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Study of Biofilm Formation and Antibiotic Resistance Pattern of Bacteria Isolated from Diabetic Foot Ulcers in Hôpital de Référence Saint Joseph, Kinshasa, Democratic Republic of Congo

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Abstract

Foot infections resulting from biofilm producers and multi-drug resistant organisms is one of the most important complications of diabetes mellitus, as it can impede the wound healing process. This study was carried out in order to determine the antibiotic resistance pattern and the biofilm production in diabetic foot ulcers isolates. Clinical samples were collected from patients suffering from diabetic foot ulcers by using sterile swabs. Antibiotic susceptibility test was done using disk diffusion method on Mueller Hinton Agar. Biofilm formation was assessed by Crystal Violet Staining Method. *Staphylococcus aureus* isolates were resistant to ofloxacin (83.3%), ciprofloxacin (75.0%), trimethoprim-sulphamethoxazole (75.0%), and gentamicin (58.8%) but very sensitive to oxacillin (100.0%) and vancomycin (91.7%). *Pseudomonas aeruginosa* isolates showed resistance to the commonly used antibiotics such as ofloxacin, cefotaxime, ampicillin (81.8%), ceftazidime and imipenem (72.7%). The majority of bacteria studied were biofilm producers. This study showed that bacteria isolated from diabetic foot ulcers were biofilm producers and presented resistance to commonly used antibiotics. Knowledge on antibiotic sensitivity pattern and biofilm phenotype of the isolates will be helpful in de-

termining the drugs for the treatment of diabetic ulcers.

Keywords

Biofilm Formation, Antibiotic Resistance, Diabetic Foot Ulcers, Democratic Republic of Congo

1. Introduction

Diabetic foot ulcers (DFUs) are a prevalent complication of diabetes mellitus (DM) and lead significant morbidity, mortality, and healthcare expenditures [1]. Africa is estimated to have 15.9 million adults living with DM making a regional prevalence of 3.1%. The annual incidence of diabetic foot ulcer worldwide is between 9.1 to 26.1 million [2].

The African continent has the greatest proportion of people with undiagnosed DM and global projections show that the continent will face even a greater burden of DM of about 156% by 2045 [3]. Around 15% to 25% of patients with DM will develop a diabetic foot ulcer during their lifetime [4]. DFU are among the most common complications for patients who have insufficiently controlled DM. It is one of the common causes for osteomyelitis of the foot and amputation of lower extremities [5]. These ulcers are usually in the areas of the foot which encounters repetitive trauma and pressure sensations [6]. When an ulcer is present, there is a clear entrance for invading bacteria. Infection can range from local infection of the ulcer to wet gangrene. Only half of infection episodes show signs of infection. In the presence of neuropathy and ischaemia, the inflammatory response is impaired and early signs of infection may be subtle. Deep swab and tissue samples (not surface callus) should be sent for culture without delay and wide spectrum antibiotics given to cover Gram positive, Gram negative, and anaerobic bacteria. Urgent surgical operation is needed in certain circumstances [7]. The ulcers often become chronic and infected with bacterial biofilm [8]. Systemic antibiotics are prescribed when the ulcer shows clinical signs of infection [9] [10] [11]. Resolution of infection after treatment of Diabetic Foot Infection (DFI) with systemic antibiotics varies widely with values ranging from 5.6% to 77.8% [12]. At a high bacterial load, the biofilm is likely to be very well established and highly tolerant to antibiotics [13] [14]. DFIs are typically colonized by bacteria similar to the surrounding skin and become more complex in microbial diversity over time and with progression of the ulcer [15] [16] [17] [18]. The common organisms seen in a DFU are Gram positive organisms such as *Staphylococcus, Enterococcus* and *Streptococcus*, Gram negative organisms such as *Enterobacteriaceae* and *Pseudomonas* sp, and anaerobes [19]. The biofilms are the main cause of many chronic infections such as DFUs, and they pave the way for the re-emergence of multidrug-resistant strains and result in treatment failure [20]. Biofilms are difficult to eradicate by using conventional antibiotics, hence the identification of biofilm producers among clinical isolates may lead to better

management of wound infections in diabetics who, in spite of repeated antibiotic treatment, fail to respond to treatment because biofilms are not being tested for routinely [20]. The aim of this study was to evaluate the antibiotic susceptibility pattern and the biofilm formation by Gram-positive and Gram-negative organisms isolated from DFUs in Hôpital de Référence Saint Joseph, Kinshasa.

2. Material and Methods

2.1. Origin of the Strains and Laboratory Procedures

The clinical samples were collected for diagnostic purposes in 2016 by the bacteriology laboratories of Hôpital de Référence Saint Joseph in Limete, Kinshasa, and were from wound secretions of DFU. Infected sites were aseptically cleaned using normal saline and sterile gauzes. Then a wound swab from each patient was collected using sterile cotton swabs. Isolated bacteria on Trypticase soy agar medium (Liofilchen, Roseto degli Abruzzi, Italy) were received in the Laboratory of Experimental and Pharmaceutical Microbiology at the Faculty of Pharmaceutical Sciences of the University of Kinshasa for biofilm formation studies. Antibiotic susceptibility tests were done to confirm the results from hospital. Pathogens studied are presented in Table 1 below.

2.2. Isolation and Identification of Bacteria

Wound swabs were inoculated into mannitol-salt and Mac Conkey agars (Liofilchen, Roseto degli Abbruzzi, Italy) and incubated at 37˚C for 24 hours. *Staphylococcus* sp. were identified by standard microbiological methods such as Gram staining, catalase tests. *S. aureus* suggestive colonies were confirmed by coagulase and DNase testing. Gram-negative bacilli were identified using microbiological conventional methods including Gram staining, oxidase tests, indole and urease production, citrate utilization, hydrogen sulphide, gas production and fermentation of sugars, phenylalanine deaminase, lysine decarboxylase (L.D.C.), ornithine decarboxylase (O.D.C.), arginine dihydrolase (A.D.H.) tests, and methyl red reaction [21]. In our laboratory Gram negative bacilli were confirmed as *Enterobacteriaceae* species using the same tests. *Pseudomonas aeruginosa* were confirmed after 24 hours incubation time into Cetrimide agar.

Table 1. Bacteria strains.

Pathogens	Bacteria species	N (%)
Gram positive cocci	*Staphylococcus aureus*	12 (41.4)
	Coagulase negative staphylococci (CNS)	1 (3.4)
Gram negative rods	*Escherichia coli*	2 (2.6)
	Klebsiella pneumoniae	2 (6.8)
	Salmonella sp.	1(3.4)
	Pseudomonas aeruginosa	11 (37.9)
TOTAL		**29 (100%)**

2.3. Antibiotic Susceptibility Testing

Antibiograms of each isolated *Staphylococcus* sp. strains using the diffusion method on Mueller Hinton Agar were realized with the following antibiotic disks (Liofilchen, Roseto degli Abruzzi, Italy): Vancomycin (30 µg), Erythromycin (15 µg), Imipenem (10 µg), Trimethoprim-sulphamethoxazole (25 µg), Ciprofloxacin (5 µg), Ofloxacin (5 µg), and Gentamicin (10 µg). Test for methicillin resistance was performed with diffusion method using Oxacillin (1 µg) on Mueller Hinton agar with 4% NaCl. Gram negative strains were tested against the following antibiotic disks (Liofilchen, Roseto degli Abruzzi, Italy): Ceftazidime (30 µg), Gentamycin (10 µg), Amikacin (30 µg), Imipenem (10 µg), Trimethoprim-sulphamethoxazole (25 µg), Ampicillin (10 µg), Ofloxacin (5 µg), and Cefotaxime (30 µg). After incubation of plates at 37˚C for 24 hours, diameters of zone of inhibition were measured. Evaluation of the results was done according to the criteria of Clinical Laboratory Standards Institute (CLSI), 2012 [22]. *E. coli* ATCC 25922, *P. aeruginosa* ATCC 28753 and *S. aureus* ATCC 25923 were used for quality control.

2.4. Biofilm Formation Assay

In the present study, we screened all the isolates for their ability to form biofilm by Crystal Violet Staining Method (CVSM). The *S. aureus* and *Enterobacteriaceae* isolates were analyzed as described previously by Stepanovic *et al.*, [23] and Ramos-Vivas *et al.*, [24] respectively with minor modifications. A suspension equivalent to the McFarland 0.5 turbidity standard was prepared in trypticase soya broth (Liofilchen, Roseto degli Abruzzi, Italy) for each strain. Accuracy of bacterial counts in the suspension was confirmed by serial dilution in log steps. Polystyrene sterile strips were inoculated with 200 µL of each calibrated bacterial suspension and incubated for 24 hours at 35˚C in a humid atmosphere. A control well was inoculated with sterile medium. Each strain was evaluated in triplicate. Medium was removed from the wells which were washed 3 times with 200 µL sterile distilled water. The strips were air-dried for 45 min and the adherent cells were stained with 200 µL of 0.1% Crystal violet solution. After 45 min, the dye was eliminated and the wells were washed 5 times with 300 µL of sterile distilled water to remove excess stain. The dye incorporated by the cells forming a biofilm was dissolved with 200 µL of 33% (v/v) glacial acetic acid and the absorbance of the well was obtained by means of enzyme-linked immunosorbent assay (ELISA) reader, at the wavelength of 540 nm. The results were expressed as variation of Optical Density (OD) 540 nm (OD540 nm sample—OD540 nm control). These OD values were considered as an index of bacteria adhering to surface and forming biofilms. For interpretation of biofilm production, the average of the three wells was calculated, and different criterions was adopted. For *Staphylococcus*, criterion proposed by Stepanovic *et al.*, [23] was adopted: non-adherent ($OD < 0.12$), moderate producer ($0.12 < OD < 0.24$) and strong producer ($OD > 0.24$). For Gram-negative bacteria criterion proposed by Ramos-

Vivas *et al.*, [24] was used: OD ≤ 0.05, non-biofilm producer; OD > 0.05 - 0.1 weak biofilm producer; OD > 0.1 - 0.3 moderate biofilm producer; and OD > 0.3 strong biofilm producer.

2.5. Statistical Analyses

GraphPad software package was used to calculate mean and standard deviation.

3. Results

3.1. Antibiotic Susceptibility

3.1.1. *Staphylococcus* Isolates

The results of the antibiotic susceptibility tests of *Staphylococcus* and Gram-negative organisms are shown in tables below. Among *S. aureus* strains studied, the highest resistance rates were observed for ofloxacin (83.3%), followed by trimethoprim-sulphamethoxazole and ciprofloxacin with a resistance rate of 75.0%, respectively. These strains were more sensitive to imipenem and vancomycin (91.7% respectively) and oxacillin (100%). The other antibiotics showed a resistance rate of 58.3% (Table 2).

3.1.2. Gram Negative Isolates

The highest rates of resistance (greater than 80.0%) against *Pseudomonas* sp. were observed for cefotaxime, ofloxacin and ampicillin followed by ceftazidime (72.7%), imipenem (72.7%) and trimethoprim-sulphamethoxazole (63.6%). The lowest resistance levels were observed for amikacin (18.2%) and gentamycin (27.3%). *E. coli* and *Klebsiella pneumoniae* isolates were fully sensitive to amikacin (Table 3(a) and Table 3(b)).

Table 2. Antibiotic susceptibility profile of *Staphylococcus* isolates.

Antibiotics	*S. aureus*			CNS		
	S	I	R	S	I	R
	n (%)	n (%)	n (%)	n (%))	n (%)	n (%)
Oxacillin	**12 (100.0)**	0 (0.0	0 (0.0)	0 (0.0)	0 (0.0)	1 (100.0)
Vancomycin	**11 (91.7)**	0 (0.0	1 (**8.3**)	1 (100.0)	0 (0.0	0 (0.0
Erythromycin	3 (25.0)	2 (16.7)	**7 (58.3)**	1 (100.0)	0 (0.0	0 (0.0
Imipenem	**11 (91.7)**	1 (8.3)	0 (0.0)	1 (100.0)	0 (0.0	0 (0.0
Trimethoprim-sulphamethoxazole	2 (16.7)	1 (8.3)	**9 (75.0)**	0 (0.0)	0 (0.0)	1 (100.0)
Ciprofloxacin	1 (8.3)	2 (16.7)	**9 (75.0)**	0 (0.0)	0 (0.0)	1 (100.0)
Ofloxacin	0 (0.0)	2 (16.7)	**10 (83.3)**	0 (0.0)	0 (0.0)	1 (100.0)
Gentamicin	2 (16.7)	3 (25.0)	**7 (58.3)**	0 (0.0)	0 (0.0)	1 (100.0)

S: Susceptible; I: Intermediate; R: Resistant.

Table 3. Antibiotic susceptibility profile of Gram-negative isolates.

(a)

Antibiotics	*Pseudomonas aeruginosa*			*Escherichia coli*		
	S	I	R	S	I	R
	n (%)	**n (%)**	n (%)	**n (%)**	**n (%)**	**n (%)**
Ceftazidime	3 (27.03)	0 (0.0)	**8 (72.7)**	0 (0.0)	0 (0.0)	2 (100.0)
Gentamicin	6 (54.5)	2 (18.2)	3 (27.3)	0 (0.0)	0 (0.0)	2 (100.0)
Amikacin	**8 (66.7)**	1 (9.0)	2 (18.2)	2 (100.0)	0 (0.0	0 (0.0)
Imipenem	1 (9.1)	2 (18.2)	**8 (72.7)**	0 (0.0)	0 (0.0)	2 (100.0)
Trimethoprim-sulphamethoxazole	3 (27.3)	1 (9.0)	**7 (63.6)**	0 (0.0)	1 (50.0)	1 (50.0)
Ampicillin	1 (9.0)	1 (9.0)	**9 (81.8)**	0 (0.0)	0 (0.0)	2 (100.0)
Ofloxacin	2 (18.2)	0 (0.0)	**9 (81.8)**	0 (0.0)	0 (0.0)	2 (100.0)
Cefotaxime	1 (9.0)	1 (9.0)	**9 (81.8)**	1 (50.0)	0 (0.0)	1 (50.0)

(b)

Antibiotics	*Klebsiella pneumoniae*			*Salmonella* sp.		
	S	I	R	S	I	R
	n (%)	**n (%)**	**n (%)**	**n (%)**	n (%)	**n (%)**
Ceftazidime	1 (50.0)	0 (0.0)	1 (50.0)	0 (0.0)	0 (0.0)	1 (100.0)
Gentamicin	1 (50.0)	0 (0.0)	1 (50.0)	1 (100.0)	0 (0.0	0 (0.0)
Amikacin	2 (100.0)	0 (0.0	0 (0.0)	1 (100.0)	0 (0.0	0 (0.0)
Imipenem	0 (0.0)	1 (50.0)	1 (50.0)	0 (0.0)	0 (0.0)	1 (100.0)
Trimethoprim-sulphamethoxazole	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	0 (0.0)	1 (100.0)
Ampicillin	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	0 (0.0)	1 (100.0)
Ofloxacin	0 (0.0)	0 (0.0)	2 (100.0)	1 (100.0)	0 (0.0	0 (0.0)
Cefotaxime	0 (0.0)	0 (0.0)	2 (100.0)	1 (100.0)	0 (0.0	0 (0.0)

S: Susceptible; I: Intermediate; R: Resistant.

3.2. Biofilm Formation

The results presented in Figure 1 showed that 8 strains of *S. aureus* were moderate biofilm producers (0.12 < OD < 0.24). Three *S. aureus* strains (1, 2 and 7) were non-biofilm producers (OD < 0.120). CNS strain was moderate biofilm producers (0.12 < OD < 0.24). Out of 11 Pseudomonas sp. isolates studied for biofilm formation, 2 isolates were strong biofilm producers (OD > 0.3), 7 isolates were moderate biofilm producers (OD > 0.1 - 0.3), and 2 isolates were weak biofilm producers (OD > 0.05 - 0.1) (Figure 2). All *Enterobacteriaceae* strains were moderate biofilm producers (OD > 0.1 - 0.3) with exception for *K. pneumoniae*2 which were strong biofilm producer (OD > 0.3) (Figure 3).

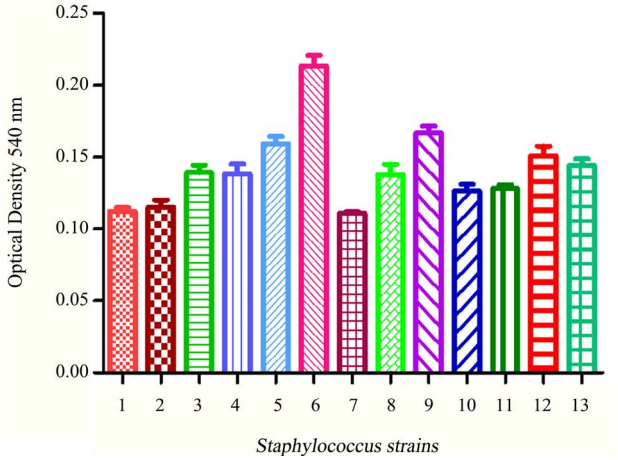

Figure 1. Biofilm formation by *Staphylococcus* isolates was performed using CVSM. Data are the mean ± standard deviation of 3 independent experiments. 1 - 12: *S. aureus*; 13: CNS. OD: optical density.

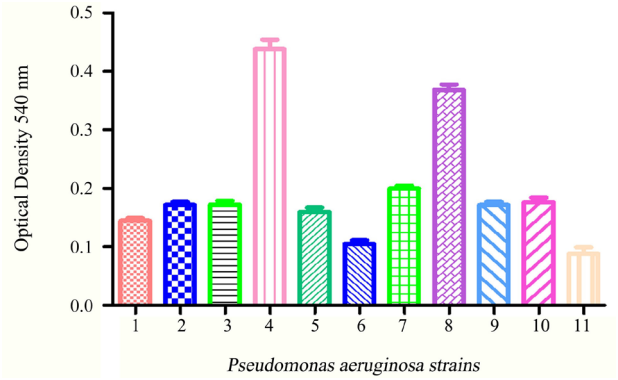

Figure 2. Biofilm formation by *P. aeruginosa* isolates was performed using CVSM. Data are the mean ± standard deviation of 3 independent experiments. OD: optical density.

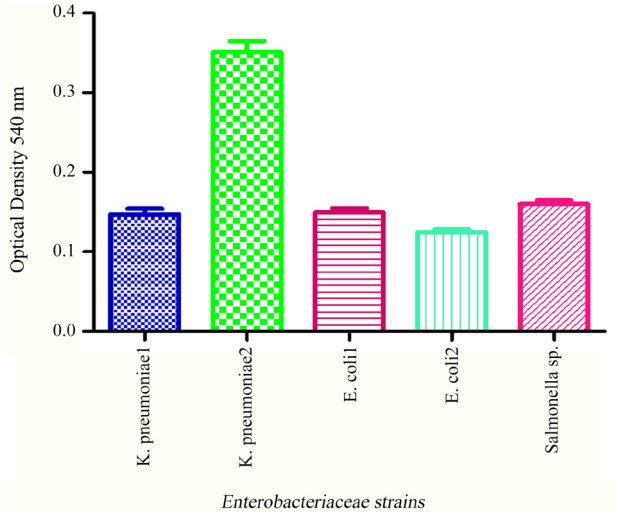

Figure 3. Biofilm formation by *Enterobacteriaceae* isolates was performed using CVSM. Data are the mean ± standard deviation of 3 independent experiments. OD: optical density.

4. Discussion

DFUs can become chronic and non-healing despite systemic antibiotic treatment. The penetration of systematically-administered antibiotics to the site of infection is uncertain, as is the effectiveness of such levels against polymicrobial biofilm [25]. Regarding the antibiograms performed, the results obtained (**Table 2**, **Table 3(a)** and **Table 3(b)**) showed that the strains of staphylococci studied were resistant to the majority of the antibiotics tested, with the exception for oxacillin, vancomycin, and imipenem. Methicillin-resistant *S. aureus* (MRSA) was not observed. Our results are consistent with a report from Kenya in which *S. aureus* was highly resistant to trimethoprim-sulphamethoxazole but sensitive to oxacillin and vancomycin [26]. *P. aeruginosa* and other *Enterobacteriaceae* strains were highly resistant to the majority of antibiotic tested, with the exception for amikacin. This is not in the line with report from Kenya in which *P. aeruginosa* was sensitive to ampicillin, ceftazidime, and trimethoprim-sulphamethoxazole [26]. Many studies have reported an increase in bacterial resistance pathogens isolated from DFI to several groups of antibiotics [27] [28] [29] [30]. The Gram-positive bacterium *S. aureus* is the most commonly found bacterial species in diabetic ulcers. Other microorganisms such as beta-hemolytic streptococci and a mixture of Gram-negative species such as *E. coli*, *Klebsiella*, and *P. aeruginosa* are also present in wounds [31]. Conventionally, bacterial infections have been treated with oral or intravenous antibiotics depending upon the severity of infection and sometimes bioabsorption of the antibiotics. However, infections of chronic wounds are canny. Wounds can become infected by bacteria that encapsulate themselves in biofilms over time or when the body's natural defense mechanisms are impaired [32]. A non-healing wound is an indicator of the presence of biofilm [33]. Biofilms cause a delay in healing by initiating an immune response leading to chronic inflammatory cycle and tissue damage due to high levels of proteases and reactive oxygen species [34] [35]. In this study, CVSM was used to evaluate the capability of bacteria from DFUs to produce a biofilm. The results have showed that the majority of *Staphylococcus* strains have produced a biofilm. A recent study has demonstrated that the staphylococcal isolates are able to form biofilm [36]. Several virulence genes are implicated in biofilm formation, like icaA and icaD, responsible for the biosynthesis of polysaccharide intercellular adhesion (PIA) molecules, containing N-acetylglucosamine, the main constituent of the biofilm matrix in the accumulation phase [37]. Staphylococcal strains studied were resistant to the majority of the antibiotics tested. Indeed, biofilms exhibit enhanced tolerance to antibiotics compared to free-living bacteria, which makes treatment of wound infections challenging [32]. A retrospective study has demonstrated that Gram-negative from DFI were found to be biofilm producers [38]. The results of the present study demonstrated that all *P. aeruginosa* and *Enterobacteriaceae* strains produced biofilms. Two *P. aeruginosa* isolates 4 and 8 (**Figure 2**) and one *K. pneumoniae* (**Figure 3**) strain produced strong biofilms. *P. aeruginosa* plays an important role in diabetic foot infections.

As a Gram-negative opportunistic pathogen, *P. aeruginosa* causes recurrent and refractory infections that are characterized by biofilm formation [39]. Extracellular matrices (ECMs) of biofilms usually consist of exopolysaccharide (EPS), extracellular DNA (eDNA), and proteins, which act as a matrix, adhesive material, and protective barrier [40] [41]. There are three identified EPSs in *P. aeruginosa* which are involved in biofilm formation: Psl (polysaccharide synthesis locus), Pel (a glucose-rich polysaccharide polymer), and alginate [42]. Quorum Sensing (QS) plays also an important role in *P. aeruginosa* biofilm formation. Indeed, QS systems not only sense population density, but also regulate a variety of traits, such as bacterial phenotype, spatial differentiation in biofilms, motility, and biofilm formation [43]. But recent data demonstrated that *P. aeruginosa* establishes a robust and persistent infection in diabetic wounds independent of its ability to form biofilm and causes severe wound damage in a manner that primarily depends on its Type III Secretion System (T3SS). The T3SS virulence structure is required for the pathogenesis of all *P. aeruginosa* clinical isolates, suggesting that it may also play a role in the inhibition of wound repair in diabetic skin ulcers [44]. *Staphylococcus*, *P. aeruginosa* and *Enterobacteriaceae* strains studied were highly resistant to the majority of antibiotic tested as demonstrated in previous studies. Results obtained by other authors have shown that multidrug resistant organisms isolated from DFU were biofilm formers [20] [45] [46]. The ineffectiveness of traditional antibiotics-based treatment of biofilm has been attributed to a combination of different factors. The multilayered defense against antibiotics includes poor penetration into biofilms, adaptive stress responses, and metabolic inactivation due to nutrient and gas limitation [47]. A negatively charged biofilm membrane may limit the penetration of positively charged antibiotics through the biofilm [48]. Even if the antibiotic molecule enters the biofilm, it has to diffuse through the aqueous matrix in order to reach the bacterial cells. Aminoglycosides and beta-lactams may be inactivated or sequestered by binding to any solutes present in the matrix, making it impossible for them to diffuse to the depths of the biofilm [49] [50], also referred to as mass transport limitation.

5. Conclusion

The present study showed that multidrug-resistant pathogens in DFUs were biofilm producers. As biofilms infections are difficult to eradicate using conventional antibiotics, it is necessary to determine the antibiotic susceptibility pattern of the biofilm producers among clinical pathogens prior to the treatment of DFI.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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