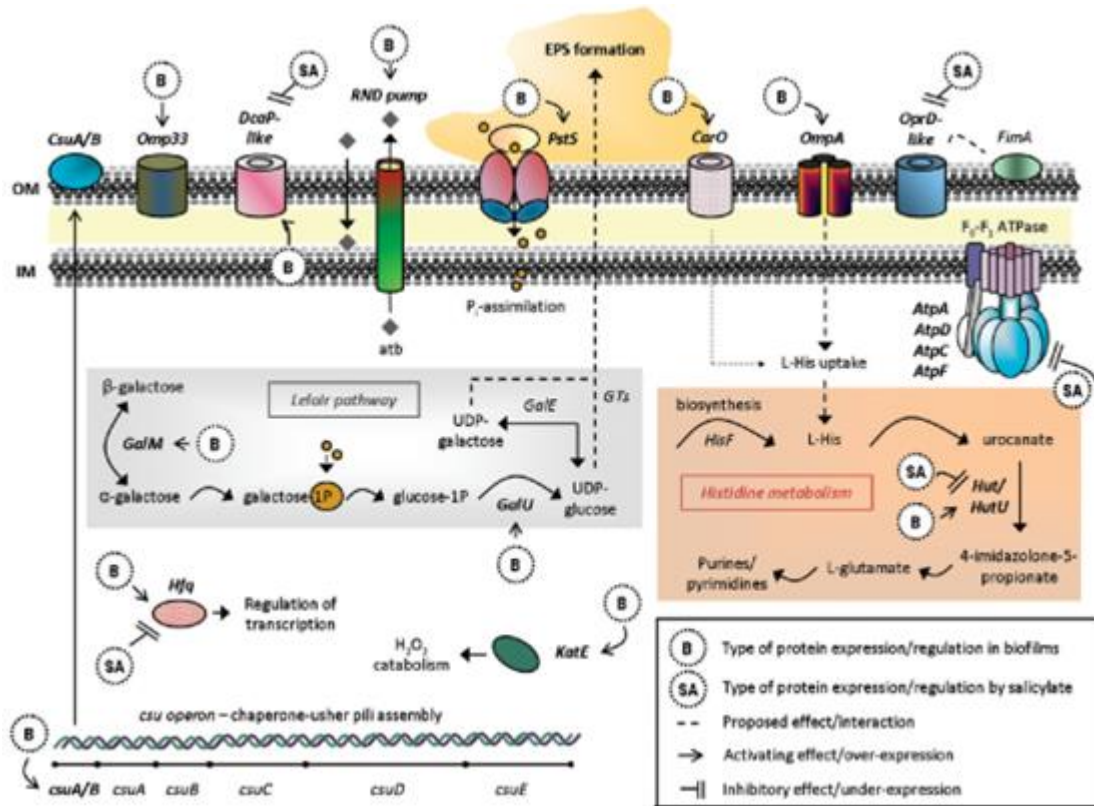|	Without Glucose Induction	Glucose Induction 0.08%	Glucose Induction 0.15%	Glucose Induction 0.2%	Glucose Induction 0.4%
Without Glucose Induction	-	0.671	0.005	< 0.0001	< 0.0001
Glucose Induction 0.08%	-	-	0.002	< 0.0001	< 0.0001
Glucose Induction 0.15%	-	-	-	< 0.0001	< 0.0001
Glucose Induction 0.2%	-	-	-	-	< 0.0001

ANOVA statistical analysis p = 0.000 (p<0.05)

Fig. 1. Leloir pathway (Cabral et al 2011).

DISCUSSION

Acinetobacter baumannii is a Gram negative coccobasil bacteria that is facultative anaerobic, pleomorphic and non-motile has become one of the bacteria that is often found in patients who are hospitalized and cause various types of infections in patients in hospitals, including pneumonia, tract infections urinary, bacteriemic, wound infection, meningitis and Ventilator-Associated Pneumonia (VAP) (Safari et al 2015, Vijayakumar et al 2016).

A 5-year retrospective study conducted at the University of Maryland Medical Center showed that 4% of the total 7925 patients underwent *A. baumannii* MDR colonization in the perianal region and had a profile including

the elderly (>50 years), patients had received previous antimicrobial therapy and has been admitted to an Inpatient Installation for 2 days or more. In these patients it is 15.2 times more likely to experience an *A. baumannii* MDR infection as evidenced by positive culture results, and has a 1.4 times higher risk of dying during ICU care. *A. baumannii* MDR colonization can develop into an infectious process in ICU-treated patients because of the use of assistive devices including intravenous catheter installation, urine catheter, naso-gastric tube insertion and mechanical ventilator use; and the condition of patients who are immunocompremised and sanitary hygiene are poorly maintained (Blanco et al 2018).

Biofilm is one of the virulence factors of *A. baumannii* which often complicates management of infection and *A. baumannii* MDR therapy which worsens the condition and prognosis of patients admitted to hospitals in general and those who are admitted to ICU in particular (Longo et al 2014, Badave & Dhananjay 2015). The National Institutes of Health reports that more than 60% of microbial infections are caused by biofilms and frequent chronic infections are repeated and are often irresponsive to antimicrobial therapy (Costerton et al 2003).

In this study, using clinical isolates of *A. baumannii* non-biofilm forming obtained from 13 non-DM patients who were admitted to the ICU dr. Soetomo Surabaya in the period April-May 2018. Most isolates were from non-DM patients who were admitted to the Intensive Care Unit with the age group 30-39 years and 60-69 years (each of 4 patients or 30.8%) and the group age of 40-49 years as many as 2 patients (15.4%). The age group> 40 years has risk factors for experiencing metabolic disorders, hemodynamic disorders or malignant processes that often require treatment in the ICU. In ICU care, patients are often attached to medical devices for therapeutic or diagnostic purposes such as intravenous lines, urine catheters, naso-gastric tubes or mechanical ventilators. This will increase the potential translocation of colonized *A. baumannii* MDR, both in patients and in the environment around the patient, thus triggering the process of *A. baumannii* MDR infection. This will be exacerbated in immunocompremised conditions in patients and poor maintenance of sanitation of patients and the surrounding environment (Khosama 2015, Haryani et al 2016, Arisma et al 2017).

According to the antimicrobial resistance, the sample consisted of 76.92% MDR *A. baumannii* and 23.08% non-MDR. This is consistent with the picture of an increase in the incidence of *A. baumannii* MDR infection in Southeast Asia up to 40-67% (Hsu et al 2017) and shows the risk factors for *A. baumannii* MDR

infection in patients admitted to the ICU (Yanong et al 2017, Anandhalakshmi et al 2017, Blanco et al 2018).

Based on the type of clinical isolate specimens obtained, 8 of 13 specimens (61.53%) were sputum aspirates from ETTs installed in patients treated in the ICU and 2 specimens (15.38%) were from blood specimens. This supports the fact that *A. baumannii* MDR is often a pathogen associated with the risk of Ventilator-Associated Pneumonia (VAP) in ICU-treated patients (Sukanya et al 2014, Anandhalakshmi et al 2017). The adhesion ability of *A. baumannii* on the surface of abiotic objects, in this case is the surface of Endo-Tracheal Tube, and on the surface of host respiratory epithelial cells to be virulence factors for these bacteria to colonize to trigger the infection process in the host (Lee et al 2007, Giannouli et al 2013). The over-expression of genes involved in the process of biofilm formation in *A. baumannii*, including chaperone-usher type I pili assembly system, OmpA, poly-ß- (1,6) -N-acetyl glucosamine (PNAG), Biofilm associated protein (BAP), triggers the production of *A. baumannii* biofilm which colonizes the surface of the ETT pipe and the surface of host respiratory epithelial cells, making it a source of chronic and recurrent infections (Zarrilli 2016).

This study showed a significant increase in biofilm production from *A. baumannii* non-biofilm forming clinical isolates treated with the addition of glucose with a certain concentration. In table 1, it can be seen that the results of reading Optical Densitry 630 nm (OD630) on TSB media without the addition of glucose inoculated with 0.5 McFarland *A. baumannii* showed no biofilm formation (OD<0.275). The formation of biofilms has not been seen in *A. baumannii* bacteria which was dinoculated in TSB media which added 0.08% glucose and 0.15% glucose. The formation of biofilms began to appear significantly in TSB media which added 0.2% glucose and increased even more at 0.4% glucose.

Addition of glucose with a concentration of 0.08%, 0.15%, 0.2% and 0.4% aimed at bacteria living in an atmosphere of the same blood sugar levels in patients. Glucose concentration is 0.08% to represent the condition of patients with normal fasting blood sugar level (80 mg/dL), while 0.15% glucose concentration represents the condition of patients with normal blood sugar level 2 hours post prandial (150 mg/dL). Addition of a concentration of 0.2% was representative of the condition of the blood sugar level which was slightly higher than the normoglycemic condition (0.08-0.15%) but in borderline hyperglycemic conditions in DM patients (200 mg/dL). 0.4% glucose addition represented a high level of patient's blood sugar level (400 mg/dL) (NICE Guiedelines).

The results of this study support the results of a similar study conducted at the RSUD dr. Soetomo Surabaya in 2017 about the effect of glucose induction on E. coli biofilm formation obtained from indwelling urine catheters. In this study showed a positive correlation between increased glucose concentration in the environment around bacteria and increased biofilm formation (Mewo 2017).

A. baumannii is not able to ferment glucose and is unable to use glucose as a carbon source or a single energy source (Kitagawa et al 1986), but this bacterium has NADH dehydrogenase (Ubiquinone), an enzyme that functions to react dehydrogenase to glucose, to produce ATP which is used as energy in the active transport system (van Schie et al 1985, Kitagawa et al 1986) and biosynthetic processes (Towner et al 1991). Glucose can function as an important carbon source for bacteria and can act as a signaling molecule that will affect the expression of various genes including those directly involved in glucose utilization. The growth of *A. baumannii* in a glucose-enriched medium will increase the bacterial sugar anabolism process so that it will trigger an increase in lipopolysaccharide biosynthesis and exopolysaccharide, so that an increase in exogenous glucose triggers the emergence of inflammatory cytokines from host macrophages and increases the bacterial hemolytic activity. Both of these allow the pathogenesis of *A. baumannii* to occur during sepsis conditions (Rossi et al 2016).

Rossi et al's study of the effect of glucose on lipopolysaccharide production and immunogenicity in *A. baumannii* showed a decrease in bacterial growth in the exponential phase in glucose-enriched media. This shows that *A. baumannii* is able to use glucose as a carbon source but is less effective (Rossi et al 2016). In the same study, transcriptomic analysis showed an increase in the expression of genes involved in primary metabolism, such as energy production (especially NADH: ubiquinone reductase multisubunit system), transcription and translation processes and cell wall biogenesis, which usually occur in the cell replication process exponential phase on the bacterial growth curve (Cox 2004). Based on these two things, it can be concluded that there is a process of other metabolic activities that occur in these glucose-rich conditions.

In the transcriptomic analysis of the above study, there was a downregulation of the NDP-sugar epimerase gene, which involved the biogenesis of outer membranes from bacterial cell walls, accompanied by upregulation of murG, pgm and galM and galU genes. The murG gene produces the main enzyme to combine sugar into peptidoglycan. The pgm gene produces the phosphoglucomutase enzyme as a catalyst for the isomerization process of sugar-6-P into sugar-1-P which is the first step in the biosynthesis process of energy-activated sugars in the EPS production process. Cabral et al argue that the galM gene encodes the galactose-1-epimerase enzyme and the galU gene encodes the UTP-glucose-1-P uridylyltransferase enzyme, wherein the Leloir pathway is involved in galactose utilization, which is associated with precrusor EPS biosynthesis in several bacteria including *A. baumannii* by process glycosyltransferase (Cabral et al 2011).

Shin et al stated proteins that are only expressed in biofilm producing cells are UDP galactose 4-epimerase (GalE), ProFAR isomerase (HisA) and GidA (Shin et al 2009). The role of the GalE protein (UDP-galactose 4-epimerase) in the Leloir pathway which functions the conversion of UDP-galactose to UDP-glucose and vice versa, which is used as the basic framework of bacterial EPS in general (Whitfield & Paiment, 2003). The HisA protein in the form of ProFAR isomerase involved in the process of histidine biosynthesis which plays an important role both in purine synthesis and nitrogen metabolism (Fani et al 2007). GidA protein has the role of repairing DNA by controlling the translation process through modification of tRNA if there is a misreading in the translation process that causes gene expression errors (Brégeon et al 2001) modification of this tRNA is believed to have an important role in the process of antimicrobial resistance and the persistent nature of *A. baumannii* in biofilms (Cabral et al 2011).

Fig. 1 shows that there appears to be an up-regulation of proteins involved in histidine metabolism, including CarO and HutU (urocanase). CarO is a protein in the outer membrane of *A. baumannii* and together with OmpA acts as a channel for the entry of L-His into bacterial cells (Cabral et al 2011). Urocanase is an enzyme that plays an important role in L-His degradation in histidine metabolism with purine and pyrimidine end results (Kapatral et al 2004). Purine and pyrimidine are the raw materials for eDNA biosynthesis, where eDNA is an important component in biofilm matrices with EPS and other proteins (Cabral et al 2011).

In addition to stimulating biofilms, an increase in exogenous levels of glucose increases the expression of genes involved in the production of LPS (Lipopolysaccharide) *A. baumannii*. In Rossi et al's study, it was shown that the increase in LPS production in media added to glucose with levels of 0.08%, 0.15% and 0.2% (Rossi et al 2016). LPS is an important characteristic of Gram negative bacteria and is a ligand for Toll-like receptors 4 (TLR4). LPS *A. baumannii* does not contain O-antigen, similar to LPS in Neisseria and Campylobacter species, so the LPS has a LOS

(Lipo-Oligosaccharide) design (Luke et al 2010). LPS can be released from the bacterial cell wall into the host bloodstream in the form of a monomer (Mattsby-Baltzer et al 1991) or as OMV (Outer Vesicle Membrane) (Beveridge 1999). Secretion of *A. baumannii* LPS as OMV contributes to virulence factors (Li et al 2015) and is a strong inducer of macrophage cytokine production in experimental animals. Increased production of LPS as a response to glucose induction is not directly related to the outer membrane biogenesis process and is not strongly bound to the surface of bacterial cells because it is easily released by rinsing EDTA. This provides an insight that *A. baumannii* in the blood will be induced to produce LPS, which has weak bonds on the surface of bacterial cells (Rossi et al 2016). If the nature of *A. baumannii* LPS is related to the ability of *A. baumannii* biofilm production on the surface of medical devices, especially IV-line catheters, then we need to be cautious if *A. baumannii* colonization is found in the device, which will produce easily released LPS following the flow blood and trigger a great increase in the production of host macrophage cytokines. LPS production in the blood and will increase up to 5 times or more in hyperglycemic sugar levels (>200 mg/dL) (Rossi et al 2016).

CONCLUSION

Biofilm density of clinical isolates of *A. baumannii* patients in ICU of Dr. Soetomo Hosspital, Surabaya, induced with glucose concentration 0.08%; 0.015%; 0.2%; 0.4% has an OD630 average successively 0.137 + 0.042; 0.272 + 0.124; 1.274 + 0.290; 3.407 + 0,269. Biofilm density of clinical isolates of *A. baumannii* patients in ICU RSUD dr. Surabaya Soetomo which is not glucose induced has an OD630 average 0.126 + 0.062. Biofilm density of clinical isolates of *A. baumannii* patients in ICU RSUD dr. Soetomo Surabaya significantly increased with glucose induction concentrations of 0.2% and 0.4%.

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