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Antibiotic resistance of biofilm-producing bacteria from sepsis patients in Prof. Dr. Margono Soekarjo Hospital, Purwokerto, Central Java

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ABSTRACT

Sepsis is a life-threatening organ dysfunction induced by the body's response to infection and is a significant cause of critical illness and death in hospitals. Bacteria are the most common pathogens that cause sepsis, and their ability to form biofilms increases their resistance to antibiotics. As a result of the failure of antibiotic administration therapy, the severity and pain of sepsis worsen. The study used a descriptive research design to determine the antibiotic resistance pattern of biofilm-producing bacteria from clinical isolates of sepsis patients. Using the BacT/Blood Culture System Alert, all patients suspected of sepsis in the intensive care unit of Prof. Dr. Margono Soekarjo General Hospital Purwokerto were examined for blood cultures between March and July 2018. These were then identified and tested for antibiotic resistance with the Vitek 2 Compact. Biofilm formation was detected utilizing the microtiter plate assay method, and the data were analyzed using a frequency distribution table. The results obtained 12 bacterial isolates, with *Escherichia coli* (41.67%), *Staphylococcus haemolyticus* (33.33%), *Klebsiella pneumoniae ssp pneumoniae*, *Enterobacter cloacae complex*, and *Acinetobacter baumannii complex* (8.33%) as the most common bacteria. All gram-negative bacteria (more than 80%) were resistant to ampicillin, cefoxitin, ceftazidime, ceftriaxone, aztreonam, and trimethoprim but were sensitive to meropenem (100%). Gram-positive bacteria were resistant to cefoxitin, benzylpenicillin, oxacillin, ciprofloxacin, erythromycin, and clindamycin (100% each). However, they were sensitive to tigecycline, nitrofuran, quinupristin, linezolid, vancomycin, and tetracycline (100% each). Gram-negative bacteria formed 50% biofilms, and 50% did not, whereas gram-positive bacteria produced 100% biofilms. In conclusion, bacteria clinical isolates of septic patients from Prof. Dr. Margono Soekarjo General Hospital Purwokerto are multiresistant to more than six types of antibiotics and produce weak to moderate biofilms, which can promote antibiotic resistance.

Keywords:
bacteria;
biofilm;
antibiotic resistance;
sepsis;
epidemiology

ABSTRAK

Sepsis adalah disfungsi organ yang mengancam jiwa yang diakibatkan oleh respon tubuh terhadap infeksi dan diyakini menjadi penyebab utama penyakit kritis dan kematian di rumah sakit. Bakteri sebagai patogen utama penyebab sepsis sering menjadi resisten terhadap antibiotik dan kemampuannya membentuk biofilm dapat meningkatkan resistensinya sehingga meningkatkan keparahan dan kesakitan kejadian sepsis karena kegagalan terapi pemberian antibiotik. Penelitian ini bertujuan untuk mengetahui pola resistensi antibiotik bakteri penghasil biofilm dari isolat klinik pasien sepsis. Desain penelitian yang digunakan deskriptif. Semua pasien terduga sepsis di ruang perawatan intensif RSUD Prof. Dr. Margono Soekarjo Purwokerto selama bulan Maret-Juli 2018 dilakukan pemeriksaan kultur darah dengan alat *BacT/Alert Blood Culture*

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System, kemudian diidentifikasi dan diuji resistensi dengan alat Vitek 2 Compact. Pembentukan biofilm dengan metode mikrotiter *plate assay*. Data dianalisis dengan tabel distribusi frekuensi. Hasil didapatkan 12 isolat bakteri dengan bakteri terbanyak, yaitu *Escherichia coli* (41,67%), *Staphylococcus haemolyticus* (33,33%), *Klebsiella pneumoniae ssp pneumoniae*, *Enterobacter cloacae complex*, *Acinetobacter baumannii complex* masing-masing (8,33%). Semua bakteri gram negatif resisten diatas 80% terhadap ampisilin, cefoxitin, ceftazidin, ceftriaxon, aztreonam, dan trimetoprim tapi sensitif terhadap meropenem 100%. Bakteri gram positif resisten terhadap cefoxitin, benzilpenicilin, oxacilin, ciprofloxacin, eritromisin dan clindamisin masing-masing 100% dan sensitif terhadap tigecyclin, nitrofurat, quinupristin, linezoid, vancomycin and tetracyclin masing-masing 100%. Dari ke 12 bakteri, 50% bakteri gram negatif membentuk biofilm dan 50% tidak membentuk biofilm, sedangkan bakteri gram positif penghasil biofilm 100%. Kesimpulan penelitian ini adalah bakteri isolat klinik pasien sepsis dari RSUD Prof Margono Soekarjo Purwokerto multiresisten terhadap lebih dari 6 jenis antibiotik dan menghasilkan biofilm lemah sampai moderat yang dapat meningkatkan resistensinya terhadap antibiotik.

INTRODUCTION

Sepsis is a potentially fatal organ dysfunction induced by the body's immune response to infection and is a vital cause of critical illness and death in hospitals.[1] The incidence is relatively high, affecting millions of individuals each year, and it is rising. According to the World Health Organization (WHO), sepsis affects more than 30 million people each year, with the potential to cause 6 million deaths,[2] which is a heavy burden for developing and impoverished countries. In Indonesia, 35 patients were studied at Prof. Dr. R. D. Kandou Manado Hospital, where 29 patients (82.8%) were diagnosed with sepsis, and 23 patients (65.7%) died.[3]The patients with sepsis experienced organ dysfunction due to irregular host body responses that can lead to septic shock and death.[4] Nearly 15% of sepsis patients are treated in Intensive Care Units (ICUs), with two-thirds developing septic shock. According to reports, sepsis is a leading cause of death among patients treated in these units.[3,5] In addition, patients with sepsis are particularly vulnerable due to low immunity, malnutrition, and exposure to medical treatment, all of which increase mortality and cost of care.[6,7]

Gram-negative bacteria such as *Escherichia coli*, *Klebsiella* spp., *Pseudomonas aeruginosa*, *Enterobacter* spp., *Proteus* spp., *Neisseria* spp., *and Acinetobacter* spp., are known to be the most common pathogens that cause sepsis, other than gram-positive bacteria (*Staphylococcus aureus* and *Coagulase-negative Staphylococci*).[3,8] Bacteria can produce clinical symptoms of sepsis because they share virulence factors with their hosts and induce an inflammatory response. Furthermore, Gram-negative bacteria can infect the host due to the presence of lipopolysaccharide and an endotoxin, but gram-positive bacteria contain an exotoxin that functions as a superantigen.[8]

Bacteria that cause sepsis are often antibiotic-resistant, worsening the condition of the patients.[9] Appropriate antibiotic selection is crucial for lowering sepsis mortality and is administered within 1-2 hours after sepsis diagnosis. In addition, the pattern of resistance differs by hospital and changes over time. A study at Moewardi Hospital in 2016, *E. coli* is resistant to ampicillin, chloramphenicol, and gentamicyn.[10] Lewis *et al.*[11] also reported *Acinetobacter resistance* to ceftriaxone and imipenem. Research conducted in the intensive care room of Fatmawati Hospital, Indonesia, showed that *P. aeruginosa*, *S. epidermidis*, and *E. coli* were resistant to meropenem, gentamicin, and levofloxacin.[12] Furthermore, in Margono Soekarjo General Hospital, the resistance of

Klebsiella to meropenem was reported.[13]

Antibiotic resistance is a primary cause of antibiotic therapy failure in patients with sepsis and septic shock, raising the mortality rate in hospitals. Biofilm formation by sepsis-causing bacteria is another factor that contributes to the ineffectiveness of sepsis patients' treatment.[14] Bacteria can produce biofilms, making them more resistant to antibiotics at least 10-1000 times than planktonic bacteria.[15] This can increase the severity and pain of the incidence of sepsis due to the failure of antibiotic therapy. Biofilms protect bacteria from antibiotics and the immune system by bacterial colonization.[16] For instance, biofilms can tolerate antimicrobial agents at concentrations of 10–1000 times that needed to inactivate genetically equivalent planktonic bacteria.[17]

Sepsis requires fast and precise handling. Proper and prompt administration of antibiotic therapy is needed to reduce sepsis mortality.[17] Information on bacterial antibiotic resistance that causes sepsis and the ability of these bacteria to produce biofilms that can increase the incidence of antibiotic resistance is needed to be used as guidelines for appropriate antibiotic therapy in the treatment of septic patients. Research on the antibiotic resistance of bacteria that causes sepsis and the ability of these bacteria to produce biofilms has never been carried out at the Prof. Dr. Margono Soekarjo General Hospital, Purwokerto. This study aimed to determine the antibiotic resistance pattern of biofilm-producing bacteria from clinical isolates of sepsis patients in Prof. Dr. Margono Soekarjo General Hospital, Purwokerto.

MATERIALS AND METHODS

Subject

The research design used is descriptive. The study sample was all bacteria isolated from adult patients suspected of sepsis treated in intensive care at Prof. Dr. Margono Soekarjo General Hospital, Purwokerto, during the period March - July 2018. Identification of bacteria and antibiotic resistance testing was carried out according to the standards in the Microbiology Division of the Clinical Pathology Laboratory Prof. Dr. Margono Soekarjo General Hospital, Purwokerto using Vitek 2 Compact. Biofilm testing was conducted at the Research Laboratory of the Faculty of Medicine, Universitas Jenderal Soedirman, Purwokerto.

Bacteria isolates

All bacteria were obtained from Prof. Dr. Margono Soekarjo General Hospital and isolated from clinical material of blood samples from sepsis patients in the intensive care unit room. As much as 5 mL blood samples were taken by skilled health workers according to the standards, then put into the BacT/Alert Blood Culture System transport media for review.

Identification and antibiotic susceptibility test

The bacteria were identified and tested for antibiotic resistance with Vitek 2 Compact.

Microtiter plate assay

The microtiter plate method was conducted as previously described[14] with modification: bacteria were grown in 3 mL of trypticase soy broth (TSB) medium for 24 h at 37°C. The initial inoculum was diluted with a ratio of 1: 100 (which concentration 10µL culture in 1 mL TSB), then put 100 µL TSB with 1.5% glucose and TSB without glucose into each of the 96-well microtiter plate wells and added 10 µL of diluted culture into each well. The plate was covered

with plastic (wrapping) and incubated at 37°C for 20 h. Planktonic bacteria were carefully removed from each well with a 200 µL pipette (gently pipette and leave the biofilm on the bottom of the well until the bottom of the well looks clear. Furthermore, the well was washed with 300 µL PBS (phosphate-buffered saline) to remove non-adherent bacteria. After that, PBS wasis discarded with a 200 µL (2 times) pipette slowly. Then the well was painted with 100 µL 1% crystal violet (try not to touch the bottom of the pipette), then save 30 min at room temperature (on the table). The well was washed again with distilled water then added 5% acid isopropanol (HCl and two propanols). Optical density (OD) was calculated at a wavelength of 450 nm. *Staphylococcus epidermidis* 12228, which is a biofilm-producing strain, was used as a positive control. Bacterial cells are categorized as very strong biofilm producers when > 4 X ODC; 2 X Odc <... ..4X Odc is moderate; or Odc <... .. ≤ 2 X Odc is said to be weak, ≤ Odc includes non-biofilm. Odc = OD negative control, Ods = OD.

Data analysis

The data were analyzed and then presented descriptively with a frequency distribution table. This research has passed the ethical review by the Medical Research Ethics Commission, Faculty of Medicine, Universitas Jenderal Soedirman, Purwokerto.

RESULTS

The patient characteristics

The results showed 12 positive blood cultures from sepsis patients: five men (41.66%) and seven women (58.33%)

Bacterial distribution

This research obtained 12 bacterial isolates from sepsis patients, namely *E. coli* (41.67%), *S. haemolyticus* isolates (33.33%), *K. pneumoniae ssp pneumoniae*, *E. cloacae complex*, and *A. baumannii complex*, respectively (8.33%). The distribution of bacteria that cause sepsis is presented in TABLE 1.

TABLE 1. Distribution of sepsis bacteria in Prof. Dr. Margono Soekarjo General Hospital Purwokerto for the period March-July 2018 (n = 12)

Microorganism	Number of isolate	Percentage (%)
Gram-negative		
▪ *E. coli*	5	41.67
▪ *K. pneumoniae ssp pneumoniae*	1	8.33
▪ *E. cloacae complex*	1	8.33
▪ *A. baumannii complex*	1	8.33
Gram-positive		
▪ *S. haemolyticus*	4	33.33
Total	12	100

Antibiotic resistance

In this study, the antibiotic resistance result, obtained automatically by a Vitek® 2 Compact. Gram-negative bacterial antibiotic resistance test results from sepsis patients are presented in TABLE 2, and the gram-positive bacterial resistance test results are presented in TABLE 3. It was found that all gram-negative bacteria are still sensitive to carbapenem antibiotics, which are meropenem and resistant to beta-class antibiotics lactam, namely ampicillin. *Escherichia coli* is still sensitive to meropenem, amikacin, ertapenem, gentamicin, tigecycline,

and nitrofuran(100%) but resistant to more than 50% beta-lactam class antibiotics. *Klebsiella* is still sensitive to carbapenem, amikacin, gentamicin, ciprofloxacin, tigecycline, and nitrofurat groups (100%) and all resistant to ampicillin, cefoxitin, ceftriaxone, and trimethoprim. *Enterobacter cloacae complex* shows sensitivity to meropenem and amikacin but is resistant to 14 other types of antibiotics. *Acinetobacter baumannii complex* is sensitive to ertapenem, meropenem, and tigecycline respectively by 100% but resistant to 12 different types of antibiotics.

Gram-positive bacteria *S. haemolyticus* is resistant to cefoxitin, benzylpenicillin, oxacillin, ciprofloxacin, erythromycin, and clindamycin 100% and sensitive to tigecycline, nitrofuran, quinupristin, linezolid, vancomycin, and tetracycline 100%. *Staphylococcus* is resistant to cefoxitin (TABLE 3).

TABLE 2. Resistance patterns of septic gram-negative bacteria in Prof. Dr. Margono Soekarjo General Hospital Period March-July 2018.

Antibiotic	Antibiotic resistance [% (n)]			
	E. coli	*K. pneumoniae ssp*	*E. cloacae complex*	*A. baumannii complex*
Ampicillin	80 (4)	100 (1)	100 (1)	100 (1)
AS	60 (3)	100 (1)	100 (1)	0
Piperacillin	20 (1)	0	100 (1)	100 (1)
Cefoxitin	60 (3)	100 (1)	100 (1)	100 (1)
Ceftazidime	60 (3)	100 (1)	100 (1)	100 (1)
Ceftriaxone	60 (3)	100 (1)	100 (1)	100 (1)
Cefepime	40 (2)	100 (1)	100 (1)	100 (1)
Aztreonam	60 (3)	100 (1)	100 (1)	100 (1)
Ertapenem	0	0	100 (1)	0
Meropenem	0	0	0	0
Amikacin	0	0	0	100 (1)
Gentamicin	0	0	100 (1)	100 (1)
Ciprofloxacin	0	0	100 (1)	100 (1)
Tigecycline	0	0	100 (1)	0
Nitrofuran	0	0	100 (1)	100 (1)
Trimethoprim	60 (3)	100 (1)	100 (1)	100 (1)

TABLE 3. The pattern of resistance of gram-positive bacteria to cause sepsis in Prof. Dr. Margono Soekarjo General Hospital Period March-July 2018.

Antibiotic	Antibiotics resistance to *S. haemolyticus* [% (n)]
Cefoxitin	100 (4)
Gentamicin	75 (3)
Tigecycline	0
Nitrofuran	0
Trimethoprim	50 (2)
Benzylpenicillin	100 (4)
Oxacillin	100 (4)
Ciprofloxacin	100 (4)
Levofloxacin	100 (4)
Moxifloxacin	25 (1)
Erythromycin	100 (4)
Clindamycin	100 (4)
Quinupristin	0
Linezolid	0
Vancomycin	0
Tetracycline	0
Rifampicin	75 (3)

Biofilm assay

The biofilm test results using the microtiter plate assay method are presented in TABLE 4. Negative control OD was obtained (Odc = 0.0128). Of the 12 bacteria, 50% of gram-negative bacteria form biofilms, and 50% do not form biofilms, while 100% gram-positive bacteria produce biofilms. *Staphylococcus haemolyticus* and *K. pneumoniae ssp pneumoniae* bacteria show the weak ability to produce biofilms (0.0128 < ODc 00.0256), while *E. coli* and *E. cloacae complex* do not produce biofilms (Odc ≤ 0.0128). Only *A. baumannii* is a moderate biofilms former (0.0256≤Odc 0.0512). The frequency of biofilm-producing bacteria and multi antibiotic resistance of bacterial isolates are presented in TABLE 5.

TABLE 4. Results of biofilm production for bacterial isolates using the microtiter plate method

Bacterial isolates	Biofilm former			
	Non-biofilm [n (%)]	Weak [n (%)]	Moderate [n (%)]	Strong [n (%)]
Gram-positive				
▪ *S. haemolyticus*		4 (33.33)		
Gram-negative				
▪ *E. coli*	5 (41.67)			
▪ *E. cloacae complex*	1 (8.33)			
▪ *A. baumannii complex*			1 (8.33)	
▪ *K. pneumoniae ssp pneumoniae*		1 (8.33)		

Non biofilm: ODc ≤ 0.0128; Weak: 0.0128 < ODc ≤ 0.0256; Moderate 0.0256 < ODc ≤ 0.0512; Strong ODc> 0.0512

TABLE 5. Frequency of biofilm-producing bacteria and antibiotic multi-resistance

Microorganism	Antibiotic resistance	Biofilm former
Gram-positive bacteria		
▪ *S. haemolyticus*	11 multi-resistance	Weak
Gram negative bacteria		
▪ *E. coli*	11 multi-resistance	Non-former
▪ *K. pneumoniae ssp pneumoniae*	8 multi-resistance	Weak
▪ *E. cloacae complex*	14 multi-resistance	Non-former
▪ *A. baumannii complex*	12 multi-resistance	Moderate

DISCUSSION

The results showed that the most common bacterial causes of sepsis in adults were gram-negative bacteria, *E. coli* (41.67%), and gram-positive bacteria *S. haemolyticus* (33.33%). This is consistent with the study in Brazil (53.2%), in Southeast Asia (5%), and in Indonesia (68,8%).[5,18,19] The research conducted in Moewardi General Hospital, Solo, and in Ethiopia shows a different result, where gram-positive bacteria were the most common causes of sepsis by 15.09% and 83.4%, respectively.[8,10] The variations of bacteria that cause sepsis in hospitals in different countries can occur due to geographical location,

epidemiological variants, the nature of the patient population, limited sample size, and limited research time span. The prevalence of infection can be affected by season; this study may have failed to show the actual prevalence of pathogen that causes it.[5,20] In this study, the research time span is only five months (March to July), therefore the pattern of bacteria is different from the other hospital.

Escherichia coli in the study was a gram-negative bacteria that was reported to be the cause of sepsis and other gram-negative bacteria, namely *Klebsiella, Acinetobacter*, and *Enterobacter*. These results are consistent with the study in Ethiopia and Brazil.[5,20] The different results found from Indonesia's research, where the gram-negative bacteria found were *Klebsiella* sp. This can occur because of differences in geographical location, causing a variety of agents causing sepsis.[19]

Gram-positive bacteria *S. haemolyticus* is the second *Staphylococcus negative coagulase* (CoNS) after *S. epidermidis*, most often isolated from clinical cases such as sepsis.[20] However, the results of the study showed *S. haemolyticus* to be the most gram-positive bacteria causing sepsis. This difference is likely due to differences in blood culture systems and media content. The amount of CoNS isolated from blood cultures needs to be considered because it is considered a contaminant in many studies. The current research of CoNS is an important pathogen for nosocomial infections and sepsis.[21,22]

Antibiotic therapy for septic patients is faced with the challenge of the emergence of antibiotic-resistant bacteria and even develops into multi-resistance. The antibiotics recommended for adult sepsis patients for gram-positive bacteria are vancomycin and gentamicin, while for gram-positive bacteria is meropenem.

Echerichia coli isolates in the study were resistant to beta-lactam antibiotics above 50% but sensitive to meropenem, amikacin, ertapenem, gentamicin, tigecycline, and nitrofuran 100%. This is according to study at Riau Hospital and Solo.[9,10] *Klebsiella* is still 100% sensitive to carbapenem, amikacin, gentamicin, ciprofloxacin, tigecycline, and nitrofuran groups, all of which are resistant ampicillin, cefoxitin, ceftriaxone, and trimethoprim. *Enterobacter cloacae complex* shows sensitivity to meropenem and amikacin but is resistant to 14 other antibiotics. *Acinetobacter baumannii complex* is still 100% sensitive to ertapenem, meropenem, and tigecycline, but resistant to 12 different types of antibiotics.

All gram-negative bacteria are resistant above 80% against ampicillin, cefoxitin, ceftazidime, ceftriaxone, aztreonam, and trimethoprim but are sensitive to 100% meropenem. Empiric antibiotic therapy for sepsis can use meropenem. Meropenem is the choice for the treatment of *Enterobacter* infection because of its immunity to destruction by beta-lactamase produced by this pathogen.[19]

Gram-positive bacteria *S. haemolyticus* are 100% resistant to cefoxitin, benzylpenicillin, oxacillin, ciprofloxacin, erythromycin, and clindamycin, and 100% sensitive to tigecycline, nitrofuran, quinupristin, linezolid, vancomycin, and tetracycline. *Staphylococcus* is resistant to cefoxitin, which means that the isolates include methicillin resistance. Many studies report *S. haemolyticus* is resistant to penicillin, cephalosporin, tetracycline quinolones, aminoglycosides, glycopeptides, and phosphomycines.[23,24] The mechanism of *S. hemolytic* methicillin resistance determines resistance to all beta-lactam antibiotics i.e. penicillin, cephalosporins, carbapenems, and monobactams. This mechanism is related to the presence of the mecA gene, encoding the PBP2a protein modified penicillin transpeptidase, which is responsible for

the synthesis of pentaglycine bridges in peptidoglycan. Another important feature of *Staphylococci* is their ability to survive in a hospital environment.[25] Vancomycin can be an empiric therapy for antibiotics against *S. haemolyticus*. However, many studies have reported cases of *Vancomycin Resistance S. aureus* (VRSA).[26]

All bacterial isolates became multiresistant because they were resistant to more than two or more types of antibiotics. Similar studies also showed the same results.[19,20] Ciprofloxacin, which became an antibiotic for gram-positive and negative bacteria, actually experienced resistance.

In this study, the susceptibility test and species identification were carried out automatically by Vitec compact 2. The advantages of this tool are practical and useful in detecting species.[16]

In addition to being resistant to antibiotics, the ability of bacteria to produce biofilms that can decrease antibiotic efficacy is a serious problem in handling septic patients. The ability of bacteria in surface attachments and the formation of multicellular communities is an important key to the infection stage and becomes a bacterial virulence factor.[14,16] Biofilm assay showed that all 100% gram-positive bacteria were able to produce biofilms even though they were weak. In gram-negative bacteria, 75% did not produce biofilms, while 25% produced weak and moderate biofilms. All isolates were multiresistant to more than six antibiotics, where 50% produced biofilms. This shows that most multi-drug resistance bacteria are biofilm producers.

The antibiotics resistance mechanisms of biofilms and planktonic bacteria are different. The biofilm is more resistant to antibiotics up to 1000 times more than free-bacteria.[16] The prevalence of bacteria that cause sepsis, most of which are multi resistant to antibiotics, needs special attention. The pattern of resistance and sensitivity to antibiotics is necessary as a guideline for empirical antibiotic therapy to treat septic patients whose immune conditions are very vulnerable. In addition, the ability of bacteria to produce biofilms that increase their tolerance in biofilms means that different therapeutic methods are needed, such as topical antibiotics.

CONCLUSION

The clinical bacterial isolates from sepsis patients at Prof. Dr. Margono Soekarjo General Hospital, Purwokerto, Central Java is multiresistant to more than six types of antibiotics and produce weak to moderate biofilms, which can increase their resistance to antibiotics

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