



A Study on Biofilm Production and Its Association with Chronicity in Isolates Obtained From Various Types of Ulcers

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Abstract

*Ulcers pose a significant affliction to our health care system. The prevalence of chronic ulcers in Indian population is much higher than that reported in the Western population. The healing of ulcers is affected by multiple systemic and local factors, among which biofilm formation by the pathogens is a therapeutic challenge. The present study was undertaken for a period of 1 year from February 2015 to find out the biofilm production status of the isolates & to determine any association between biofilm production by the isolates and chronicity of the ulcer. In the present study, 66.9% of the total isolates from various types of ulcers were biofilm producers. ³⁷Gram positive bacteria were better biofilm producers in our study. Most frequent biofilm producers among Gram positives were *Staphylococcus aureus* & among Gram negatives it was *Pseudomonas aeruginosa*. 74% of isolates from ulcers of duration of >3 months were biofilm producers which was found to be a statistically significant association.*

Keywords: ulcers, biofilm, chronicity.

Introduction

Ulcers are defined as wounds with a “full thickness depth” and a “slow healing tendency” which mainly occurs by endogenous mechanisms associated with predisposing conditions like Diabetes, venous insufficiency. In ulcers there is complete loss of the epidermis, portions of the dermis and even subcutaneous fat. Chronic ulcers are defined as wounds that have failed to proceed through the orderly process that produces satisfactory anatomic and functional integrity or that have proceeded through the repair process without producing an adequate anatomic and functional result¹⁻⁵. Major types of chronic ulcers include. Diabetic foot ulcers,

Venous ulcers & Pressure sores/Decubitus ulcers⁶. Healing of an ulcer is a dynamic process & is mediated by interactive reactions of parenchymal cells, soluble mediators, blood elements, and extracellular matrix. This normal course is affected by various systemic as well as local factors which delays the healing and determines the chronicity of an ulcer⁷⁻¹².

Another important mechanism by which bacteria interfere with the healing process by forming **biofilms**. Biofilms are bacterial populations that are enclosed in a matrix of extracellular polymeric substances (EPS). Biofilms may form from the accumulation of a single bacteria (monomicrobial

aggregation) or from the accumulation of numerous species (polymicrobial aggregation)¹³. Chronic ulcers provide an ideal environment for biofilm formation. The necrotic tissue & debris present in chronic ulcers allow bacterial attachment which form biofilms.¹⁴ Biofilms in ulcers produce an inflammatory response resulting in an infiltration of neutrophils & macrophages surrounding the biofilm. It is hypothesised that the initiated inflammatory response is in favour of biofilm. By inducing an ineffective inflammatory response, the biofilm protects the bacteria contained in it and the increased exudate production serves as a source of nutrition, thereby helping to perpetuate the biofilm³⁵.

The resistance of biofilm producing bacteria to antimicrobial agents starts from the attachment phase and increases with the development of the biofilm. The mechanisms involved include the following

1. Blocking-the matrix acts as a diffusion barrier to smaller molecules like antimicrobial agents.
2. Mutual protection- Different species of bacteria which in a biofilm can confer protection to each other.
3. By transfer of genes that confer antibiotic resistance between bacteria of same or different species.
4. Hibernation- another strategy of bacteria within the biofilms is going in to a state of hibernation or remaining quiescent. Most of the antimicrobial agents target on rapidly growing bacteria. Therefore hibernating bacteria in biofilms are unaffected by antibiotics. In the presence of antibiotics this antibiotic resistant strains will be selected out, which will multiply to become the dominant type among the bacterial population.

Thus, the treatment of ulcers infected with biofilm producing bacteria is a therapeutic challenge. The non-healing of such ulcers affect the patient adversely, both socially as well as financially.

Relevance

Epidemiological data available from the studies shows that the prevalence of chronic ulcer in Indian

population is much higher than that seen in the Western population. In our institution also chronic ulcers is a significant burden. 44% (377/849) of the total samples from the surgery department is from ulcers.

Biofilm formation by bacteria is an important feature in chronic ulcers. The biofilms prevent phagocytosis; they also act as diffusion barriers to penetration of antibiotics which contribute to the drug resistance exhibited by the infecting bacteria & can adversely affect the treatment. Therefore, the presence of bacterial biofilms in chronic ulcers may help us to explain the chronicity of ulcers and also, why an ulcer doesn't heal despite adequate antibiotic treatment as well as treatment for the underlying condition.

The morbidity & mortality associated with such chronic ulcers can be dramatic. It is also associated with a high cost of healthcare, loss of productivity, and reduced quality of life and for a developing country like India this will cause severe health care burden. Therefore, prompt and appropriate management of the condition is needed to alleviate the consequences which can occur following a non-healing of an ulcer; both medical as well as social. Studies on biofilm formation will give way to new arenas of research and may help us to develop new treatment modalities for tackling the same.

Objectives

1. To assess biofilm production of pathogenic bacteria associated with different types of ulcers.
2. To determine association between biofilm production and duration of ulcer.

Materials & Methods

Study Design: Descriptive Study (Prevalence study)

Study Setting: Department of Microbiology & Department of Surgery

Study Period: 1 Year from January 2015/all samples collected during this period.

Sample Size: In a study done by Rahim et al to study the antimicrobial resistance among bacterial pathogens isolated from chronic ulcers in a tertiary

care hospital in Pakistan, 50.9% of the ulcers were positive for bacterial isolation. We had calculated sample size for our study using this prevalence in the formula $N=4pq/D^2$
 $P= 50.9\%$ $q=49.1\%$.

Using an absolute precision (D) of 10%, sample size was calculated to be **99**. We had included all patients admitted in surgical wards with ulcers during the study period. Even though the minimum sample size calculated for our study was 99, we had included all 103 patients meeting the inclusion criteria, in our study.

Study Population: All patients admitted with ulcers in the surgical wards were included in the study.

Inclusion Criteria: A patient with ulcer was included only once during the period of study

Exclusion Criteria: Patients with malignant ulcers and ulcers associated with burns and post-operative wound infections were not included in the study.

Sampling Methodology: Universal sampling

Ethical Clearance: Obtained from the Institutional Ethical Committee before commencement of the study (IEC 11/2014).

Sample collection and Transport

Several studies have demonstrated significant correlation between swab cultures & biopsy specimen cultures in patients with ulcers.^{16,17} Hence we adopted swab cultures for our study.

Surrounding skin of the ulcer was disinfected with **alcohol swabs**. Ulcer area was thoroughly cleaned with sterile saline. Two sterile swabs were used for each patient.^{18,19} Samples were collected by making firm rotatory movements, covering entire area of the ulcer, holding both the swabs together. The swabs were then transported to the laboratory in **sterile test tubes** and processed within 2 hours.²⁰⁻²³

Processing of the specimen

Swabs with the specimens were inoculated on to **culture media** by streak culture method & thioglycollate broth. Identification of the isolates up to species level was done as per standard microbiological methods. Antibiotic sensitivity

testing was done for the above isolates using Kirby – Bauer Disc Diffusion technique according to standard microbiological methods. All the above isolates were subjected to the test for biofilm detection. We had chosen the Tissue culture plate method for detecting biofilm production by the isolates.

Tissue culture plate method

- Bacterial isolates were sub-cultured on to nutrient agar.
- Incubated the same for overnight at 37⁰C
- From the growth on nutrient agar obtained after overnight incubation a loopful of the isolate was inoculated into Trypticase soy broth with 1% glucose.
- The broth was incubated at 37⁰ C for 24 hours
- After incubation 1 ml of broth was diluted in 100ml of sterile un-inoculated TSB with 1% glucose
- 0.2ml from the diluted broth culture was transferred into each well of 96 well sterile flat bottom tissue culture plate (Nest, Tarson).
- 0.2 ml each of positive and negative control (treated like the isolates) were also included every time the test was done.
 - Positive control used was Klebsiella spp & negative control was coagulase negative staphylococci
 - The controls used were obtained from a pilot study done on bacterial isolates from ulcers. These were tested for biofilm production repeatedly and the results were consistent. Hence, we had used these as the positive & negative controls for each test batch
- 0.2 ml of un-inoculated sterile TSB with 1% glucose was inoculated into the well of each plate, which served as the control to check sterility and non-specific binding of the media.
- Tissue culture plates were covered with the lid provided & incubated at 37⁰C for 24 hours.
- After 24 hours broth in the plates were

decanted off into a discard jar containing disinfectant.

Each well was washed three times with approximately 300 µl of phosphate buffered saline

Then 0.25 ml of methanol was added to each well & kept at room temperature for 15 minutes.

- Wells were then stained by adding 0.2ml of crystal violet (0.1% w/v) to each well and left for 20 minutes- Adherent bacterial cells were uniformly stained with crystal violet.
- Excess stain was removed by repeated washing (3 times) using approximately 300µl of sterile distilled water for each well
- Plates were then dried by keeping it in an inverted position on absorbent paper
- Finally, 0.2 ml of 33% glacial acetic acid was added to each well to extract the absorbed stain from the adherent cells
- The optical density (OD) of the stained wells were then measured using a Bio Rad ELISA reader at 490 nm
- All the isolates including controls were tested in duplicate and average of the OD values taken.
- To compensate for background absorbance, average of OD readings from sterile medium were taken and subtracted from all test values to get the final OD value.
- Depending on these OD values, bacteria were considered as biofilm producer or non-producer.²⁴

The cut off OD value to assess biofilm producing status is given in the table below

Table 1: Cut off optical density value for biofilm production^{25,26}

Od Value	Biofilm Status
<0.12	Non/weak producer
>0.12	Producer

Test was repeated for all isolates to prove reproducibility of the test method.

Data entry

History and clinical details obtained from each patient was numerically coded and entered into

Microsoft excel spread sheet. The status of biofilm production by the isolates were also coded & entered into excel spread sheet.

Data analysis

Chi square test & Fishers exact test were used in the analysis of study variables. The level of statistical significance was taken as p Value < 0.05. Statistical significance was analysed using Statistical package for Social Sciences (SPSS) software 16.0.

Results

The present study was conducted at the Departments of Microbiology & Surgery over a period of one year starting from 1st of February 2015. A total of 103 samples were collected from patients with different types of ulcers. The mean age of patients who were included in the study was calculated to be 55+/- 12 years. Males were predominant in our study population compared to females.

Category of Ulcers in the Study

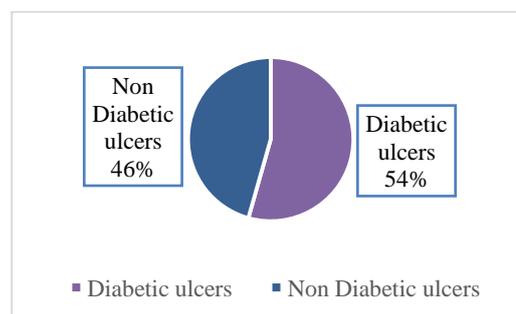


Figure 1: Pie diagram showing the proportion of types of ulcers in the study population

- Of the 103 cases 56 patients had diabetic ulcers, out of which 10 had smoking & 8 had alcoholism as an additional risk factor.
- *Non-diabetic ulcers were 47 in number which included,*
 - 15 varicose ulcers of which 4 had diabetes & 11 had alcoholism, also as a predisposing factors.
 - 11 decubitus ulcers.

Table 2 : Table showing the frequency of various isolates obtained from major types of ulcers in the study

Bacteria	Varicose ulcer	Diabetic ulcer	Decubitus ulcer
Staphylococcus aureus	8	20	4
Pseudomonas aeruginosa	7	10	4
Enterococcus spp	0	8	0
Escherichia coli	1	5	1
Proteus spp	0	6	1
Klebsiella pneumoniae	1	6	2
Acinetobacter spp	0	3	1
Corynebacterium spp	1	8	1
Serratia spp	0	3	0
Morganella Morgagni	2	1	0
Flavobacterium spp	0	1	0
Enterobacter spp	0	2	0
Streptococcus spp	0	4	0
Coliform bacilli	1	3	0
CoNS	0	2	0

- Monomicrobial infection in 37 cases (66%) & polymicrobial infection was seen in 19 cases (34%)
- In monomicrobial infections Gram positives were present in 51% & Gram negative in 49% of patients
- In polymicrobial infections Gram positive & Gram-negative infection were present in 45% & 55% respectively.
- Commonest Gram-positive isolate in both polymicrobial & monomicrobial infection was Staphylococcus aureus & Pseudomonas aeruginosa being the commonest Gram-negative isolate in both types.

Bacterial Flora in Diabetic Ulcer

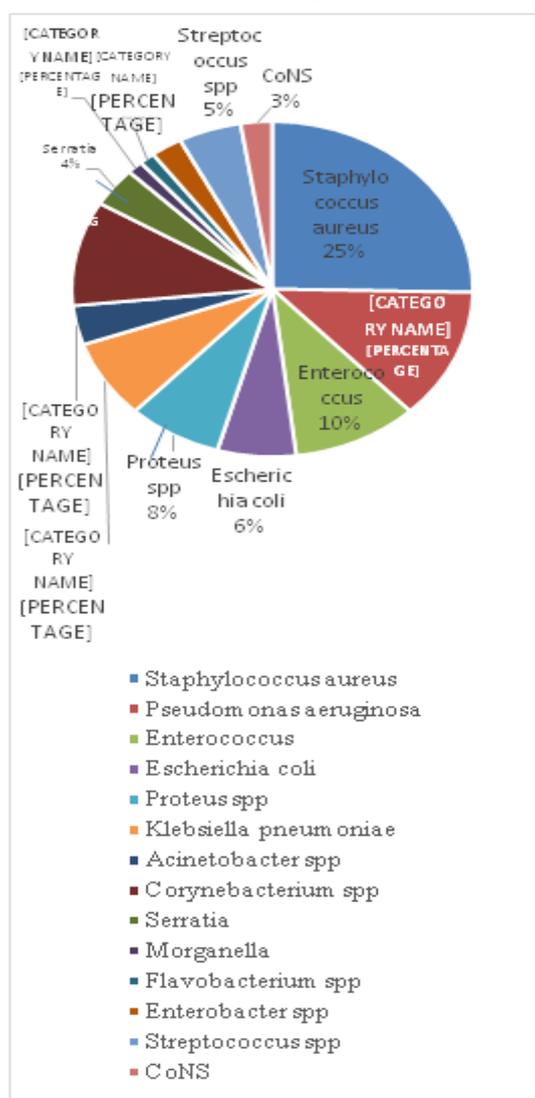


Figure 2: Bacterial flora obtained in patients with diabetes ulcer

Biofilm Production by Isolates

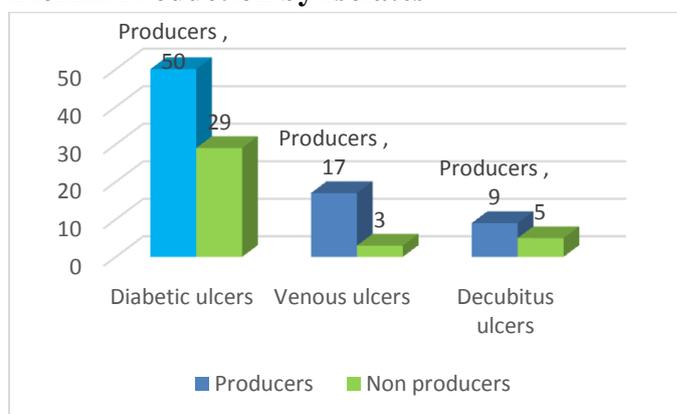


Figure 3: Biofilm production status of isolates from various types of ulcers

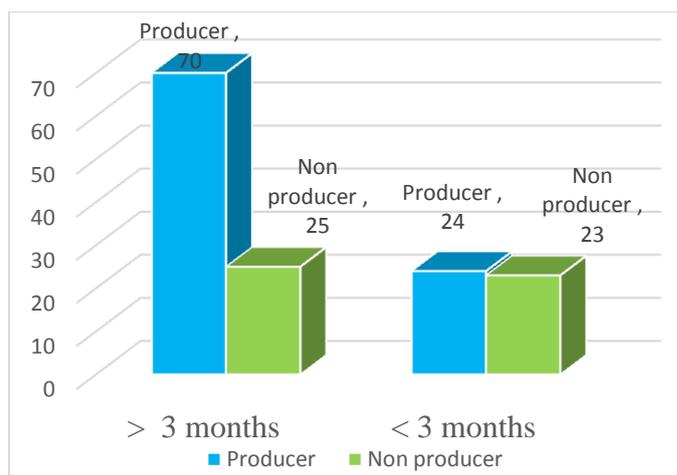


Figure 4: Bar diagram showing association between Biofilm Status & Duration of ulcer

- 63% of isolates from diabetic ulcers, 85% of venous ulcer isolates & 64 % of isolates from decubitus ulcers were biofilm producers.
- There was *no statistically significant association between type of ulcers & biofilm production by isolates* obtained from them which was assessed using Chi square test and p value obtained was 0.102. (>0.05)
- The association between *biofilm status and duration of ulcer* was assessed using Chi Square test and the p value obtained for the same was 0.002(<0.05) and this association was found to be *significant statistically*.

Discussion

Ulcers especially chronic ulcers pose a significant health care burden worldwide. It also causes major social as well as economic problem to the affected individual. The non-healing of such ulcers could be due to multiple factors among which bacterial infections are the forerunners. Infection with MDR bacteria as well as biofilm production by bacteria prevent healing process in ulcers

The present study was conducted in the Department of Microbiology & Department of Surgery over a period of 1 year starting from February 1, 2015, since regional studies comparing the flora of the major types of ulcers were few & literature search didn't reveal any studies assessing the biofilm status of the bacteria isolated from ulcers from our region. The principal aim of this study was to identify the predominant bacteriological flora, to find out the biofilm production status of the isolates & to determine any association between biofilm production by the isolates and chronicity of the ulcer

The study population in the present study included patients from the age of 25 to those up to the age of 87. Maximum number of patients were from the age group 51- 60. This data was in accordance with the observation from other studies²⁷⁻³³. The mean age in other studies on ulcers also lies in the range of 55 +/- 13 years.

We had a male predominance among our study population. This was in accordance with the studies

on ulcers from India²⁸⁻³⁴ but studies from outside India especially from the West^{27,35} had reported that women were twice likely to get affected with ulcers than men.

Biofilm production by bacteria impedes healing of an ulcer which contribute to the chronicity of an ulcer. Non-healing can be because of the evasion of the host immune response by the isolate as well as poor penetration of antibiotics. In the present study 66.9% of the total isolates from ulcers were biofilm producers. In other studies assessing biofilm status also, > 60% of the isolates were found to be biofilm producers.^{28,36,37} Gram positive bacteria were better biofilm producers in our study which was in discordance with other studies which have reported Gram negative bacteria as better biofilm producers³⁷. In the present study 72.7% of MRSA were biofilm producers whereas in a study by Rahim et al at Pakistan had shown that all MRSA isolates were biofilm producers³⁶. Most frequent biofilm producers among Gram negatives were *Pseudomonas aeruginosa* & among Gram positives was *Staphylococcus aureus* in our study. Study by Swarna et al³⁷ also has similar findings, but Zubair et al had reported *Proteus vulgaris* as the predominant producer.²⁸ Other isolates in our study like *Enterococcus* spp, *Proteus* spp, *Escherichia coli*, *Klebsiella pneumoniae*, *Serratia*, *Morganella*, *Streptococcus* & CoNS which were lesser in number were also biofilm producers.

In our study 74% of isolates from ulcers of duration of >3 months were biofilm producers which was found to be a statistically significant association. This was in agreement with study done by Zubair et al²⁸ who also reported a statistically significant association between duration of ulcer & biofilm status. In the study by James et al³⁸ at Texas, they had found that more than 60% of chronic ulcers were harbouring biofilm producing bacteria.

More multi centric studies should be conducted to assess the biofilm production status of isolates from ulcers to know the extent of the problem. Increased prevalence of biofilm producing isolates would necessitate development & inclusion of new strategies to prevent biofilm formation like

prevention of attachment & prevention of development of biofilms in the treatment plan of chronic ulcers.³⁹

Limitations of the Study

ATCC control strains for biofilm production was not used as positive & negative controls as they were not available for purchase at the time of study; instead an in house positive & negative controls were used which gave consistent results on repeated testing.

Conclusion

- 63% of isolates from diabetic ulcers, 85% of venous ulcer isolates & 64 % of isolates from decubitus ulcers were biofilm producers.
- There was ***no statistically significant association between type of ulcers & biofilm production by isolates*** obtained from them which were assessed using Chi square test and p value obtained was 0.102. (>0.05)
- Majority of the biofilm producing bacteria were isolated from ulcers with duration more than 3 months & this association was statistically significant. This observation was shared by another similar study by Zubair et al at Aligarh.

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