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RESEARCH ARTICLE

The effect of biofilm formation on the outcome therapy of diabetic foot infections (DFIs) patients in the outpatient clinic and inpatient ward of Dr Sardjito General Hospital Yogyakarta

Ika Puspitasari[1], Titik Nuryastuti[2], Rizka Humardewayanti Asdie[3], Hemi Sinorita[3], Nusaibah Umaroh[4], Wahyu Tri Hapsari[4]

[1]Department of Pharmacology & Clinical Pharmacy, Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia

[2]Department of Microbiology, Faculty of Medicine, Public Health & Nursing, Universitas Gadjah Mada, Indonesia

[3]Department of Internal Medicine, Faculty of Medicine, Public Health & Nursing, Universitas Gadjah Mada, Indonesia

[4]Magister of Clinical Pharmacy Program, Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia

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Correspondence
Ika Puspitasari
Department of Pharmacology & Clinical Pharmacy
Faculty of Pharmacy
Universitas Gadjah Mada
Sekip Utara Yogyakarta
Indonesia
ika.puspitasari@gmail.com

Abstract

Introduction: Diabetes is a non-communicable disease with incidence rate of about 1.5 – 2.3% per annum with the most complication is Diabetic Foot Infections (DFIs). **Objective:** This research was conducted to describe the bacteria responsible for biofilm formation and its ability to cause DFIs in biofilm formation at Dr. Sardjito General Hospital as well as the therapy outcome. **Methods:** This research was conducted from September to November 2017. Specimens of samples were obtained from wound swabs of DFIs patients who met the inclusion and exclusion criteria (31 outpatients and 15 inpatients), and were then tested for culture and sensitivity and their ability to form biofilms. **Results:** The DFIs with the biofilm-producing bacteria (weak to moderate) have a different outcome compared to DFIs patients without biofilms.

Introduction

Diabetes is one of the chronic diseases caused by metabolic disorders of the body. This disease is one of the non-communicable diseases that continue to spread among individuals between 1.5% - 2.3% per annum (Al-Rubeaan *et al*., 2015). One of the complications that often occur in diabetes is Diabetes Foot Infections (DFIs). The number of DFIs patients is about 15% of the number of patients with diabetes (Aumiller & Dollahite, 2015). Nearly 85% of people with DFIs end up with amputation, whereby 40% of them can prevent the amputation through appropriate therapy and treatment. The management of DFIs includes reducing pressure on the foot (offloading), debridement and antibiotics administration. One of the challenges faced in managing DFIs is the formation of biofilms from bacteria that cause ulcers. Biofilms are thought to reduce the effectiveness of antibiotic use through several mechanisms, which ultimately leads to antibiotic resistance and delay in antibiotic penetration (Abbas *et al*., 2013; Banu *et al*., 2015). The management of diabetic ulcer antibiotic therapy with the biofilm-producing bacteria requires a specific strategy so that antibiotics are able to eradicate the infection-causing bacteria and accelerate wound healing (Abbas *et al*., 2013). This study was conducted to

describe the biofilm-producing bacteria and their ability to cause DFIs in Dr Sardjito General Hospital Yogyakarta (SGHY) and the outcome therapy to obtain the appropriate management therapy in overcoming bacterial infections in DFIs with biofilms formation.

Methods

This study was an observational study with a prospective cohort design which was conducted from September to November 2017 in the polyclinic and inpatient ward of Dr Sardjito General Hospital Yogyakarta (SGHY). The subject was the outpatients and inpatients who were diagnosed with DFIs. The inclusion criteria in this study were patients diagnosed with DFIs during the study period, aged ≥18 years old, who were examined for their DFIs age and had a complete medical record. Patients with malignancy and immune disorders were excluded from this study. The subjects involved in the study voluntarily agreed to take part, and informed consent was signed. A total of 31 patients in the outpatient clinics and 15 patients in the inpatient ward met the inclusion and exclusion criteria. The wound swab samples were taken when the wound was opened; after that, the culture and sensitivity tests were conducted to determine the profile of wound infecting bacteria and their sensitivity to antibiotics in accordance with the Clinical and Laboratory Standard Institute (CLSI) guidelines. In addition to culture and sensitivity tests, bacteria found in wound swab samples were tested for their ability to form biofilms in the laboratory. The culture and sensitivity tests and biofilm formation were carried out in the Microbiology laboratory of the Faculty of Medicine, Public Health and Nursing of Gadjah Mada University. The results of the culture and sensitivity tests and the biofilm formation ability were then given to the doctor who treated the subject as consideration for therapy. The subject was monitored for the development of the DFIs and their treatment until the wound improved, or the study was completed. Outcome assessment or wound repair was determined by the doctor who took care of the patient. The duration of wound repair was calculated from the time the patients were taken for basic ulcer swabs until the ulcer improved. The study was approved by the Medical and Health Research Ethics Committee of the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia (KE/FK/0838/EC/2017 and KE/FK/1041/EC/2017).

Results

In this study, for three months, samples were collected from 31 patients in the outpatient clinics and 15 patients in the inpatient ward. The age range of subjects in this study was between 36 and 80 years, with an average age of 58.7 years (outpatient) and being 57.9 years old (inpatient), with a balanced number of male and female patients. The number of patients below 60 years of age and over 60 years was balanced in the outpatient care. In contrast, the inpatient care had more patients who were below 60 years old. The demographic data of the patients can be seen in Table I. In both outpatient and inpatient wards, most patients had a BMI of ≤ 25 Kg/m^2, suffering from diabetes with an average of more than ten years and the average occurrence of DFIs being less than six months. Almost all patients also experienced complications of Peripheral Arterial Disease (PAD).

Table I: Demographic profile of patients

Parameter	Outpatient	Inpatient
Average age (year)	58.8	57.9
Age ≤ 60 years old (%)	16 (51.6)	10 (66.7)
Age > 60 years old (%)	15 (48.4)	5 (33.3)
Male (%)	16 (51.6)	8 (53.3)
Female (%)	15 (48.4)	7 (46.7)
Average BMI (Kg/m^2)	23.9	23.5
BMI ≤ 25(%)	19 (61.3)	10 (66.7)
BMI > 25 (%)	12 (38.7)	5 (33.3)
Average DM duration (year)	12.2	10.1
≤ 12 years (%)	20 (64.5)	8 (53.3)
> 12 years (%)	11 (35.5)	7 (46.7)
Average wound duration (month)	5.8	3.2
≤ 6 months (%)	24 (77.4)	11 (73.3)
> 6 months (%)	7 (22.6)	4 (26.7)
Comorbidity*		
Total PAD (%)	28 (100)	15 (100)
Total hypertension (%)	20 (64.5)	7 (46.7)
Total Eye disease (%)	10 (32.3)	1 (6.7)
Total Kidney disease (%)	10 (32.3)	4 (26.7)
Total cardiovascular disorder (%)	6 (19.4)	0
Total stroke (%)	2 (6.5)	0
Total DVT (%)	2 (6.5)	1 (6.7)
Total other infection (%)	2 (6.5)	0

* Patients who suffered from more than one comorbidities

There were 27 bacterial isolates found in this study from outpatients and 14 bacterial isolates from inpatients, as presented in Table II. The ability of biofilm formation occurred in 8 of 27 bacteria in outpatients (29.6%) at a weak to moderate level. In inpatients,

there were 5 out of 14 bacteria with an ability to form biofilms at a weak level (35.7%). Almost all the biofilm-producing bacteria are Gram-negative bacteria (Table II).

Table II: The ability of bacteria to form biofilm and DFIs patient outcome therapy

Age of ulcer (month)	Bacteria isolated from ulcer	Biofilm strength				
1	*Pseudomonas aeruginosa*and*Klebsiella pneumonia* (MDR)	weak	Ceftazidime and metronidazole	Meropenem; Amikacin; Fosfomicyn	19	Bad
1.5	*Pseudomonas aeruginosa*(MDR)	weak	Ceftazidime and clindamycin	Amikacin	14	Bad
2.5	*Actinobacillussp*	weak	Ceftazidime and metronidazole	Meropenem; Fosfomicyn	17	Bad
5	*Klebsiellapneumoniae*	weak	Ceftazidime and metronidazole; Clindamycin	Amikacin and meropenem	17	Bad
12	*Morganellamorganii, Proteus mirabilis* (MDR)and*Klebsielaoxytoca*	weak	Ceftazidime and metronidazole;	Amikacin and meropenem	16	Good
					16.6±1.8	

The duration of the ulcer did not have any effect on the level of the biofilm, as shown in Table III. In outpatients, the longer the duration of DFIs, the broader the range of biofilm formed, from weak to moderate. Meanwhile, in inpatients who have suffered from DFIs for 5-48 months, the biofilms formed are all weak. There is no significant difference in the duration of healing between DFIs with and without biofilm-producing bacteria in outpatient. However, there is a significant difference in the duration of healing between DFIs with biofilm-producing bacteria and those with the non-biofilm-producing bacteria in hospitalized patients (10.1±3.5 days versus 16.6±1.8 days).

Table III: Statistical analysis

Outpatient	**Antibiotic duration (days)**	***p*-value**
DFIs with no-biofilm-producing bacteria	22.9±6.3	0.05
DFIs with biofilm-producing bacteria (weak-moderate)	22.0±3.9	
Inpatient	**Antibiotic duration (days)**	***p*-value**
DFIs with no-biofilm-producing bacteria	10.1±3.5	0.03
DFIs with biofilm-producing bacteria (weak-moderate)	16.6 ±1.8	

Discussion

Ageing can increase the risk of DFIs by two to four times. On the other hand, younger people have higher mobility than the older ones, who are at risk of getting new trauma or injuries. Age affects the duration of DFIs healing. Older patients have a longer healing time associated with a decrease in the inflammatory response, such as not immediately infiltrating T cells in the wound due to a disruption in chemokines production and decreased capacity of macrophage phagocytosis (Guo & Dipietro, 2010).

The risk of DFIs in women tends to be lower because they maintain and take care of their feet than men; besides, they have a lower risk of neuropathy than men (Al-Rubeaan *et al*., 2015). Wound healing also depends on hormones such as estrogen, testosterone and dehydroepiandrosterone (DHEA). Estrogen is related to matrix production, regeneration, inhibition of proteases, epidermal functions and is associated with genes related to inflammation so that their presence has an effect on wound healing (Horng *et al*., 2017).

Body mass index (BMI) can affect the speed of wound healing. In this study, the majority of patients with DFIs has a BMI of less than 25. The increase in BMI is directly proportional to the increase in the risk of DFIs in patients, as every 20 kg increase in weight can increase the risk of DFIs by 20%. This is because the fatter the patient, the greater the foothold and pressure on the feet compared to a slim patient (Sohn *et al*., 2010). In patients with obesity, adipose tissue secretes various molecules that can cause vascular disorders, including PAD. In addition, there is an increase in the working of the heart, which improve tissue perfusion; if the heart fails to perform perfusion, it can cause necrosis of the

tissue, which prolongs the healing process. In addition, patients with obesity are at risk of hyperventilation which can cause low oxygen level around the wound, leading to damage. In patients with PAD, perfusion can occur, which results in low antibiotic concentrations in the lower extremities, so that the healing process of DFIs is inhibited (Vella *et al*., 2016). The aggressive revascularisation therapy can increase reperfusion and accelerate wound healing in these patients.

A kidney disorder is a concomitant disease commonly found in patients with DFIs. Patients with renal impairment also discovered that PAD had a worse prognosis because PAD in patients with chronic kidney disorder was a poor predictor of wound healing (Prompers *et al*., 2008). In this study, there were 32.3% of patients with kidney disorder and PAD in outpatient care and 26.7% in inpatient care.

The average duration of the DFIs patients in this study suffered from diabetes mellitus was more than ten years, with an average duration of the wound being more than three months. In the prolonged duration of diabetes, the risk of complications, including diabetic ulcers, increased (Zoungas *et al*., 2014). The wound healing process in patients with diabetes was disrupted due to hypoxia, fibroblast and epidermal cells dysfunction, angiogenesis and neovascularization disorders, high metalloprotease level, neuropathy, and decreased immune resistance from the host (Guo & Dipietro, 2010). The duration of diabetes is also associated with the presence of complications in the form of neuropathy. The longer the duration of diabetes, the higher the patient's risk of developing neuropathy. In patients with neuropathy, neuropeptide level is lower; this decrease in neuropeptide level causes a long process of wound healing (Ackermann & Hart, 2013). The duration of the wound can also affect the speed of wound healing. Chronic (prolonged) wounds are associated with chronic inflammatory activity, ageing of fibroblasts, and growth of bacteria in wounds (Bosanquet & Harding, 2014).

Based on this study, in outpatient's clinics, the number of monomicrobial and polymicrobial bacteria tends to be similar. However, for the inpatients, the number of polymicrobial bacteria is higher. This may be due to the fact that the outpatients involved in the study were patients who have a more regular (weekly) check-up to the outpatient clinic, which prevents the accumulation of the growing bacteria, as there is more intensive debridement provided with a medical check-up at the clinic. One of such therapies to combat biofilm formation is using an agent that is capable of disrupting the multicellular structure of the biofilm (Deepigaa, 2017; Mendes *et al*., 2014). However, there is still a need for further research related to the effect of intensive debridement as a multicellular structure disrupter on the recovery of DFIs. The type of bacteria will also determine the level of the biofilm formed. *Pseudomonas aeruginosa, Citrobacter sp., E. coli, Proteus sp* and *Klebsiellaoxytoca* are known to be Gram-negative bacteria that are capable of forming biofilms in DFIs and Multi-Drug Resistance (MDR) organism (Abbas *et al*., 2013; Banu *et al*., 2015). The type of Gram-negative bacteria is in accordance with the results of the study.

Diabetes-associated foot ulcer infections are predominantly polymicrobial. Several bacterial can be part of the DFIs microbial, namely Staphylococcus, Pseudomonas, Streptococcus, Enterococcus, Corynebacterium, Acinetobacter, Prevotella, Porphyromonas, and members of the family Enterobacteriaceae. The predominant Gram-positive and Gram-negative species present in DFIs are *Staphylococcus aureus* and *Pseudomonas aeruginosa*, respectively (Abbas et al., 2013; Banu *et al*., 2015; Lipsky *et al*., 2008). In infected DFIs, because of deficient vascularization, antibiotics frequently reach the local ulcer microenvironment only at sub-therapeutic concentrations (Lipsky *et al*., 2008). Even when topically applied, antibiotics rarely reach bacteria that reside within mature biofilms at therapeutic concentrations (Lipsky *et al*., 2004). In addition, some antibiotics such as aminoglycoside contribute to the increase in the formation of biofilm by *P.aeruginosa* and *E.coli,* so that a strategy is needed to deal with this (Hoffman *et al*., 2005). The microbial cells growing within a biofilm are physiologically distinct from planktonic cells of the same strain. The overall resistance level in biofilms is distinct from the one observed at a cellular level (Stewart & Costerton, 2001). As a consequence, the antimicrobial concentration required to inhibit biofilms can be up to hundreds or even a thousand times higher than the corresponding concentration necessary to eliminate free-living bacterial cells (Ceri *et al*., 1999). The resistance of biofilms formed by Gram-positive strains was low against azithromycin and imipenem. Imipenem was the least affected by biofilms formed by Gram-negative bacteria. Vancomycin is unable to fight *S.aureus* and *Enterococcus faecalis* (LaPlante & Mermel, 2009). In addition, Ciprofloxacin was unable to eradicate the biofilm of *S.aureus*, *E.coli* and *P.aeruginosa* (measured by the ratio of MBEC/MIC expressed by ≥ 90% of the tested isolates) (Ceri *et al*., 1999). Several novel therapeutic strategies, namely bacteriophages, probiotics and antimicrobial peptides (AMP), are recently explored as potential alternatives to eradicate bacterial biofilms in DFIs. Antibiofilm agents in combination with antibiotics, for example, Ciprofloxacin, may be useful to overcome the high biofilm resistance to antibiotics. The synergistic effect of potential antibiofilm agents with Ciprofloxacin appears in several strains, namely *Acinetobacterbaumanii,* including

ambroxol, piroxicam, Manuka honey and grape vinegar (Abbas *et al*., 2013). An interesting observation from this study is the discovery of *Bulkholderia pseudomallei* bacteria, which can form biofilms at weak to moderate levels. *Burkholderia pseudomallei* are the causative agents of melioidosis, an infection common in Southeast Asia and other parts of the world. Clinical manifestations vary and may be entirely absent or may include acute septic shock and abscesses. Acute septic shock syndrome is common in patients with melioidosis and diabetes or chronic renal failure. The immune status of the host is an important factor in infection by B. pseudomallei. Susceptibility to melioidosis was found in hosts who were immunocompromised and/or had diabetes mellitus and other conditions *(Currie et al*., 2010). The stimulation of B. pseudomallei to produce biofilms resulted in upregulation of some genes to be more resistant to antimicrobial agents (Lee *et al*., 2010). B. pseudomallei in biofilm cells are highly resistant to ceftazidime, doxycycline, imipenem, and trimethoprim sulfamethoxazole. However, the drug resistant mechanism of biofilm is still unclear (Currie *et al*., 2010; Korbsrisate *et al*., 2005).

Improvement was observed in the outcomes of all the outpatients, while the inpatients had bad outcomes as the biofilms have been formed. The patient severity index has not been mapped in this study which is likely to complement the results of the study. The absence of differences in the duration of healing between the DFIs and biofilm-producing bacteria in outpatient is likely due to routine debridement of the ulcer. In addition to removing necrotic tissue, debridement also reduces bacterial colonization of the ulcer and damages the biofilm physically (Aumiller & Dollahite, 2015). In the future, DFIs can use combination of antibiofilm agents and antibiotics to improve the patients' therapeutic outcome.

Conclusion

Most of the bacteria that cause infection in DFIs at the Clinic of SGHY are Gram-negative bacteria. A total of 29.6% of bacteria in outpatients have an ability to form biofilms with a weak to moderate nature, while 35.7% of bacteria in inpatients have an ability to form biofilms with weak nature. In hospitalised patients, the presence of biofilms will prolong the healing of patients with DFIs.

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