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The Prevalence of Biofilm in Diabetic Foot Patients in Zagazig University Hospital, Egypt

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Prevalence of biofilm in diabetic foot patients

Abstract: ***Background: Biofilms have been associated with chronic infections in diabetic foot leading to resistance to host mechanisms and antimicrobial interventions. Objective: The Aim of this study is to estimate the prevalence of biofilm in diabetic foot patients and identification of the causative bacteria. Methods: 75 patients suffering from Diabetic foot were admitted to the surgical department during the one year period of the study, 48 males and 27 females, aged 37 - 80 ys. Swab samples from the foot lesions were studied for Bacteriological culture, antibiotic sensitivity, detection of Biofilms by Tissue Culture Plate test, Scanning Electron Microscopy and routine blood investigations. Results: Out of 75 diabetic foot patients, 58 (77.3%) cases were biofilm positive, while 17 (22.7%) cases were biofilm negative so, the prevalence of biofilm formation was 77.3%. 130 isolates from pus swab of 75 patients were obtained, an average of 1.73 organisms per specimen was estimated. The association between the number of isolated microorganisms and biofilm production is statistically significant (p= 0.026). Most of cases 44 (58.6%) showed mixed infection. The Gram negative bacilli were highly prevalent (60%) than Gram positive cocci (40%). The Multi Drug Resistance isolates were present in 100 (76.9%) of organisms, the majority of them were Gram negative bacilli mostly Escherichia coli and Klebsiella pneumoniae. As regard Gram positive cocci, Staphylococcus aureus considered the highest organism producing Multi Drug Resistance pattern. Conclusion: The prevalence of Biofilm among diabetic foot patients in Zagazig University Hospitals was 77.3 % , with predominance in gram negative bacilli. All debrided tissue samples upon their examination by SEM showed biofilm formation, so it is a good confirmatory method to the positive cases already diagnosed first with TCP test.***

Keywords: biofilm, Tissue Culture Plate test, Scanning Electron Microscopy, Multi- Drug Resistance

1. Introduction

Diabetes mellitus (DM) is a one of most common chronic diseases in nearly all countries, it continues to increase in number and significance **[29]**. It is a disease as old as mankind itself and is a major health care challenge [**24]**. The global prevalence of DM in the year 2010 among adults was estimated to be 6.4% , while it is estimated that by the year 2030, Egypt will have at least 8.6 million adults having diabetes **[29].** The Global diabetes incidence is increasing rapidly, this rise in prevalence of DM is likely to bring a concomitant increase in its complications among diabetic patients **[12].**

Infected foot lesions are a major medical social and economic problems ,which are the leading cause of hospitalization for patients with DM worldwide **[34]** as , diabetic foot ulcers are a disastrous complications of D.M that may end up to leg amputation **[4]** .

In Egypt, prevalence of diabetic foot ulcers has been found to be high. The reasons commonly stated for this high prevalence includes inappropriate footwear and the lack of knowledge regarding diabetic foot problems. The latter is very pertinent to Egypt since more than 90% of the people having diabetes do not receive education on diabetic foot problems **[18].** Bacterial infection, tissue ischemia and poor wound management can cause diabetic foot ulcers to heal slowly and to transform it to chronic wounds **[16].** Impaired circulation in patients with diabetic foot limits the access of phagocytes favoring development of infection. Escherichia coli spp., Klebsiella spp., Pseudomonas spp., Staphylococcus aureus and Enterococcus spp. are the most frequent pathogens contributing to progressive and wide spread tissue destruction **[17].**

As diabetic foot infections are often polymicrobial **[36],** This increase association of multi-drug resistant organisms (MDROs) with diabetic foot ulcers resulting in high risk of limb amputation **[37].** Infection with MDROs is also responsible for increasing duration of hospitalization, cost of management, morbidity and mortality of the diabetic patients **[17].** Biofilms are the natural phenotype of bacteria. These typically consist of polymicrobial populations of cells, which are attached to a surface and encase themselves in hydrated extracellular polymeric substances. “Microbial populations that have attached to a biological or non-biological surface” is the most basic description of a medical biofilm. Thus, most chronic infections, including bacteria that are associated with chronic wounds exist as a biofilm communities **[20].**

Bacteria growing in biofilms often display a variety of phenotypic differences from the same strains growing in planktonic culture. The phenotypes pave way for the emergence of multi-drug resistant ability of a microorganism to form biofilm, which is an important virulence factor protecting them from many traditional therapies **[15].** So, physical removal of the biofilm is one of the most successful strategies for management of biofilm-related conditions, through frequent debridement of the diabetic foot ulcers **[14].**The recognition of bacterial biofilm in chronic wounds may give the opportunity to explain many of the characters of the chronic wound. As , it may explain why chronic wound does not heal despite adequate treatment of underlying condition and can give a new path of research

that may lead to new treatments **[8]**. As, both systemic and topical antibiotics alone are unable to eradicate biofilm infections **[3]**, there is an increasing interest in their etiological role. So ,there is an increasing clinical need to identify biofilms in these wounds **[2].**

The Aim of the present study was to estimate the prevalence of biofilm in diabetic foot patients in Zagazig University Hospital, EGYPT and identification of the causative bacteria.

2. Subjects and Methods

The study was carried out in Departments of Clinical Pathology and General Surgery, Faculty of Medicine, Zagazig University, and Faculty of Science, Zagazig University, during the period from June 2013 to May 2014. It was conducted on 75 patients who had foot pathology on top of diabetes with signs of infection swelling, exudates, surrounding cellulitis, bad odour, tissue necrosis and crepitation. Each patient was included only once in the study.

All Patients was subjected to:

- Full clinical history taking and thorough clinical examination .
- Routine Laboratory investigations including CBC, fasting and postprandial blood sugar and HbA1C .
- -Swab samples from the foot lesions were collected under aseptic conditions. All the samples were transported either immediately to the Clinical Pathology Department laboratories or on nutrient broth media as a transport media for investigation in the following ways :

1) All the bacterial isolates were identified to the species level using Routine standard identification techniques. That was done using direct microscopic examination of gram stained smear, cultivation of the specimen on a suitable medium and observation of cultural characters of the organism growth and its biochemical reactions **[30].**

2) Disc diffusion antibiotic sensitivity testing (antibiogram) was performed for all plankitonic bacterial isolates **[35].**

3) ***Biofilm Detection by:***

a) Tissue Culture Plate (TCP) method [31]:
The Tissue Culture Plate is a quantitative and reliable method to detect biofilm forming microorganisms. TCP can be recommended as a general screening method for detection of biofilm producing bacteria in laboratories. It is most widely used and is considered the gold-standard test for detection of biofilm formation **[22].** Strains were classified as no biofilm producer; Weak biofilm producer, Moderate biofilm producer and Strong biofilm producer according to **Table A.**

Table A: Classification of Biofilm producer

Biofilm Formation	Classification of biofilm formation by TCP method **Mean OD values**
Non	<0.120
Weak	0.120-0.240
Moderate	0.240-0.480
Strong	>0.480

b) Scanning Electron Microscopy (SEM):
Soft tissue samples were collected with sterile tools from selected patients when undergoing deep debridement in the operating theatre . Then Samples were prepared for SEM examination using a JEOL-JSM-T100 scanning electron microscope. SEM-micrographs at various magnifications were used to elucidate the biofilm morphological features of the studied species. Depending upon the size of organisms, magnification power between 2000 and 5000× was selected with the aim of showing the finest possible detail in biofilm **[7].**

Statistical Analysis
The collected data were computerized and statistically analyzed using SPSS (statistical package for social sciences) version 16. Data were expressed as number and percentage for Qualitative variables and mean (x) ± standard deviation (SD) for Quantitative variables. Student "T" test, paired"T" test and Chi-square (X^2) where used when indicated to assess significance. A result was considered statistically significant when the significant probability was less than 5% ($P<0.05$).

3. Results

This study included 75 patients with Diabetic foot 48 males and 27 females aged 37-80 years.

Table 1. Age distribution among the included diabetic foot infected (DFI) patients (biofilm positive and negative cases). It shows age distribution and percentage among the total 75 patients included in this study. Peak was at age range 41-60years followed by age above 60 years as both showed statistically significance P-value <0.001 in relation to biofilm production while the least was at age below 40 years.

Table 2. Sex distribution among the included DFI patients (biofilm positive and negative cases). It shows that majority 48 (64%) of patients were males. Out of 48 male patients, biofilm positive cases were detected in 36 patients and biofilm negative cases were in 12 patients. Regarding female patients biofilm positive cases were found in 22 patients and biofilm negative cases were in 5 patients.

Table 3. Clinical data of DFI patients in biofilm positive and negative cases. It shows that majority of the subjects 55(73.3%) had lesions for >1 month before presentation at the hospital. The association between ulcer duration and biofilm production is statistically significant (P=0.0014).

Table 4. Diabetic history of DFI patients in biofilm positive and negative cases . It shows that majority 57(76%) of subjects had T2DM.The association between diabetes types and biofilm production is statistically significant (P<0.001). Majority of patients 48(64%) were hypertensive followed by forty-five patients (60%) had neuropathy which its association with biofilm production is statistically significant (P<0.006)

Table 5. Detection of biofilm formation for various isolates from swabs. It shows the ability to form biofilm on plastic surfaces by various isolates from pus swab of diabetic foot in TSB medium.70.8% (92/130) isolates were positive biofilm

producers and 29.2% (38/130) were negative biofilm producers.

Table 6. Bacteriological profile of the isolates from pus swab of DFI patients (N=130 isolates). It shows bacteriological profile of 130 isolates from pus swab of 75 patients with diabetic foot infection. An average of 1.73 organisms per specimen was obtained in total DFI patients. The Gram negative bacilli were highly prevalent (60%) than Gram positive cocci (40%).

Table 7. Biofilm formation versus multi-drug resistance organisms. It shows Correlation of multidrug resistant organisms (MDRO,s) with respect to biofilm. The association between the MDR and biofilm production is statistically significant (p= <0.001).
There were 27 strong biofilm producing organisms isolated from 21 patients. Debrided tissues collected from these patients were examined for biofilm using Scanning Electron Microscopy as shown in Figures 1,2,3,4.

Table 1: Age distribution among the included diabetic foot infected (DFI) patients (biofilm positive and negative cases).

Age distribution (years)	*Biofilm positive cases 58 (77.3%)*	*Biofilm negative cases 17 (22.7%)*	*Total diabetic foot patients 75(100%)*	*P-value*	*OR (95%CI)*
<40	4 (5.3%)	5 (6.6%)	9 (12%)		1
41-60	38 (50.7%)	8 (10.6%)	46 (61.3%)	<0.001*	38(3.75-935)
>61	16 (21.3%)	4 (5.3%)	20 (26.7%)	<0.001*	32(2.51-931)
Mean ± SD	56.5 ± 8.5	48.6 ± 11.7	54.8 ± 9.9		
Range of age	40-70 years	37-80 years	37-80 years		

*Highly significant difference (P < 0.01)

Table 2: Sex distribution among the included DFI patients (biofilm positive and negative cases).

Sex	Total N=75	Biofilm positive cases 58 (77.3%)	Biofilm negative cases 17 (22.7%)	P-value	OR (95%CI)
Male	48 (64%)	36 (75%)	12 (25%)	0.51	0.68 (0.18-2.49)
Female	27 (36%)	22(81.5%)	5 (18.5%)		

Table 3: Clinical data of DFI patients in biofilm positive and negative cases

N=75	**Total**	**Biofilm + (n=58)**	**Biofilm – (n=17)**	**P-value**	**OR (95%CI)**
Size of ulcer				0.49	2.17 (0.39-15.7)
≤4 cm2	15 (20%)	13	2		
>4 cm2	60 (80%)	45	15		
Duration of Ulcer					
< 1month	20 (26.7%)	10	10	0.0014*	6.86 (1.83-26.7)
>1 month	55 (73.3%)	48	7		
Hospital stay(days)	6.5 ± 2.9	6.7 ± 3.1	5.8 ± 2.0		
≤7	47 (62.7%)	35	12	0.31	X^2=2.29
8-10	21 (28%)	16	5		
>10	7 (9.3%)	7	0		
Treatment					
conservative	45 (60%)	32	13	0.11	0.38 (0.09-1.47)
amputation	30 (40%)	26	4		

*Highly significant difference (P < 0.01).

Table 4: Diabetic history of DFI patients in biofilm positive and negative cases

N=75	**Total**	**Biofilm + (n=58)**	**Biofilm – (n=17)**	**P-value**	**OR (95%CI)**
Type of Diabetes					
Type 1	18 (24%)	8	10	<0.001*	10.18 (2.61-41.83)
Type 2	57 (76%)	50	7		
Diabetes duration(years)	12.5 ± 7.8	12.3 ± 7.7	13.3 ± 8.1	0.15	X^2=3.79
<5	10(13.3%)	10	0		
5-10	27(36%)	21	6		
>10	8(50.7%)	27	11		
Complications					
neuropathy	45 (60%)	30	15	0.006*	0.14 (0.02-0.75)
nephropathy	23(30.6%)	19	4	0.46	1.58 (0.4-6.69)
retinopathy	27 (36%)	23	4	0.22	2.14 (0.55-8.93)
hypertension	48 (64%)	34	14	0.07	0.3 (0.06-1.32)
osteomyelitis	23(30.6%)	21	2	0.054	4.26 (0.8-29.89)
HbA1c					
7-8 % (fair control)	1(1.3%)	1	0	0.51	Undefined
>8 % (poor control)	74 (98.7%)	57	17		

*Highly significant difference (P < 0.01).

Table 5: Detection of biofilm formation in various isolates from swabs

Microorganisms		Strong biofilm producers	Moderate biofilm producers	Weak biofilm producers	Non biofilm producers
Gram Positive cocci (52)	***S.aureus* (25)**	1 (4%)	4 (16%)	8 (32%)	12 (48%)
	***Enterococcus spp* (17)**	3 (17.6%)	4 (23.5%)	2 (11.8%)	8 (47.1%)
	CONS (10)	0	1 (10%)	3 (30%)	6 (60%)
	Total (52)	**4 (7.7%)**	**9 (17.3%)**	**13 (25%)**	**26 (50%)**
Gram Negative bacilli (78)	***E.coli* (25)**	2 (8%)	14 (56%)	4 (16%)	5 (20%)
	***P.aeruginosa* (23)**	15 (65.2%)	6 (26.1%)	0	2 (8.7%)
	***K.pneumoniae* (14)**	3 (21.4%)	8 (57.1%)	2 (14.3%)	1 (7.2%)
	***Proteus spp* (8)**	2 (25%)	4 (50%)	1 (12.5%)	1 (12.5%)
	***Acinetobacter spp* (6)**	0	4 (66.7%)	0	2 (33.3%)
	***Citrobacter spp* (2)**	1 (50%)	0	0	1 (50%)
	Total (78)	**23 (29.5%)**	**36 (46.2%)**	**7 (8.9%)**	**12 (15.4%)**
Total	**130**	27 (20.8%)	45 (34.6%)	20 (15.4%)	38 (29.2%)
Patients	**75**	**21 (28%)**	**28 (37.3%)**	**9 (12%)**	**17 (22.7%)**

Table 6: Bacteriological profile of the isolates from pus swab of DFI patients (N=130 isolates).

Microorganisms	N (%)	No of MDR isolate
Gram positive cocci (GPC)	**52 (40%)**	**25 (48.1%)**
1-Staphylococcus aureus	25 (19.2%)	14 (56%)
2-Enterococcus spp	17 (13.1%)	9 (52.9%)
3-Coagulase negative Staphylococcus (CONS)	10 (7.7%)	2 (20%)
Gram negative bacilli (GNB)	**78 (60%)**	**75 (96.2%)**
1-Escherichia coli	25 (19.2%)	25 (100%)
2-Pseudomonas aeruginosa	23 (17.7%)	20 (86.9%)
3-Klebsiellapneumoniae	14 (10.8%)	14 (100%)
4-Proteus spp	8 (6.2%)	8 (100%)
5-Acinetobacterspp.	6 (4.6%)	6 (100%)
6-Citrobacter spp.	2 (1.5%)	1(50%)

Table 7: Biofilm formation versus multi-drug resistance organisms

Total No. of isolates (130)	Biofilm positive isolates 92 (70.8%)	Biofilm negative isolates 38 (29.2%)	X2	P Value
MDRO positive (100)	82 (89.1%)	18 (47.4%)	26.42	<0.001*
MDRO negative (30)	10 (10.9%)	20 (52.6%)		

*Highly significant difference (P < 0.01).

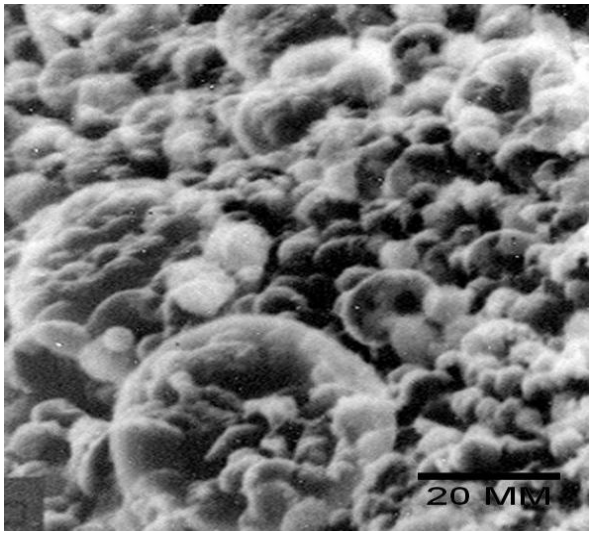

Figure 1: SEM image of debrided tissues taken from infected diabetic foot wound during a deep debridement procedure showing small islands of of various shaped cells of bacterial biofilm species colonized the tissues**.** Scale bar = 20 MM , 5000 x magnification.

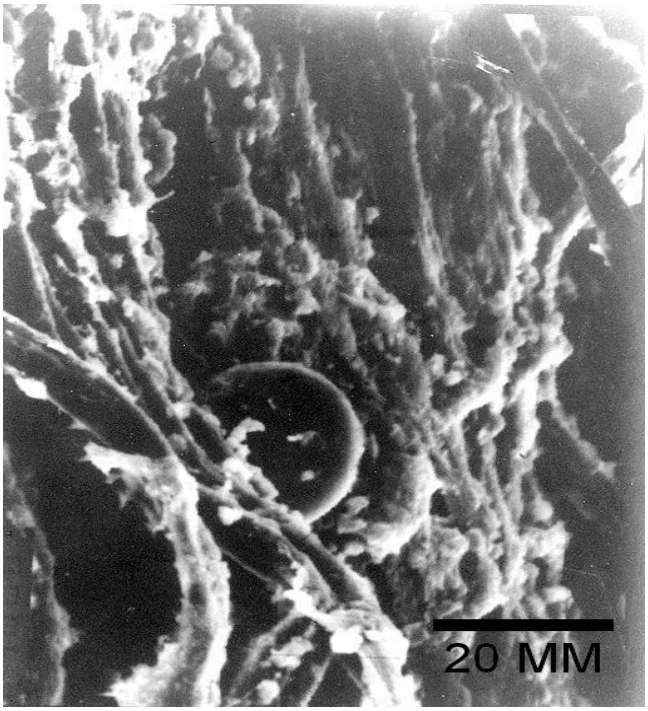

Figure 2: SEM image of debrided tissues taken from infected diabetic foot wound during a deep debridement procedure showing mature biofilm attached to the tissues and embedded within amorphous slime with scattered fiber reminants of glycocalyx. Scale bar = 20 MM, 2000 x magnification.

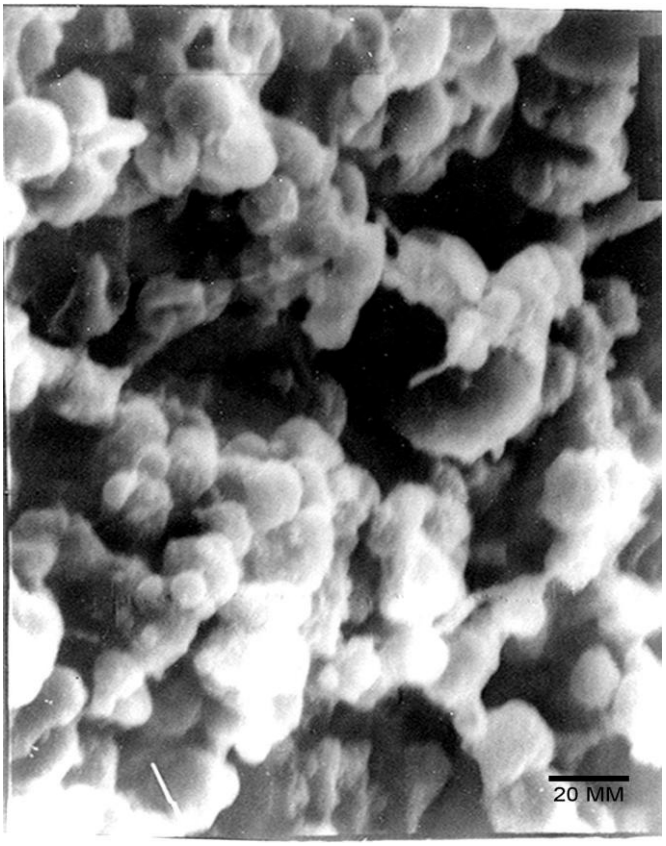

Figure 3: SEM image of debrided tissues taken from infected diabetic foot wound during a deep debridement procedure showing clusters of cocci attached to the tissues and embedded within amorphous slime in form of biofilm**.** Scale bar = 20 MM, 5000x magnification.

Figure 4: SEM image of debrided tissues taken from infected diabetic foot wound during a deep debridement procedure showing groups of bacterial cells and remnants of glycocalyx in form of tower-shaped structure composed of multiple layers of bacteria with scattered fiber remnants of glycocalyx in a biofilm structure. Scale bar = 20 MM, 3000 x magnification.

4. Discussion

In chronic wounds, biofilm infects host tissue for extended periods of time. Although biofilm infected wounds did not show marked differences in wound closure, the repaired skin demonstrated compromised barrier function. This observation is clinically significant because it leads to the concept that even if a biofilm infected wound is closed as observed visually, it may be complicated by the presence of failed skin which is likely to be infected and/or further complicated post-closure **[26]**.

This study was a comprehensive clinical and microbiological profile of infected diabetic foot with study of biofilm production in the bacterial isolates from hospitalized patients of Zagazig University Hospitals during the period from June 2013 to May 2014.

With the rise in the prevalence of diabetes mellitus there is increasing problem of infections, especially foot infections. According to some studies, patients with diabetic foot infections account for 20% of hospital admissions **[28].** In our hospital 75 DFI patients included during the period of the study form 25% of inpatient cases of General Surgery Department. The prevalence of diabetic foot ulcers among male subjects in the present study was found to be 64% against 36% in female i.e. a ratio of 1.7:1. This agrees with **[38]** who attributed that to be due to higher level of outdoor

activity among males compared to females**.** In present study, we found polymicrobial etiology in 44/75 (58.7%) and monomicrobial in 31/75 (41.3%) patients. **[38], [10], [39]** have reported 33%, 66% and 83% of polymicrobial infections and 56.6%, 23% and 16.2% monomicrobial infections respectively. The findings of our study are similar to **Citron** study in which higher percentages of polymicrobial infections were also found.

Tissue culture plate method is considered the gold standard method for biofilm detection **[22]**. The correlation of the PCR test with phenotypic tests occurred with the TCP test was done by **[13]** who found that TCP tests showed results that were significantly correlated with the molecular analysis. In our study, the biofilm formation was detected by this method revealing that 70.76% (92/130) of the isolates are biofilm producers and gram negative bacilli seem to be potent biofilm producers 84.6% (66/78) compared to the gram positive cocci 50% (26/52). This is coordinated with **[31]** opinion in which the ability, quality and quantity of biofilm production seem to reflect the nutritional status of the culture medium as gram negative bacteria produce more biofilm in nutrient-poor medium ,while gram positive bacteria produce same in nutrient rich medium**.**

In the present work debrided tissues collected from 21 patients infected with strong biofilm producing bacteria were examined for biofilm using scanning electron microscopy. Our choice of SEM usage as a confirmatory method to biofilm production depends on that many of the conclusions about biofilm development, composition, distribution, and relationship to substratum have been derived from SEM **[9],** which allows visualization of surface structures with a three dimensional appearance and at different resolutions **[19].** In the present study all samples upon their examination by SEM showed biofilm formation and this was a confirmatory method to the positive cases already diagnosed first with TCP method in this study**.**

Gram negative bacilli in our patients were more prevalent (60%) than gram positive cocci (40%). These findings correlated well with those of **[23]** who reported that 76% of the organisms which were isolated were gram negative bacilli and with the study of **[6]** which was done in Kuwait, they reported that more gram-negative pathogens (51.2%) were isolated than gram-positive pathogens (32.3%).In the present study, *S.aureus* (19.2%), *E.coli* (19.2%), and *P.aeruginosa* (17.7%) were the most predominantly isolated species followed by *Enterococcus spp* (13.1%), *Klebsiella spp* (10.8%) *and CONS* (7.7%).These findings correlated well with **[1][10][38]** who reported *Staphyloccus aureus* as the predominant pathogen, which comprised 57.2%, 28% and 26.2% of their isolates respectively. **[38]** reported *Escherichia coli* (26.6%) and *Pseudomonas aeruginosa* (10.6 %) as the predominant gram negative isolates. The predominant bacterium in wound infection is *Escherichia coli* which are reported by **[5]**. *Enterococcus spp.*, considered low-virulence commensal organisms, except in diabetic and other compromised patients, were identified in 20.4% of patients **[32]**. In contrast, **[23]** reported that *Pseudomonas* to be the predominant pathogen (23%), followed by *Staphylococcus aureus* (21%) in Indian patients. The most common isolate in our study was *S. aureus*, which was also reported in many other studies to be the predominant microorganism (40–60% of the total microorganisms) isolated from different types of wounds **[21].**

[23] reported increasing resistance to more than one group of drugs (MDRO)**.** This agrees with our results in which isolates resistant to 3 or more than three groups of antibiotics (MDRO) were noticed in 48.1 % of gram positive cocci (25/52) and 96.2% of gram negative bacilli (75/78). Multidrug resistance is observed more among gram negative bacterial isolate compared to the gram positive isolates. Most of the other international studies that have reported a similarly high percentage of MDR organisms in gram negative bacteria **[39].**In the present study, prevalence of MDRO among all isolated organisms accounts for 76.9%. This is coordinate with **[33]** study on DFU in which 72% of the isolates were reported as MDRO**.** Regarding gram negative bacilli in our study, *K.pneumoniae* considered the best as out of 14 isolates tested 13 strains (92.8%) were positive for biofilm formation. Followed by *P.aeruginosa*, *Proteus spp* and *E. coli*, 21 of 23 (91.3%), 7 of 8 (87.5%) and 20 of 25 (80%) of clinical strains tested were observed to form biofilms respectively. In *Acinetobacter spp*, 4 of 6 isolates tested (66.7%) while in *Citrobacter spp*, 1 of 2 isolates tested (50%) were determined to be positive for biofilm formation.

This correlated partly with the findings of **[27]** study which showed that in *K. pneumoniae*, 41 of the 54 isolates tested (76%) were determined to be positive for biofilm formation, in *P. aeruginosa* and *Acinetobacter spp.*, 30 of 36 (83%) and 29 of 53 (55%) of clinical strains tested were observed to form biofilms, respectively. In contrast **[11]** showed that *E. coli* was the weakest biofilm forming group with only 5 of the 39 strains (13%) capable of forming biofilms**.**

Regarding gram positive cocci in our study, of the 17 *Enterococcus spp* isolates tested 9 strains (52.9%) were positive for biofilm formation. Followed by *S. aureus* and CONS, 13 of 25 (52%) and 4 of 10 (40%) of clinical strains tested were observed to form biofilms respectively. In contrast **[25]** showed that out of the 23 *S. aureus* isolates tested, 21 strains (91%) were positive for biofilm formation. Bacteria forming biofilms play a major role in developing multi-drug resistance in chronic infections. Biofilm mediated infection are difficult to eliminate resulting in treatment failure. It is suggested that the development of biofilm in chronic wounds are associated with increased synthesis of exoploysaccharides that leads to poor penetration of antibiotics **[8].**The prevalence of biofilm producing MDRO could be considered serious because the choice of antibiotic treatment is limited and may lead to poor outcome. Therefore, routine screening for detection of biofilm and MDR are currently needed to reduce the incidence of morbidity and amputations in diabetic patients with foot ulcer **[27].** The presence of biofilm phenotype bacteria on the surface of the wound is now well established.

Biofilm phenotype bacteria is an excellent model to explain what is observed in chronic, non-healing wounds and their responses to antibiotics and other wound care treatments. This knowledge opens up exciting new possibilities for the management of wound biofilm and the improvement of

outcomes. Both gram positive cocci and gram negative bacilli caused diabetic foot infections and this study showed a preponderance of gram negative bacilli. Up till now the effective method for treatment is removal of biofilm by tissue debridement once biofilm formation is proved. We concluded that the prevalence of Biofilm among diabetic foot patients in Zagazig University hospitals in the period of the study was 77.3 % , there was an association between the number of isolated microorganism and biofilm production with gram negative bacilli predominance mostly E.Coli and Klebsiella . All debrided tissue samples upon their examination by SEM showed biofilm formation So , it was a good confirmatory method to the positive cases already diagnosed first with TCP test **.**

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