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Study on Bio-film forming bacterial pathogens in diabetic foot ulcer

Sharnita Nadella¹, Dr. Sheetal Harakuni²

¹MBBS Student, Jawaharlal Nehru Medical College, KAHER University, Belagavi 590010

²Professor, Department of Microbiology, Jawaharlal Nehru Medical College, KAHER University, Belagavi 590010

Abstract:

Purpose: Biofilms refer to a group of microorganisms that adhere irreversibly to a surface. Biofilms increase the chronicity of infection and are not penetrable by antibiotics. Strategies preventing biofilm formation in a diabetic foot ulcer patient may help in early healing and cure from infection; along with preventing the severe complications that arise from these infections.

Method: The ulcer was cleaned from slough; pus was collected using sterile swab. Gram staining was performed. Samples were inoculated on Blood agar and Mac Conkey medium. Predominant colonies were identified and antibiogram was performed as per standard protocol. Biofilm production by isolated colony was measured by microtiter spectrophotometric method.

Result: 32 patients with chronic diabetic foot were included in the study. From these patients, 25 isolates were processed. Three samples grew no bacteria; 4 grew skin commensals, on culture. 32 percent of the isolates were biofilm-producing by the microtiter plate method. *Staphylococcus aureus* was the predominant pathogen producing biofilm followed by *Escherichia coli*. Gram positive isolates showed maximum resistance to doxycycline and gram negative isolates to ceftazidime and gentamycin. **Conclusion:** Biofilm provides a media enabling normally antibiotic sensitive bacteria to exhibit resistance against the same antibiotics. Gram negative bacteria (*Escherichia coli* predominantly) and *Staphylococcus aureus* among the gram positive bacteria isolated were identified as predominant biofilm forming isolates.

Keywords: Diabetic foot ulcer, antibiotic sensitivity resistance, biofilm

*Author for Correspondence. E-mail: sharnitanadella8@gmail.com

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Introduction:

A biofilm consists of any syntrophic consortium of microorganisms resulting in cells sticking to each other as well as a surface. Extracellular polymeric substances comprise a slimy extracellular matrix in which are found adherent, the cells of a biofilm [1]. A biofilm is formed by microbes in response to quorum sensing triggered by sites on a surface. Antibiotics fail to penetrate the biofilm and the chronicity of infection is increased by the formation of biofilms [2]. This greatly limits the healing and changes the mode of treatment.

Amid over 62 million diabetics in India, over 20% develop a diabetic foot ulcer (DFU), over half of which get infected and about 20% require amputation and hospitalization.[3]

India is near the top of the list of the most expensive countries for the treatment of diabetic foot ulcers in the world concerning the income of an average patient.

The identification of bacterial pathogens most commonly found in a diabetic foot, their antibiotic susceptibility and virulence factors, may help doctors to treat the infection at hand and shorten the timeline of the treatment significantly.

Hence the present study was undertaken to identify the prevailing pathogens in cases of chronic diabetic foot ulcers along with the antibiotic susceptibility patterns. The study intended to identify the biofilm-forming pathogens so that it can be assigned as a virulence trait in pathogens of DFU. Diabetes mellitus is a metabolic disorder with deranged carbohydrate metabolism. Like all humans, diabetics are prone to injuries; however in diabetics, contaminating bacteria establish themselves in the open wounds due to higher blood sugar levels and microangiopathy.[4] Diabetic foot ulcer is the most common complication of uncontrolled diabetes. [5]

Gram-positive organisms such as *Staphylococcus aureus*, *Enterococcus*, and gram-negative organisms such as *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella*, *Proteus* species, and anaerobic organisms cause infection in ulcers of diabetic patients. They also exhibit multidrug resistance. There is great range of possibility of further infection caused by organisms colonizing the wound surface [6].

This wide variety of microorganisms may occur as a unit or exist independently thereby leading to the formation of micro-communities that exist within a matrix of extracellular polymeric substances called biofilms. [7]

The ability to form biofilms is a virulence factor of microorganisms of great importance as it contributes to the chronicity of a diabetic ulcer and enables the organisms to survive against antibiotics while maintaining a suitable environment for them.

It also establishes a way for the emergence of multidrug-resistant strains and causes failure in the treatment process. The microorganisms forming biofilms are difficult to treat with antibiotic therapy which implies that identifying factors that cause biofilm formation allows for better management of the infections caused in diabetic foot patients in whom treatment fails despite repeated use of antibiotics as a result of infrequent testing of biofilms. [8]

In most cases of diabetic foot infections (DFIs), patient is hospitalized and administered with broad spectrum antibiotics for treatment along with immediate surgical intervention. It is an absolute necessity to perform regular microbiological background checks to ensure that adequate antibiotic dosage and coverage is being provided. This helps detect antibiotic sensitivity pattern of the DFI, the pathogens within it and facilitates the provision of adequate treatment [9].

Detection of biofilm:

The biofilm production can be detected by phenotypic and genotypic methods. The phenotypic methods are reliable methods for characterization of biofilm production. [10-19] Screening for biofilm formation, along with the usual antibiogram needs to be performed as an absolute necessity as a routine procedure which helps to develop effective treatment strategies for patients with chronic diabetic foot ulcers. Debridement of wound regularly and stringent antibiotic use also needs to be established. This results in a decrease in recurrences and various complications of the same.

Materials and Methods

The research work was carried out in the Department of Microbiology of Jawaharlal Nehru Medical College, KAHER. Thirty-two patients with chronic diabetic foot were included in the study. The study group comprised of 25 males and 7 females. The patient's age ranged from 28-71 yrs. The study was conducted over a period of one month. This study was approved by the Indian Council of Medical Research (ICMR) and follows all ethical standards of ICMR and the institution.

Processing of specimens: The details about the demographic details of the patient, duration of diabetes, treatment, stages of the ulcer were documented. Samples were taken from patients with diabetic foot ulcers between grade 3 and grade 4. The ulcer was cleaned from the slough. The base of the ulcer was pressed; fresh exudate that oozes out was collected on a sterile swab. The swabs were transported to Laboratory. Gram staining was performed. Samples were inoculated on Blood agar and Mac Conkey medium. The predominant colony was processed as per the standard protocol^[20].

Biofilm Assay:

Biofilm production by an isolated colony was measured by the microtiter spectrophotometric method [21]. The cultures were sub cultured to obtain the isolated colonies. The isolated colony was emulsified in 1 ml of saline. Then the emulsion was diluted in the trypticase soy broth, 1:10. 300µL of the diluted broth was pipetted into flat bottom sterile microtitre plate well. Each isolate was tested in triplicates. The plates were incubated aerobically at 37°C for 24hrs. After incubation, the content of wells was poured off. The wells were washed thrice with sterile distilled water. To fix the biofilm produced in every well, it was filled with 250 µl of methanol after which it was drained off. The wells were stained with 250µl of 1% solution of crystal violet for five minutes. Excess stain was removed, air-dried, and dye was solubilized with 250µl of glacial acetic acid. The optical density (OD) was measured in an ELISA reader with a 490 nm filter. The cut-off

OD was determined as three standard deviations above the mean OD of the negative control. Based on the results isolates were designated as biofilm producers and non-biofilm producers [22].

Antibiotic sensitivity testing was performed on Mueller Hinton agar by Kirby Bauer disc diffusion method. Gram-positive isolates were tested for sensitivity against erythromycin, clindamycin, cotrimoxazole, Doxycycline, penicillin, ampicillin, gentamycin, oxacillin/ colistin, Ceftazidime. Gram-negative bacteria were screened for sensitivity against Tobramycin, gentamycin, ceftazidime, amoxiclav, imipenem, amikacin, piperacillin-tazobactam, and doxycycline.

Data was entered in Microsoft excel and are expressed in descriptive statistics, percentages. The reports were intimated to the clinician. The study subjects were the hospitalised patients, who underwent debridement and adequate treatment for foot ulcer. The patient recovery was followed during their stay in hospital.

Results

A total of 32 patients were included in the study. The predominant pathogens were isolated only in 25 samples, whereas 3 samples showed no growth and 4 samples grew skin commensals. The isolates were found to be *Staphylococcus aureus* (32%), *Escherichia coli* (24%), *Pseudomonas aeruginosa* (12%), *Proteus mirabilis* (8%), *Klebsiella pneumoniae* (8%), *Citrobacter freundii* (8%), *Acinetobacter sp* (4%), *Enterococcus species* (4%). The isolates were labelled as biofilm forming if the average optical density (OD) was equal to or more than OD of negative control +3 standard deviation. 32% of the isolates were biofilm-producing by the microtiter plate method. *Staphylococcus aureus* was the predominant pathogen producing biofilm followed by *Escherichia coli*, *Proteus mirabilis*, and *Pseudomonas aeruginosa*. Whereas *Citrobacter freundii*, *Klebsiella pneumoniae*, *Acinetobacter sp*, *Enterococcus* were identified as non-biofilm producers (Table 1).

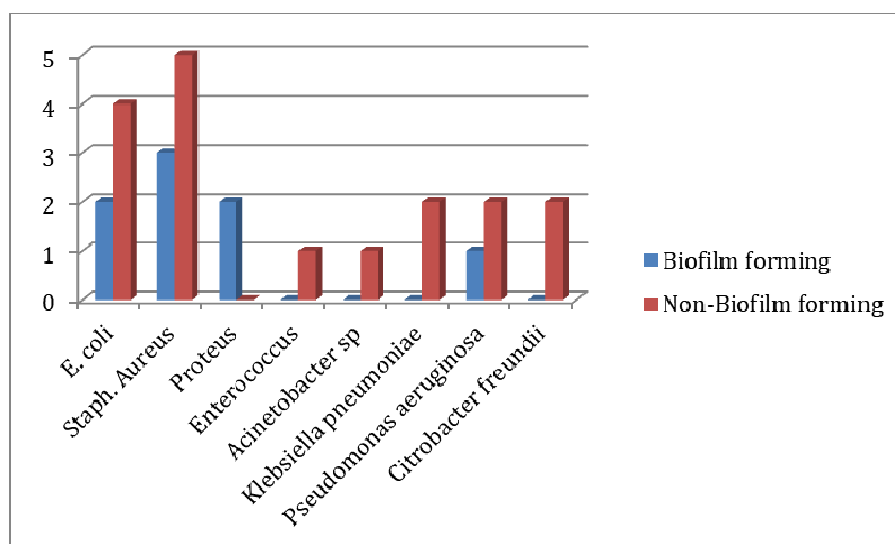
Table 1: Biofilm characteristics of isolates tested in the study

ISOLATES	BIOFILM PRODUCERS	GRAM STAIN
<i>Staphylococcus aureus</i> (32%)	+	POSITIVE
<i>Enterococcus species</i> (4%)	-	POSITIVE
<i>Escherichia coli</i> (24%)	+	NEGATIVE
<i>Pseudomonas aeruginosa</i> (12%)	+	NEGATIVE
<i>Proteus mirabilis</i> (8%)	+	NEGATIVE
<i>Klebsiella pneumoniae</i> (8%)	-	NEGATIVE
<i>Citrobacter freundii</i> (8%)	-	NEGATIVE
<i>Acinetobacter sp</i> (4%)	-	NEGATIVE

Gram positive isolates were sensitive to clindamycin and cotrimoxazole, whereas they were resistant to erythromycin (62%), doxycycline (77%), penicillin (66%), ampicillin (66%), gentamycin (33%), ceftazidime (66%). 33% of *Staphylococcus aureus* were methicillin resistant. Gram negative bacteria were resistant to tobramycin (62.5%), gentamycin (81%), ceftazidime (81%), amoxiclav (50%), imipenem (50%), amikacin (37.5%), piperacillin-tazobactam (31.2%), and doxycycline (18.7%).

Resistance forming pattern amongst biofilm producing isolates is variable among organisms and can be caused by various factors. Factors aiding the antibiotic resistance exhibited by biofilm forming organisms include oxygen and nutrient depletion due to biofilm formation and possession of biofilm specific antimicrobial resistance genes. [13,14,15,16]

Fig.2: Comparison of biofilm-forming clinical isolates



Discussion

A diabetic foot ulcer is the commonest complication of diabetic patients. The infection of these ulcers adds up to the non-healing complication. Thus the study was carried out to determine whether biofilm production is one of the virulence factors exhibited by DFU isolates. This would add to the diagnostic tool to effectively treat DFU.

The study was carried out in a period of one month and over the short period of study; the observations that are analysed are comparable with the similar type of studies conducted on biofilm-producing isolates from diabetic foot ulcer patients.

Of the isolates 64% were gram-negative, and 36% were gram-positive. This corresponds to the findings of other investigators [23].

In our study *Staphylococcus aureus* is the most predominant gram positive biofilm forming isolate which exhibits maximum resistance to doxycycline followed by penicillin, ampicillin and ceftazidime. *E. coli* and the 2 isolates of *Proteus mirabilis* were also found to be biofilm-forming, which is similar with the outcome of the study done by Raja (2007) [24]. *Proteus mirabilis* and *Pseudomonas* are the biofilm forming gram negative isolates that exhibit maximum resistance to gentamycin, ceftazidime and tobramycin. Biofilm formation is a tenacious character of a bacterium and can be considered as a virulence factor. Antibiotic sensitive bacteria behave as resistant when they survive in a biofilm. Hence, the biofilm provides a media enabling normally antibiotic sensitive bacteria to exhibit resistance against the same antibiotics. This does not occur due to the gain of any virulence factors but as a result of the formation of biofilms with bacteria exhibiting quorum sensing to adequately establish their growth on the wound surface.

Proper treatment of ulcer avoiding biofilm formation may bring about the healing of chronic DFU. Further, culture for bacterial pathogen would help to identify factors that enable the cessation of biofilm formation on wound surfaces. The limitation of the study is the small sample size, findings to be confirmed by a large group of isolates.

Conclusion:

In our study Gram negative bacteria are predominating pathogens isolated. *Escherichia coli* was found to be the predominant gram-negative biofilm forming isolate and *Staphylococcus aureus* was found to be the predominant gram-positive biofilm-forming isolate. The use of more niche antibiotics with the help of adept and regular antibiotic sensitivity testing of microbiota is an important factor which if implemented can result in faster healing while simultaneously decreasing the recurrence of infections.

Conflict of Interest: There are no conflicts of interest to declare.

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