HUBUNGAN PEMBENTUKAN BIOFILM OLEH BAKTERI GRAM NEGATIF DENGAN RESISTENSI ANTIBIOTIK PADA WANITA DIABETES MELITUS TIPE 2

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Abstrak

Diabetes melitus (DM) memiliki efek jangka panjang terhadap sistem genitourinari yang menjadi faktor predisposisi terjadinya infeksi saluran kemih (ISK) pada pasien wanita dan umumnya bersifat asimtomatik. Bakteri gram negatif merupakan mikroorganisme penyebab tersering yang dapat membentuk biofilm sehingga sering menyebabkan resistensi antibiotik. Penelitian ini bertujuan untuk membuktikan hubungan antara pembentukan biofilm bakteri gram negatif dengan resistensi antibiotik pada wanita diabetes melitus tipe 2 (DMT2). Menggunakan desain penelitian *cross sectional* dengan teknik *total sampling*. Jumlah sampel penelitian adalah 45 orang wanita DMT2 anggota Program Pengendalian Penyakit Kronis (PROLANIS). Pada semua responden dilakukan kultur urin porsi tengah, uji sensitivitas dan pemeriksaan biofilm terhadap isolat bakteri yang teridentifikasi. Hasil kultur urin menunjukkan bakteriuria signifikan 14 (31%) responden. Identifikasi koloni menunjukkan bakteri penyebab ISK antara lain *Escherichia coli* (35,7%), *Klebsiella pneumoniae* (35,7%), *Enterobacter sp* (21,5%) dan *Citrobacter sp* (7,1%). Uji sensitivitas dijumpai 8 (57%) isolat resisten terhadap antibiotik, yaitu 2 (14,2%) isolat terhadap ciprofloksasin dan 6 (42,8%) isolat terhadap TMP-SMX. Bakteri gram negatif yang diisolasi 100% mampu membentuk biofilm dengan kategori *weak*. Berdasarkan analisis bivariat dengan uji *Rank Spearman correlation* dapat disimpulkan bahwa terdapatnya hubungan yang sangat lemah antara pembentukan biofilm oleh bakteri gram negatif dengan resistensi antibiotik ciprofloksasin dan TMP-SMX.

Kata kunci: bakteri-gram-negatif; biofilm; DM; resistensi-antibiotik

RELATIONSHIP BETWEEN THE FORMATION OF BIOFILM BY GRAM NEGATIVE BACTERIA WITH ANTIBIOTIC RESISTANCE IN WOMEN WITH TYPE 2 DIABETES MELITUS

Abstract

Diabetes Mellitus (DM) has a long-term effect on the genitourinary system that predisposes to urinary tract infection (UTI) in female patients and is generally asymptomatic. Gram-negative bacteria are the most common cause of microorganisms that can form biofilms that often cause antibiotic resistance. This study aims to prove the relationship between the formation of biofilms of gram-negative bacteria and antibiotic resistance in women with type 2 diabetes mellitus (DM T2). Using cross sectional research design with total sampling technique. The number of research samples is 45 women DM T2 PROLANIS member. In all respondents, the middle portion of urine culture, sensitivity test, and biofilm examination on identified bacterial isolates were identified. Result of urine culture showed significant bacteriuria 14 (31%) respondents. Identification of colonies showed that UTI bacteria were Escherichia coli (35,7%), Klebsiella pneumoniae (35,7%), Enterobacter sp (21,5%) and Citrobacter sp (7,1%). Sensitivity test found 8 (57%) isolates resistant to antibiotic, ie 2 (14,2%) isolate to ciprofloxacin and 6 (42,8%) isolate to TMP-SMX. Gram-negative bacteria isolated 100% are able to form biofilms with the weak category. Bivariate analysis with Rank Spearman correlation test on biofilm formation relationship with antibiotic resistance can be concluded that there is very weak relation ($r < 0,25$; $p > 0,05$) between biofilm formation by gram negative bacteria with antibiotic resistance of ciprofloxacin and TMP-SMX.

Keywords: gram-negative-bacteria; biofilm;DM; antibiotic-resistance

Pendahuluan

Diabetes is a major cause of morbidity and mortality and is one of the four priorities of non-communicable diseases. The World Health Organization (WHO) estimates the number of DM patients worldwide to increase to 642 million by 2040[1,2]. DM disease has long-term effects on the genitourinary system, thus predisposing to urinary tract infection (UTI) in DM patients[3].

Urinary tract infections are more common in women than men with a percentage of 52.8% and 47.2%, respectively. This is because anatomically, women have a shorter urethra than men, urethral orifice and vagina is also an area that easily becomes a place of bacterial colonization[4]. Urinary tract infections in DM women are reported to be more frequent asymptomatic UTIs[3,5]. The prevalence of asymptomatic UTI is about 3-4 times higher in diabetic women (ranging from 15% - 30%) than women without DM by 2-5% [15].

Gram-negative bacteria is the most commonly microorganism isolated from urine of diabetic patients as the cause of UTI from gram-positive bacteria with gram-negative ratio of 63.64% while 36.36% gram-positive. The most common cause of gram-negative bacteria is *Escherichia coli*, followed by other Enterobacteriae such as *Klebsiellapneumoniae*., *Proteusspp*., dan *Enterobacterspp* [6,7]. Gram-negative bacteria are able to form biofilms coated by uroplakin so that their pathogenicity increases. This ability causes urinary tract infections to become persistent, recurrent and leads to bacteria more resistant to phagocytosis and 100-1000 times more resistant to antibiotics[8].

Methods

The research design used was analytical descriptive with cross sectional approach. The samples in this study were diabetes mellitus type 2 / DMT2 PROLANIS member (Chronic Disease Control Program) at Mon Geudong Lhokseumawe Public Health Center amounted to 45 people, using total sampling with inclusion criteria: 1) Female patient with DM T2 PROLANIS member who has been diagnosed by physician as a DM patient and routinely seek treatment at Puskesmas and willing to be a respondent, 2) Have no symptoms of UTI such as dysuria, frequency, urgency, fever etc. 3) No use of antibiotic therapy in the last 2 weeks, 4) Not pregnant. Exclusion criteria are 1) DM patients with accompanying diabetic nephropathy and chronic renal disease, 2) Inpatient history and urinary catheterization in the last 3 months.

Mid-stream urine is collected sterile, then cultured on Mc Conkay and CLED (Cysteine Lactose Electrolyte Deficient). Samples with significant bacteriuria (> 10^5 cfu / ml urine) will be continued for identification of gram-negative bacteria by biochemical test. The sensitivity test of antibiotic ciprofloxacin and trimethoprim-sulfamethoxazole (TMP-SMX) was done by Kirby Bauer

disc diffusion method, biofilm formation test using Tissue Culture Plate method and reading with 570 nm wavelength ELISA reader (OD570nm). Bivariate analysis using Rank Spearman test with 95% significance level (α = 0,05).approval of ethical clearance by the Medical Research Ethics Commission (KEPK) of the Faculty of Medicine, University of North Sumatra.

Result

Table 1 Characteristics of Respondents withand without asymptomatic UTI

Characteristics of Respondents	Urinary Tract Infection			
	Negative (n)	%	Positive (n)	%
Age				
< 50 years	5	50	5	50
≥ 50 years	26	74,3	9	25,7
History DM				
< 5 years	15	65,2	8	34,8
≥ 5 years	17	73,9	6	26,1
BMI (kg/m2)				
Non overweight	13	76,5	4	23,5
Overweight	18	64,3	10	35,7
Sexual Activity				
No	13	76,5	4	23,5
< 3 times a week	16	64	9	36
≥ 3 times a week	2	66,7	1	33,3
Abdominal Circumference				
≤ 80 cm	2	66,7	1	33,3
> 80 cm	29	69	13	31
Menstruation				
Still	8	61,5	5	28,1
Stop	23	71,8	9	28,2

Table 1 shows the respondents with asymptomatic UTIs having age ≥ 50 years is 25.7%, history / duration of DM <5 years 34.8%, overweight BMI 35.7%, sexual activity <3 times per week 36%, abdominal circumference> 80 cm 31%, had stopped menstruation (menopause) 28,1%. In respondents without asymptomatic UTI, the history / duration of DM was ≥ 5 years with 77.3% percentage**.**

Table 2 Distribution Frequency of Patient withAsymptomatic UTI

Asymptomatic UTI	Frequence	Percentage
	N	%
Positive	14	31
Negative	31	69
Total	45	100

Table 2 shows that there were 14 urine samples (31%) with significant bacteriuria> 105 cfu / ml urine and 31 other urine samples (68.9%) found no bacterial growth at all. The incidence of asymptomatic UTI in women DM T2 was found to be 31%.

Table 3. Profile of Gram Negative Bacteria Causes of Asymptomatic UTI

Bacterial Profile	Subjects with Significant Bacteriuria (n = 14)	
	N	%
E. coli	5	35,7
K. pneumoniae	5	35,7
Enterobacter sp	3	21,5
Citrobacter sp	1	7,1
Total	14	100

Table 3 shows the dominant bacteria causing asymptomatic UTI in this study were *E. coli* (35.7%) and *K. pneumoniae* (35.7%), followed by *Enterobacter sp* (21.5%) and *Citrobacter sp* (7.1%).

Table 4 Sensitivity of Antibiotics Ciprofloxacin and TMP-SMX

Type of Bacteria	n	Sensitivity of Antibiotics Based on CLSI					
		Ciprofloksasin			Trimethoprim-Sulfametoksazol		
		S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
E.coli	5	5 (100)	0 (0)	0 (0)	3 (60)	0 (0)	2 (40)
K. pneumoniae	5	3 (60)	1 (20)	1 (20)	2 (40)	0 (0)	3 (60)
Enterobacter sp	3	3 (100)	0 (0)	0 (0)	2 (66,7)	0 (0)	1 (33,3)
Citrobacter sp	1	0 (0)	0 (0)	1 (100)	1 (100)	0 (0)	0 (0)
Total	14	11 (78,5)	1 (7,1)	2 (14,2)	8 (57,1)	0 (0)	6 (42,8)

Table 4 shows the results of the antibiotic sensitivity test showed that there were 8 (57%) isolates resistant to antibiotics, ie 2 (14.2%) isolates resistant to ciprofloxacin and 6 (42.8%) isolates resistant to TMP-SMX. There were 11 (78.7%) isolates sensitive to ciprofloxacin, 8 (57%) isolates sensitive to TMP-SMX and 5 (17.9%) isolates sensitive to both antibiotics.

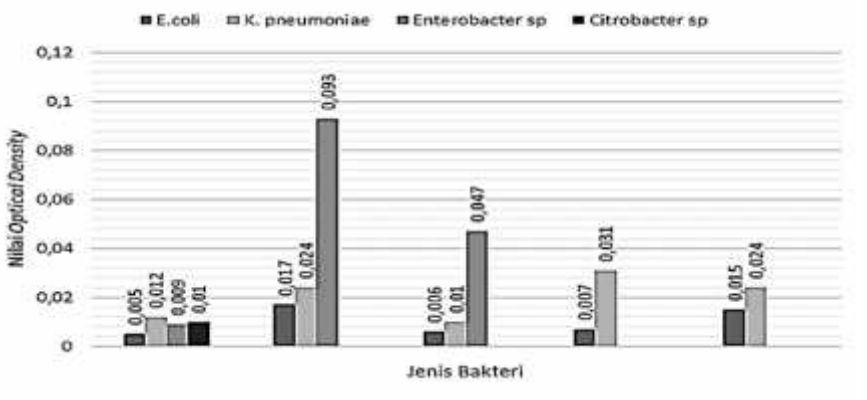

Figure 1 shows the results of examination of biofilm formation using Tissue Culture Plate method with ELISA readings obtained the range of optical density (OD) value of 14 isolates is 0.005-0.093 (<0.120) so 14 isolates are categorized non / weak ie having weak ability in forming biofilm.

Table 5 Relationship of Biofilm Formation with Antibiotic Resistance Ciprofloxacin

Biofilm Gram Negative Bacteria	Diameter of Ciprofloxacin Inhibitory Zone (mm) Based on CLSI						*P value*	r
	≥ 20 mm (n)	%	16-20 mm (n)	%	≤ 15 mm (n)	%		
<0,120	11	78,6	1	7,1	2	14,3		
0,120-0,240	0	0	0	0	0	0		
>0,240	0	0	0	0	0	0	0,501	-1,96
Total	11	78,6	1	7,1	2	14,3		

Table 5 shows 11 (78.6%) isolates that are sensitive, 1 (7,1%) intermediate and 2 (14,3%) resistant to ciprofloxacin antibiotics and have weak (weak) ability to form biofilms. Based on bivariate analysis with Rank Spearman correlation test got correlation coefficient value equal to -1,96 (r <0,25) with value of significance / p-value equal to 0,501. Because the value of correlation coefficient -1,96 (<0,25) hence relation of formation of biofilm of gram negative bacteria with ciprofloksasin antibiotic resistance is very weak, insignificant and counterclockwise (if stronger biofilm formation then ciprofloksasin antibiotic resistance will be low or vice versa).

Table 6 Relationship of Biofilm Formation with TMP-SMX Antibiotic Resistance

Biofilm Gram Negative Bacteria	Diameter of TMP-SMX Inhibitory Zone (mm) Based on CLSI						*P value*	r
	≥ 16 mm (n)	%	11-15 mm (n)	%	≤ 10 mm (n)	%		
<0,120	8	57,1	0	0	6	42,9	0,721	0,105
0,120-0,240	0	0	0	0	0	0		

>0,240	0	0	0	0	0	0
Jumlah	8	57,1	0	0	6	42,9

Table 6 shows 8 (78.6%) isolates that are sensitive, and 6 (14.3%) are resistant to TMP-SMX antibiotics and have a weak (weak) ability to form biofilms. Based on bivariate analysis with Rank Spearman correlation test got correlation coefficient value equal to 0,105 (r <0,25) with value of signifikansi / p-value equal to 0,721. Because the correlation coefficient value of 0.105 (<0.25) then means the relationship of biofilm formation of gram-negative bacteria with TMP-SMX antibiotic resistance is very weak, not significant and unidirectional (if the stronger biofilm formation, the higher the TMP-SMX antibiotic resistance).

Discussion

In this study, the age of respondents with asymptomatic UTIwere above 50 years. The results of this study are consistent with the study conducted by Reddy et al. (2013) and Sakyi et al., (2013), who found the incidence of UTI in patients with DM occurred on ages over 50 years and 9 respondents (25.7%) has stopped menstruation (menopause). It is associated with hormonal factors in postmenopausal conditions that cause vaginal atrophy, so the normal vaginal flora is reduced, pH increases and facilitates colonization of pathogenic bacteria in the urinary tract [8].

The results obtained 8 respondents (34.8%) with UTI suffering DM <5 of the year. These results are in line with the Saptaningsih (2012) and Srinivas et al., (2014) studies which reported no association with duration of DM with UTI[8,9]. The results of different studies reported by Divyashree and Yadav (2015) and Boyko et al. (2005) found a relationship between the duration of DM and the incidence of UTI. The duration of DM can not be an indipendent factor of UTI, but is closely related to hyperglycaemia as experienced by respondents in this study who have high fasting blood sugar level and without fasting. The condition of hyperglycemia causes neurogenic bladder resulting in the unreliability of urinate and a good medium for bacterial growth and development[11, 12, 7].

There were 10 respondents (35,7%) with UTI having BMI overweight. These results are in line with research by Ariwijaya and Suwitra (2007) and Al-Rubeaan et al (2013) who reported an association between BMI overweight and UTI in DM patients. The condition of overweight / obese leads to insulin resistance, coupled with the state of hyperglycemia so that pancreatic beta cells are unable to produce sufficient insulin resulting in glycosuria which will be a good growth medium and breeding for pathogenic bacteria causing infection [9,4,13]. In addition, the patient's abdominal circumference>80 cm. An abdominal circumference of>80 cm indicates a buildup of perivesical fat. Visceral fatty acid causes inflammatory epithelium causes urinary tract symptoms such as increased

frequency and urinary urgency, coupled with the symptoms of polyuria in DM patients that facilitate pathogenic microorganisms to enter the urinary tract, then colonize and cause UTI [14].

The results of this study also illustrated that 9 respondents (36%) with the UTI sexual intercourse <3 times a week. The frequency of sexual intercourse> 9 times in the last 1 month has a chance of 10 times having a UTI, while the frequency of sexual intercourse 4-8 times a month has 5-6 times experienced UTI. Ariwijaya and Suwitra (2007) study reported that UTIs in DM women were more common in sexually active respondents. During intercourse microorganisms present in the urethra or around the perineum may migrate into the urinary tract [4,9].

Prevalence asymptomatic UTI in women DM T2 was found to be 31%. This proportion is in accordance with a study reviewed by Renko et al. (2011) which states the incidence is 15% -30%. The results of this study support the theory that UTI in women DM T2 is asymptomatic (no symptoms) [15]. Among the mechanisms that contribute to UTI in women DMT2, such as shorter urethra, high urinary glucose levels that facilitate the growth of pathogenic bacteria, low levels of IL-6 and IL-8 (as the body's defense mechanisms to destroy pathogenic bacteria), Tamm- Horsfall plays an important role in preventing the attachment of pathogenic bacteria to uroepithelial and virulence factors from pathogenic bacteria itself[7,16].

The results of this study are in line with research conducted by Genghesh et al., (2009) in Libya which gained as much as the proportion of *E.coli* and *K. pneumoniae* as a cause of predominant UTI [17]. The bacteria *Escherichia coli* and *Klebsiella pneumoniae* are gram-negative bacteria enterobactericeae class which is one of the normal flora in the colon that can colonize in the urinary tract [18]. Both bacteria have the ability to colonize in the urinary tract with the help of adherence factor called adhesin. Adhesin will increase the ability of bacterial attachment to the urinary tract mucosa and also increase its virulence [18]. Shorter urethral size, sexual activity and lack of hiegine may be a predisposing factor of bacteria causing bacteria to colonize the vaginal introitus and then enter the urinary tract through the urethra and then ascending infections to the bladder, ureter, and renal parenchyma [18].

Most of the microorganisms isolated in this study are still sensitive to ciprofloxacin and show that these antibiotics are still effective as first-line therapy in treating urinary tract infections. The results of this study in accordance with research conducted Nigussie and Amsalu (2017) in Ethiopia obtained a high level of resistance to TMP-SMX (64.7%), and low resistance to ciprofloksasin (23.5%) by isolated gram-negative bacteria in this study [19]. In this study there were no bacterial isolates resistant to both antibiotics (ciprofloxacin and TMP-SMX), these findings are different from previous studies that found high resistance to more than one antibiotic by the gram-negative bacteria

causing UTI, including research in Ethiopia (93.9%), Gondar (91.7% -95%), and Addis Ababa (92.34%) [19].

Gram-negative bacterial resistance to TMP-SMX antibiotics in this study may occur because these antibiotics are widely available first-line therapy and are often used in public health services such as Puskesmas, for either UTI or other infectious diseases. There is also the role of causal microorganisms capable of producing PABA and excessive dihydrofolate reductase enzymes that are able to inhibit the action of the TMP-SMX drug that causes resistance [19]. Ciprofloxacin resistance may occur due to mutations that cause target changes in DNA gyrase enzymes and DNA topoisomerase IV as well as plasmid-mediated resistance that can transmit resistant genes so that the initially resistant bacteria become resistant [20]. Antibiotic resistance is a global public health problem that can occur due to the availability of abundant antibiotics in health facilities as well as the use of irrational antibiotics, such as improper doses, inappropriate indications, incompatible with causal microorganisms, and pathogenic microorganisms that are able to form biofilms [19].

The results of this study differ from studies conducted by Sayal et al. (2016) to obtain the results of the formation of biofilms that vary (non / weak, moderate and strong) [21]. Biofilms can be formed by almost all (99.9%) microorganisms on various surfaces (biological and non-biological), with different optical densities. The ability of gram-negative bacteria to form biofilms depends on several adhesion factors and their virulence genes such as fimbriae, toxin, LPS (Lipopolysaccharide) , protein secretions, capsules, etc. Fattahi et al. (2015) study found that gram-negative bacterial isolates expressing *pap C* and *fimH* virulence gene encoded by type P and type 1 fimbriae were able to form a strong category biofilm compared with those not expressing the virulence genes. The involvement of bacterial virulence gene expression in attachment and biofilm formation, which indicates that bacteria is capable of forming strong / moderate biofilms are more pathogenic than non-weak in forming biofilms [22]. Another study by Wood (2009) found that the excess of c-GMP protein secretion by bacteria was associated with biofilm formation, this protein enhances bacterial ability to organize/ coordinate bacterial changes from planktonic form to sessile (biofilm) [23].

The result of this study are not in line with research conducted Priyadharshini et al (2008) in Pakistan who found that bacteria isolated from DM patients had high levels of resistance against TMP-SMX antibiotics with moderate sensitivity to ciprofloxacin antibiotics. Of the resistant E.coli isolates 40% of the isolates were able to form biofilms [24].Another study by Priyadharshini et al., (2014) in India found from 56 isolated bacteria, 37 (66%) isolates were able to form biofilms, 15 (40%) isolates resistant to antibiotics. While 19 other isolates that were unable to form biofilms were still sensitive to all antibiotics used in the study [24]. A study by Alves et al (2014) found that of 58 isolates capable of forming biofilms, 44.8% isolates were resistant to some antibiotics compared with

28.6% isolates with resistance from 98 isolates that were unable to form biofilms. This incident shows that biofilm formation increases the incidence of resistance to antibiotics[25].

In this study found all the bacteria isolated to form biofilm with the category of weak (non/ weak). Biofilm is one of the bacterial self-defense mechanisms that is also associated with antibiotic resistance, but the bacteria that make up the strong category of biofilm are more resistant to antibiotics than the non/ weak categories. This is related to the expression of virulence genes (adhesion factor genes) of each bacteria that determine its ability to form biofilms. Some studies suggest that gram-negative bacteria that cause UTIs expressing the FimH gene and papC (type 1 and type P fimbriae) are able to form biofilms of moderate to strong categories, compared with those not expressing the gene. Fimbriae type 1 and type P is a filamentous structure composed of proteins located on the surface of bacterial cells and encoded by the genes of chromosomes. This structure can stimulate uroepithelial cell inflammation, facilitate bacterial invasion and form biofilm. It is also aided by the presence of c-GMP protein in the production of bacteria that organizes / coordinates bacterial changes from planktonic form to sessile [22,23].Conventional antibiotics TMP-SMX and ciprofloxacin show a high percentage of resistance even in isolates that are unable to form biofilms. This may be possible because of the excessive and inappropriate use of antibiotics that accelerate bacterial evolution and provide a bacterial response to survive so that bacteria that are initially sensitive to an antibiotic, are less sensitive or even insensitive (resistant). Bacterial factors such as the presence of genetic mutations or the expression of resistant genes that result in the change of antibiotic "target site" against bacteria and the difference of virulence genes associated with biofilm formation causing antibiotic resistance[25,26] .

Conclusion

1. There is a weak association between biofilm formation by gram-negative bacteria with TMP-SMX antibiotic resistance and ciprofloxacin.
2. The bacterial profiles of asymptomatic UTIs found in this study were E. coli (35.7%), K. pneumoniae (35.7%), Enterobacter sp (21.5%) and Citrobacter sp (7.1%) . All 100% isolated bacteria were able to form biofilm in the non / weak category with an OD value of 0.005-0.093 (<0.120)
3. There were 8 (57%) isolates resistant to antibiotics, ie 2 (14.2%) isolates to ciprofloksasin caused by K. pneumoniae (1 isolate) and Citrobacter sp (1 isolate) and 6 (42.8%) isolates against TMP-SMX caused by E. coli (2 isolates), K. pneumoniae (3 isolates) and Citrobacter sp (1 isolate).

4. The incidence of asymptomatic UTI in DMT2 women in the work area of Puskesmas Mon Geudong Kota Lhokseumawe by 31%.

Suggestion

1. Considering high number of asymptomatic UTI cases in DMT2 women, need for screening as a first step to detect UTI disease to prevent from developing the disease into symptomatic UTI and other kidney disease complications arise.
2. Although identified bacterial isolates have the ability to form weak category biofilms, High levels of resistance of TMP-SMX antibiotics as first-line therapy of UTI. Utilizing appropriate adjustment of random antibiotics is appropriate, appropriate dose, delivery method, indication in accordance with the causal bacteria infection based on bacterial culture sensitivity test. Using TMP-SMX antibiotics against newly exposed patients to first UTI and ciprofloxacin antibiotics to patients with recurrent UTIs to minimize the occurrence of resistance.

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