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Research Article
Beta-lactamases and Virulence Factors Characterization in Gram-negative Bacterial Species Isolated from Diabetic Foot Ulcer Patients

[1]Neelum Meraj, [1]Haleema Tahir, [3]Muhammad Malik, [3]Alia Begum, [3]Adnan Khan, [3]Sana Sikander, [1]Inam Ullah, [1]Muhammad Yasin, [2]Muhammad Haroon, [1]Shamim Saleha and [3]Waheed Ullah

[1]Department of Biotechnology and Genetic Engineering, Kohat University of Science and Technology, Kohat, Pakistan
[2]Khyber Teaching Hospital, Peshawar, Pakistan
[3]Department of Microbiology, Kohat University of Science and Technology, Kohat, Pakistan

Abstract

Background and Objectives: Diabetic Foot Ulcers (DFU) are most frequent foot abrasion which leads to lower extremity amputation. Diverse microbes are associated with DFU infections which make treatment difficult due to their strong resistant nature. Bacterial resistance is being linked with the production of beta-lactamases, expression of resistant genes and there is also strong correlation of virulence factors etc. Keeping in view the significance of Gram-negative pathogens association with DFU patients and their antibiotic resistance mechanisms, current study was designed to elaborate Gram-negative resistant bacteria for their resistant mechanisms and linkage with virulence as it may have some strong correlation with resistance. **Materials and Methods:** Gram-negative bacterial isolates (n = 37) that have proven resistance against various drugs through simple disc diffusion method were subjected for detection of Extended-Spectrum Beta-Lactamases (ESBL), Metallo-Beta-Lactamases (MBL) and AmpC-Beta-Lactamases. Further these isolates were subjected for detection of CTX-M beta-lactamase genotype. Further all Gram-Negative bacterial strains were screened for evaluation of their virulence factors. **Results:** In this study total of (n = 37) where *Pseudomonas aeruginosa* was the dominant bacteria followed by *Escherichia coli* and *Klebsiella pneumoniae*. The prevalence of beta-lactamases were as AmpC (72.5%) the most prevalent one, followed by MBL (67.5%) and ESBL incidence was (59.5%). Higher incidence of beta-lactamases were found in *Pseudomonas aeruginosa*. **Conclusion:** The severity of resistance in Gram-Negative bacteria to multiple antibiotics may be due to the co-production of beta-lactamases along with virulence factors that could be the leading cause of bacterial pathogenesis in such patients.

Key words: Diabetic foot ulcers, beta-lactamase, antibiotics, virulence factors, biofilm

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Corresponding Author: Waheed Ullah, Department of Microbiology, Kohat University of Science and Technology, Kohat, Pakistan

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Diabetic foot is one of the most severe, devastating and costly problems of diabetes, defined as an ulceration of foot, accompanying peripheral arterial disease and neuropathy of the lower limb in diabetes patients[1]. Diabetic foot problems are associated with hospital admittances, mortality and lower limb amputation worldwide[2]. In diabetic population the prevalence of DFU is 4-10% and this situation is more prevalent in older patients[3]. Around 5% of all patients with diabetes have a history of foot ulceration, while the lifetime threat of diabetic patients developing DFU[4] is 25%.

Several studies have investigated the relationship between the types of infections and the number and types of organisms recovered from DFU wounds. Some researchers reported that acute Diabetic Foot Infections (DFI) are usually triggered by Gram-Positive cocci, but chronic wounds often inhabit aerobic Gram-negative bacteria and the flora is often polymicrobial[5-7] but the prevalence of Gram-negative bacteria is dominant[8]. A study carried out in India reported that *Pseudomonas aeruginosa* and *Staphylococcus aureus* biofilm forming Gram-Negative are the dominant bacterial isolates recovered from DFU patients[9].

Microbes associated with DFU patients became resistant to many antibiotics leads to prolong the stay of patients at hospital will increase management costs, bring additional morbidity and in certain cases lead to greater mortality. Several factors are involved in the development of Multi Drugs Resistant (MDR) nature in many DFU related bacteria are due to production of Extended Spectrum Beta-Lactamase (ESBL), Metallo Beta-Lactamase (MBL) and AmpC[10-13]. These beta-lactamases harbor resistant to different groups of antibiotics. As most of the pathogenic have adopted beta-lactamases and also other factors like outer membrane protein and efflux pumps contribute to MDR nature. Beside beta-lactamases many bacterial species express virulence factors like capability of biofilm formation, hemolysin, protease, lipase, amylase activities and capsule formation that are responsible for long stay of infections. Due to these factors such infection may prolong their span and will help in dissemination of pathogenic strains in hospitals and community. As a result, it creates problems for clinicians in patients treatment and will also limit treatment choices with presently available antibiotics[14,15].

The current investigations were conducted to characterize beta-lactamase producing bacterial species isolated from DFU patients and their association with virulence factors. As it is presume there may be strong correlation between resistance and expression of virulence factors and as result it will lead to invasion and spread of infections in DFU patients. Additionally such pathogens will pose serious concerns for clinicians as it will complicate treatment of such patients.

MATERIALS AND METHODS

Bacterial culturing: This work was conducted on bacterial isolates that have been screened from DFU patient samples admitted at Pakistan Institute of Medical Sciences (PIMS), Pakistan during year May, 2015 to June, 2016. All these bacterial isolates were already characterized and their antibiogram profile was also analyzed. Isolates preserved in glycerol stock were cultured for further studies both liquid and solid media. All the chemicals and media were purchased from Oxide UK.

Detection of beta-lactamases: Beta-lactamases are the main mechanisms through which bacteria pose resistance to various drugs. All these DFU isolates were subjected for detection of different types of beta-lactamases using appropriate methods.

Extended-Spectrum Beta-lactamase (ESBL): Double disk synergy test was performed for the phenotypic detection of ESBL production. Briefly, 50 µL of culture was mixed with 1 mL of distilled water and inoculated on Mueller Hinton Agar-plates (MHA). In this process Antibiotic discs of amoxicillin/clavulanic acid (AMC 20/10 µg), cefotaxime (CTX 30 µg), ceftriaxone (CRO 30 µg), aztreonam (ATM 30 µg) and ceftazidime (CAZ 30 µg), were placed at a distance of 15 mm apart from each other and zone of inhibition were measured[16]. All the antibiotic disks were purchased from Oxide UK.

AmpC disc test: AmpC test was done for the detection of Plasmid-Mediated AmpC beta Lactamases. MHA was inoculated with bacterial suspension using standard disc diffusion method. A Cefoxitin (FOX 30 µg) disc was hydrated with a drop of Tris- EDTA and air dried. This disc and a blank of 30 µg FOX disc was placed on MHA media at a distance of 15 mm from each other and incubated for overnight and results were recoreded[17].

Metallo beta-lactamase (MBL) test: Test organisms were inoculated on MHA and two Imipenem (IMP 30 µg) discs were placed on the plate and then 10 µL EDTA solution was poured on one disc for the formation of IMP-EDTA. The

inhibition zones of IMP and IMP-EDTA discs were compared after 16-18 h of incubation. If zone of inhibition of IMP disc is more than 7 mm and also more than IMP-EDTA, the results were considered positive[18].

Molecular characterization of CTX beta-lactamase gene: DNA extraction was done by using chloroform phenol method[19]. The extracted genomic DNA was confirmed on agarose gel. Already used primers were used for detection of CTX beta-lactamase gene and PCR amplification of the gene was performed using the designed primers. PCR product was run on 1.5% agarose gel along with a ladder of 1 kb as a marker. After running for appropriate time the gel was placed under gel documentation system for analysis[20].

Detection of virulence factors: After completion of beta-lactamases characterization, these isolates were studied for virulence factors like biofilm formation, protease, lipase, amylase, hemolysin and bacterial capsule formation. For the detection of virulence factors selected media were used and the isolates were inoculated on these media. Briefly, the biofilm formation was detected by using the modified method used by Stepanovic *et al.*[21] and protease, lipase and amylase were detected in 1.5% agar media of 1% skimmed milk, 1% tributyrin and 2% starch by dot inoculating the selected isolates on these media and zones of clearance after incubation were observed. Hemolysin was detected using 5% sheep blood agar and after incubation clear zone of lysis producing isolates were considered positive while for capsule formation, pure culture of each bacterium were inoculated on a slide, mixed with nigrosin and methylene blue for 2 min and observed under light microscope. The nigrosin stain provides a dark color to unstained capsule and methylene blue stain provides blue color bacteria[22].

RESULTS

Isolation and identification: From the glycerol stock bacterial species were sub cultured for further analysis. These isolates were dominated by *P. aeruginosa* (57.5%) followed by *E. coli* (25%) and *K. pneumoniae* (10%) as shown in Table 1.

Results of this study revealed the production of different beta-lactamase in these isolates as shown in Table 2. In studied isolates 59.5% were analyze positive for three types of beta-lactamases. Among them *P. aeruginosa* (n = 12), *E. coli* (n = 16) and *K. pneumoniae* (n = 15) were found positive for beta-lactamase production. The AmpC beta-lactamase were detected in 72.5% isolates while remaining isolates were consider negative for AmpC production. Their prevalence were as (n = 17, 8, 4) for *P. aeruginosa*, *K. pneumoniae* and *E. coli*, respectively. Similarly, all isolates were evaluated MBL beta-lactamase production and 67.5% isolates were consider as MBL positive. MBL predominantly were found in *P. aeruginosa*. Among different bacterial isolates, *E. coli* had the highest prevalence of ESBL production while *P. aeruginosa* had high prevalence of MBL production

Table 1: Percentages of Gram-negative bacterial species isolated from DFU patients

Bacteria isolates	Number	Percentage
Pseudomonas aeruginosa	23	57.5
Escherichia coli	10	25.0
Klebsiella pneumonia	4	10.0

Table 2: Production of ESBL, AmpC and MBL in bacterial species isolated from DFU patients

Bacteria species	ESBL positive	AmpC positive	MBL positive
Pseudomonas aeruginosa	-	-	+
Pseudomonas aeruginosa	-	+	+
Pseudomonas aeruginosa	-	-	+
Escherichia coli	+	+	+
Pseudomonas aeruginosa	+	+	+
Klebsiella pneumonia	+	+	-
Pseudomonas aeruginosa	+	+	+
Pseudomonas aeruginosa	+	+	+
Pseudomonas aeruginosa	+	+	-
Escherichia coli	-	-	+
Pseudomonas aeruginosa	+	+	+
Pseudomonas aeruginosa	+	+	+
Klebsiella pneumonia	-	-	-
Pseudomonas aeruginosa	-	+	-
Klebsiella pneumonia	+	+	-
Escherichia coli	+	+	+
Escherichia coli	+	+	+
Pseudomonas aeruginosa	-	-	+
Pseudomonas aeruginosa	+	-	+
Pseudomonas aeruginosa	+	-	+
Pseudomonas aeruginosa	+	+	+
Pseudomonas aeruginosa	-	+	-
Escherichia coli	+	+	+
Escherichia coli	+	+	+
Escherichia coli	+	+	+
Escherichia coli	+	+	+
Pseudomonas aeruginosa	+	+	+
Pseudomonas aeruginosa	-	+	-
Pseudomonas aeruginosa	-	+	-
Escherichia coli	+	-	+
Pseudomonas aeruginosa	-	+	-
Pseudomonas aeruginosa	+	+	-
Pseudomonas aeruginosa	+	+	+
Pseudomonas aeruginosa	-	+	-
Pseudomonas aeruginosa	-	-	+
Escherichia coli	-	+	-
Klebsiella pneumonia	-	+	+

+: Isolate that exhibit beta-lactamase, -: Isolate that exhibit no beta-lactamase

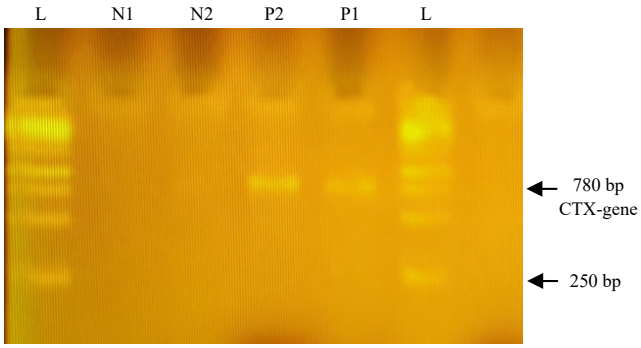

Fig. 1: PCR amplified product of CTX gene having size of 780 bp compared with 1 Kb ladder
L: Ladder, P1: Positive control, P2: Positive sample, N1: Negative control, N2: Negative sample

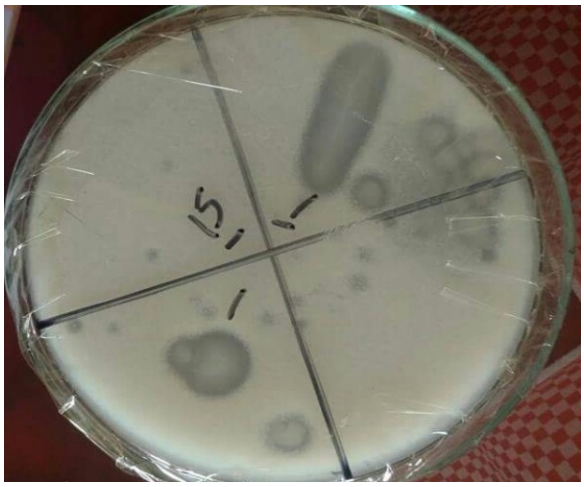

Fig. 2: Zone of hydrolysis of some isolates on skimmed milk agar

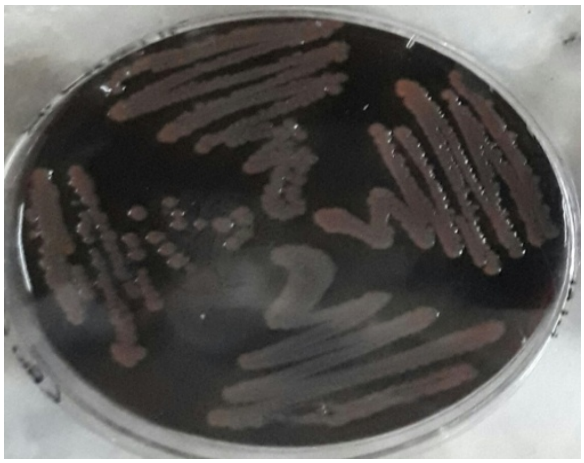

Fig. 3: Biofilm producing bacterial growth on Congo red media

Table 3: Percentage of isolates producing different virulence factors in DFU patients

	Isolates (%)		
Virulence factors	*P. aeruginosa*	*E. coli*	*K. pneumoniae*
Protease	83	70	75
Biofilm formation	74	50	100
Hemolysin	60	80	0
Amylase	87	80	50
Lipase	70	40	25
Capsule formation	100	100	100

as shown in Table 2. In *P. aeruginosa* (n = 8) isolates coproduce all 3 types of beta-lactamase while (n = 4) *E. coli* revealed coproduction of beta-lactamases. Similarly (n = 2) isolates of *P. aeruginosa* harbored both AmpC and ESBL phenotype while some isolates of *K. pneumonia* coproduce both ESBL and MBL beta-lactamase as shown in Table 2.

CTX beta-lactamase gene detection: Phenotypically ESBL/MBL/AmpC positive strains were subjected for CTX beta-lactamase genotype. CTX beta-lactamase gene was amplified in 62% of the isolates. A band of approximately 780 bp was observed while some isolates didn't show any appearance of bands means lacking CTX gene as shown in Fig. 1. Overall most of the isolates harbor CTX beta-lactamase gene it may be responsible for the paramounting resistance in bacterial isolates. Those that did not exhibit CTX gene may have other genotypes which need further screening of these isolates.

Virulence factors characterization: All beta-lactamase producers strains were screened for the expression of virulence factors. The virulence factors were characterized in three different kinds of isolates i.e., *P. aeruginosa*, *E. coli* and *K. pneumonia*.

Protease production: For protease production 83% *P. aeruginosa*, 70% *E. coli* and 75% *K. pneumonia* were positive as shown in Table 3. The positive isolates form clear zone of hydrolysis on skimmed milk agar as shown in Fig. 2.

Biofilm formation: The isolates were grown on Congo red media for biofilm formation which revealed that 74% *P. aeruginosa*, 50% of *E. coli* and 100% *K. pneumoniae* were positive as shown in Table 3. The positive isolates make darker colonies on Congo red media as shown in Fig. 3.

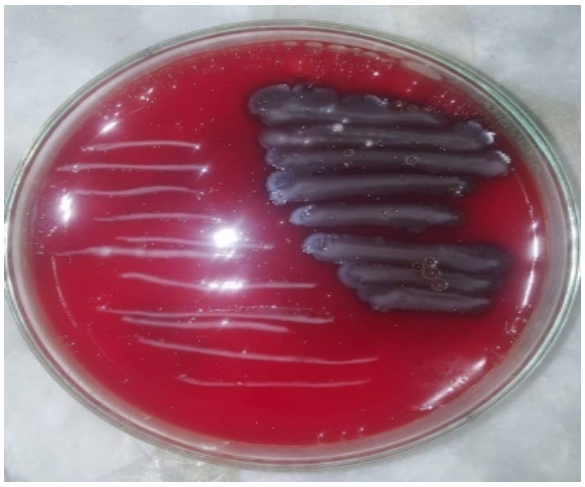

Fig. 4: Hemolytic activity on blood agar media

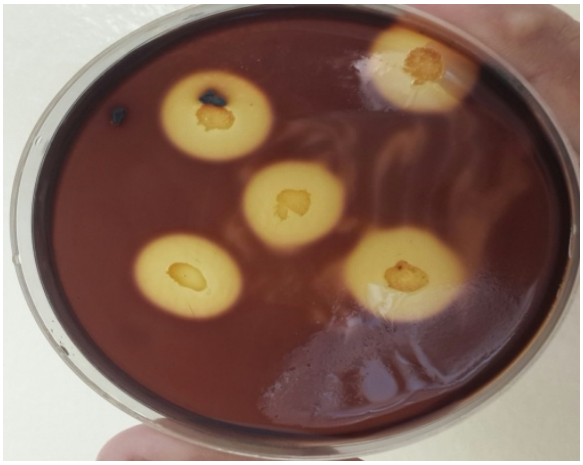

Fig. 5: Zone of hydrolysis on starch and tributyrin media by isolates from DFU patients

Hemolysin activity: For hemolysin 60% *P. aeruginosa*, 80% of *E. coli* and 0% *K. pneumoniae* were observed to be positive as shown in Table 3. Those isolates that were carrying hemolytic activity, shown hemolysis of red blood cells as shown in Fig. 4.

Amylase and lipase activities: For amylase activity 87% *P. aeruginosa*, 80% *E. coli* and 50% *K. pneumoniae* were observed positive while for lipase 70% *P. aeruginosa*, 40% *E. coli* and 25% *K. pneumoniae* were positive as shown in Table 3. Amylase activity has been confirmed by hydrolysis of starch as shown in Fig. 5.

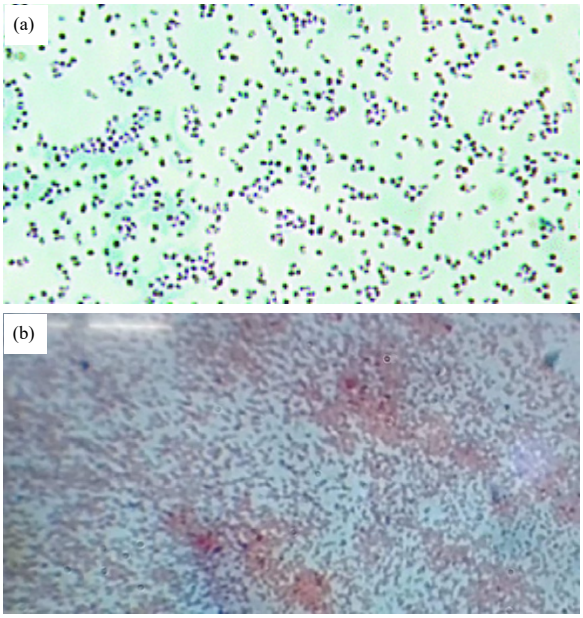

Fig. 6(a-b): (a-b) Capsule staining showing bacterial isolates forming capsules

Capsule formation: By using negative staining with Indian ink, direct examination of capsule under 100X microscope was done which showed that all *P. aeruginosa*, *E. coli* and *K. pneumonia* were capsulated bacterial species as shown in Fig. 6.

DISCUSSION

In the present study (n = 37) Gram-negative bacterial species were characterized that were isolated from DFU patients. In all isolates the most dominant strains were *P. aeruginosa* (n = 23) followed by *E. coli* (n = 10) and *K. pneumoniae* (n = 04), respectively. Foot ulcer and lower limb amputations bring considerable morbidity and mortality in patients having complaint of diabetes. Diabetic foot ulcer is a serious threat around the globe[16]. Bacterial infections are most common in developing countries and serious concerns are there for such infections. As in India the most of isolated bacterial species from DFU patients were characterized as Gram-negative pathogens. It revealed that most dominant bacterial species were *S. aureus* followed by *P. aeruginosa* suggested these two species are often found in DFU patients[17]. In most cases DFU patients confront Gram-Negative bacterial pathogens mostly comprise of *Pseudomonas aeruginosa*, *E. coli* and *S. aureus*. It has been acclaimed from recent findings that DFU patients are the easiest target of nosocomial pathogens[11,19,23].

The production of beta lactamases is an important feature of drug resistance in many bacteria. In recent findings production of ESBL, AmpC and MBL beta-lactamase were there in most of the studied isolates. But similar incidence of bacterial infections were reported in DFU patients but overall the prevalence of ESBL were lesser as compared to these findings. This indicated a slight increase in resistance pattern in bacterial pathogens[24]. But in another case the prevalence of beta-lactamases were in line with current findings which suggest there may be some risk factors which can help in establishing of such infetions[12]. In *P. aeruginosa* the incidence of MBL were higher in as compare to findings of this study which show high resistance to carbapenems drugs[25]. Higher production of ESBL and AmpC were reported in our case as compared to the previous findings[26]. The production of different number of ESBL, AmpC or MBL may be due the result of different environment, selection of specimens and use of different antibiotics. CTX gene has also an important role in the resistant behavior of microbes particularly against cefotaxime and ceftazidime[27]. Findings of current study are in correspondence with a study of Chaudhry *et al.*[11], who have amplified different antibiotic resistant genes including CTX from DFU patient's microbial flora.

The virulence factors play key role in bacterial pathogenesis. In most cases expression of virulence factors along with drug resistance increase infections in DFU patients. Virulence factors like biofilm formation, haemolysin, protease, amylase, lipase and capsule formation were uncover in most of studied isolates. *Pseudomonas aeruginosa* were found strong biofilm producers and this capability provides extra strength to microbes to withstand the effect of drugs. Similar study was conducted on MDR *P. aeruginosa* where biofilm was found one of the key factors in strengthening of their resistant behavior[28]. Similarly Protease, lipases, haemolys in have important role in establishing of bacterial infections in DFU patients and all these virulence factors were been found in isolates that were being screened from individuals that have complaint of DFU. Their findings are in agreement with recent results which stipulate that expression of virulence factors has strong potential to halt the healing process in infected individuals. Most of resistance strains nurture more than one virulence factors and combination these factors lead to complication of infections. Lipase and haemolysin are also regarded as main factors that contribute in pathogenesis of MDR isolates[29].

CONCLUSION

From this study it is deduce that MDR *P. aeruginosa* are most prevalent in DFU patients. After screening it has been established that ESBL are most common beta-lactamases producers in these isolates. ESBL producers were common in *E. coli* while MBL were dominantly found in *P. aeruginosa*. CTX beta-lactamase were detected in 62% of the isolates which revealed that these isolates have strong potential to inhibit action of ceftriaxone. Biofilm were strongly associated with *P. aeruginosa* and *K. pneumonia* while protease activity was shown by all Gram-negative pathogens. Similarly other virulence factors have strong association with *E. coli, K. pneumoniae*. The expression of virulence factors by these pathogens may lead to increase host damage and pathogenesis. The results of the present study may provide useful insights for developing new drugs to minimize beta-lactamases mediated resistance problem DFU patients.

SIGNIFICANCE STATEMENT

Current study revealed emerging situation and management of MDR pathogens in DFU patients. As Most of the pathogens exhibit the production of beta-lactamases and some isolates coproduce more than one beta-lactamase showed strong resistant nature of these bacteria. It insists that proper diagnosis may be maintained before prescription of any antibiotics to patients. As biofilm have strong potential to withstand the effect of drug, most pathogens were found as biofilms producer. To avoid spread of such pathogens in hospitals and community proper hygiene of DFU may be adopted. Prescription should be done after proper diagnosis so that dissemination of such pathogens may be discouraged.

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