ORIGINAL ARTICLE

Microbiological Profile, Biofilm Formation and Antimicrobial Resistance Pattern of Pathogenic Bacteria Isolated from Chronic Ulcerative Lesions

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ABSTRACT

Background: *Chronically infected wounds represent a major public health problem which is associated with increased morbidity and mortality, particularly with the global threat of antimicrobial resistance.* ***Objectives:*** *Isolation of pathogenic micro-organisms from chronic ulcerative lesions, identification and evaluation of their biofilm formation and antimicrobial resistance pattern.* ***Methodology:*** *This study was carried out on 50 specimens, collected from chronic ulcerative lesions of patients admitted to the Inpatient and Outpatient Surgical Units, Tanta university hospitals. Pathogenic organisms were isolated and identified. Antimicrobial susceptibility was determined, and biofilm formation assay was performed using a tissue culture plate method.* ***Results:*** *The most frequently isolated organisms were Pseudomonas spp. (26.42%), Klebsiella spp. (24.52%) then E. coli (16.98%). All E. coli isolates showed resistance to aztreonam and Ampicillin /sulbactam. Also, all Klebsiella isolates showed resistance to ciprofloxacin, levofloxacin and Ceftazidime. While 92.86% of Pseudomonas isolates showed resistance to Ceftazidime. About 58.5 % of isolated pathogens were biofilm producers with Pseudomonas spp. were the most frequently biofilm producers.* ***Conclusion:*** *The isolated pathogens showed high rate of resistance to most of the tested antimicrobial agents, with high rate of biofilm formation among most of isolates.*

INTRODUCTION

Chronic ulcer is a common infectious skin disease, characterized by an unhealed long-term wound and local bacterial infections[1]. Chronicity is often the result of an underlying medical condition such as diabetes, blood flow disorders or a delayed presentation of the clinic. Long persisting infected wounds cause morbidity and suffering and are typically associated with large expenditures, e.g., on medication and affect economic productivity[2]. Chronically infected wounds, such as venous or arterial ulcers, diabetic foot ulcers, pressure ulcers, and non-healing surgical wounds, have a substantial impact on the quality of life of patients, are a leading cause of morbidity and mortality, and significantly increasing healthcare costs. [3] Pathogenic microorganisms considerably slow the healing process due to tissue destruction that leads to an exacerbated immune response state, characterizing chronic wounds[4]. The class of microorganisms known as ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter spp.*.) are among the most common bacteria in cutaneous infections [5]. In this sense, microbial infections are highlighted as the most important causes of chronic wounds and are usually associated with biofilm formation, which are notoriously resistant to conventional antibiotics[6,7]. Biofilms delay the inflammatory and maturation phases of chronic wound healing [3]. Antimicrobial resistance is a critical global hazard that is causing increasing worry among humans. Multidrug-resistant (MDR) micro-organisms have been reported to be isolated from patients with moderate to severe diabetic foot infections. The biofilm-forming ability of bacteria has been associated with antibiotic resistance and chronic recurrent infections in diabetic foot patients [8]. Patients with burn infections caused by multidrug-resistant strains of *Pseudomonas aeruginosa*, usually affected by sepsis and suffer from elevated morbidity and mortality [9].

The aim of this study was isolation of pathogenic micro-organisms from chronic ulcerative lesions, identification and evaluation of their biofilm formation and antimicrobial resistance pattern.

METHODOLOGY

This study was carried out on 50 samples collected from patients admitted to the Inpatient and Outpatient Surgical Units of Plastic and Reconstructive Surgery Department, Tanta University Hospitals, during the

period of research from (November 2020) to (November 2021). An informed consent was obtained from all participants in this research before sample collection. Ethical approval for this study was provided by Ethics and Research Committee (no. 34065/8/20), Faculty of Medicine, Tanta University.

Specimen collection and transport:

The exudate from the lesion was taken with a sterile swab under complete aseptic conditions, transferred into a sterile container with Amies transport medium inside and then was transported immediately to the laboratory of Medical Microbiology and Immunology Department for microbiological analysis and processing. Each sample was assigned a code number to ensure participant privacy and data confidentiality.

Processing of specimens and identification of the isolates:

All samples were cultured on nutrient agar, MacConkey agar, blood agar, Sabaroud dextrose agar, mannitol salt agar and bile eschulin agar (Oxoid UK). Gram-stained smears were prepared and microscopically examined after culturing the plates to avoid the possibility of contaminating the samples. All cultivated plates were incubated at 37°C for 24 hr. After incubation, the plates were observed for growth and the isolated colonies were identified phenotypically by morphological and biochemical characteristics[10].

Antimicrobial susceptibility testing:

All bacterial isolates were subjected to antibiotic susceptibility testing by Kirby-Bauer disc diffusion method on Muller Hinton agar plate (Oxoid, UK), results were interpreted according to the clinical laboratory standard institute guidelines [11]. Gram positive organisms were tested against the following: Cefoxitin (30 µg), Ciprofloxacin (5 µg), Doxycycline (30 µg), Erythromycin (15 µg), Fusidic acid (10 µg), Gentamycin (10 µg), Linezolid (30 µg), Oxacillin (1 µg), Penicillin G (10 units), Teicoplanin (30 µg), Vancomycin (30 µg). Gram negative organisms were tested against the following: Amikacin (30 µg), Ampicillin-sulbactam (10/10 µg), Aztreonam (30 µg), Cefepime (30 µg), Cefotaxime (30 µg), Cefoxitin (30 µg), Ceftazidime (30 µg), Ciprofloxacin (5 µg), Colistin (10 µg), Gentamycin (10 µg), Imipenem (10 µg), Levofloxacin (5 µg), Meropenem (10 µg), Piperacillin - Tazobactam (100/10 µg), Tobramycin (10µg).

Biofilm Formation Assay:

Biofilm production was determined for isolates by using tissue culture plate method. Biofilm formation was examined by the semi-quantitative determination of biofilm formation in 96-well flat bottom plates. Briefly, fresh bacterial suspensions were prepared in brain heart infusion broth **(Oxoid, UK)** from overnight cultures and adjusted to OD570(optical density) of 0.1 (~ 10^7 CFU/mL). 100 µL aliquots of bacterial suspension were then inoculated into individual wells of a 96-well microtitre plate and incubated overnight at 37°C for 48h. Following overnight incubation, plates were gently washed with distilled water and stained with 100 µL of 0.1% Crystal Violet (CV) for 30 min at room temperature. Excess crystal violet was removed by washing, and biofilm was quantified by measuring the corresponding OD570 nm of the supernatant following the solubilization of CV in 95% ethanol. For each clinical strain tested, biofilm assays were performed in triplicate and the mean biofilm absorbance value was determined. Strains that formed biofilms ≥ OD570 of the positive control were positive for biofilm formation [12].

Statistical analysis:

Statistical analysis of data was done using the Statistical Package for the Social Sciences (SPSS), version 20 (Armonk, NY: IBM Corp). Quantitative variables were presented by the mean and standard deviation (SD). Categorical variables were described by the number and the percentage. The statistical significance of the differences of quantitative variables among the study groups was determined by the student's t-test. Chi-square test was used for qualitative variables. All P-values less than 0.05 were considered statistically significant. When P-value was less than 0.001, it was considered highly significant. P-values above 0.05 were considered statistically not significant.

RESULTS

Among the 50 specimens included in this study as shown in (table 1), there were 26 females and 24 males distributed among cases as following in the form of numbers and percentages. The mean age of diabetic foot ulcer cases was 62.73± 10.66, pressure sore cases 48.50± 6.42, venous ulcer cases 48.67±9.30, surgical site infection cases 36.17±5.49, burn cases 21.50±9.05 and in chronic traumatic wound infection cases 19.40±9.42, with statistically significant difference between them ($p<0.001$). The mean age of all cases was 44.04 ± 13.23 with age ranged from 10 to 75 years.

Table 1: Age and gender distribution among studied cases.

Specimen		Sex				Age			
		Female		Male		Range		Mean	SD
		N	%	N	%				
Diabetic foot ulcer (N=11)		7	26.9	4	16.7	47	75	62.73	10.66
Pressure sore (N=10)		5	19.2	5	20.8	40	62	48.50	6.42
Venous ulcer (N=12)		8	30.8	4	16.7	25	63	48.67	9.30
Surgical site infection (N=6)		1	3.8	5	20.8	30	45	36.17	5.49
Burn infection (N=6)		3	11.5	3	12.5	12	35	21.50	9.05
Chronic traumatic wound infection (N=5)		2	7.7	3	12.5	10	35	19.40	9.42
Total (N=50)		26	52%	24	48%	10	75	44.04	13.23
Tests	X^2/ f	4.946				28.055			
	P-value	0.422				<0.001**			

The type of bacterial growth was shown in figure 1

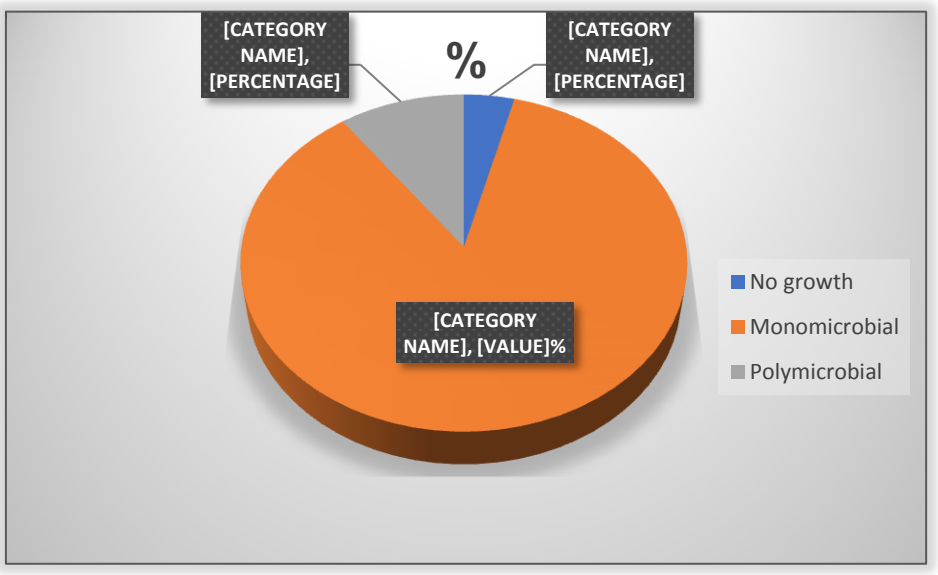

Fig. 1: Type of growth in all specimens., in total 4% of specimens showed no growth, 10% showed polymicrobial growth and monomicrobial growth was detected in 86% of specimens.

Polymicrobial growth was detected in 5 specimens., one of them was isolated from diabetic foot ulcer (*E. coli & Candida spp.*), two were isolated from pressure sores (*E. coli & Candida spp., Staph. aureus & Enterococcus spp.*) and two from venous ulcers (*Pseudomonas spp. & Enterobacter spp., Pseudomonas spp. & Staph. aureus*) as shown in table 2

Table 2: Distribution of polymicrobial growth among different specimens.

Poly microbial growth	Diabetic foot ulcer	Pressure sore	Venous ulcer
Number of specimens	1	2	2
Organisms in first specimen	*E. coli &Candida spp.*	*E. coli &Candida spp.*	*Pseudomonas spp. & Enterobacter spp.*
Organisms in second specimen	-	*Staphylococcus aureus &Enterococcus spp.*	*Pseudomonas spp. & Staphylococcus aureus*

The organisms were isolated from specimens of 6 different categories of chronic skin ulcers., diabetic foot ulcers (12 organisms) with *E. coli* the most frequently isolated, pressure sores (12 organisms) with *Klebsiella spp.*, venous ulcer (14 organisms) with *Klebsiella spp.* the most frequently isolated, surgical site infections (6 organisms), burn (6 organisms) with predominance of *pseudomonas spp.* and chronic traumatic wound infections (3 organisms). Among all specimens, the most frequently isolated organism was *Pseudomonas spp. (*26.42%). The result is shown in table (3)

Table 3: Distribution of Organisms isolated from different chronic ulcerative lesions.

Organism	Type of specimen													
	Diabetic foot ulcer		Pressure sore		Venous ulcer		Burn infection		Surgical site infection		Chronic traumatic wound infection		Total	
	N	%	N	%	N	%	N	%	N	%	N	%	N	%
E. coli	5	41.67	2	16.67	0	0	0	0	1	16.67	1	20.0	9	16.98
Klebsiella spp.	1	8.33	3	25.00	7	50	0	0	1	16.67	1	20.0	13	24.52
Candida spp.	1	8.33	1	8.33	0	0	0	0	0	0	0	0	2	3.8
Pseudomonas Spp.	3	25.00	2	16.67	4	28.57	5	83.33	0	0	0	0	14	26.42
Staphylococcus aureus	0	0	2	16.67	1	7.14	1	16.67	2	33.33	1	20.0	7	13.21
Enterobacter spp.	0	0	0	0	2	14.29	0	0	0	0	0	0	2	3.8
Proteus spp.	2	16.67	1	8.33	0	0	0	0	0	0	0	0	3	5.66
Enterococcus spp.	0	0	1	8.33	0	0	0	0	2	33.33	0	0	3	5.66
Total	12	100.0	12	100.0	14	100.0	6	100.0	6	100.0	3	100.0	53	100

Klebsiella spp, when comparted to other Gram-negative isolates, showed the highest resistance to imipenem (92.31%), meropenem (84.62%), ceftazidime (100%), ciprofloxacin (100%), piperacillin tazobactam (92.31%), cefotaxime (92.31%), cefoxitin (84.62%), cefipime (92.31%), and levofloxacin (100%). *E. coli* showed the highest resistance to aztreonam (100%), ampicillin/sulbactam (100%), and colistin (44.44%). While *Pseudomonas spp* showed the highest resistance to amikacin (71.43%), tobramycin (85.71%) and gentamycin (78.57%). As shown in table (4)

Table 4: Antibiotic resistance pattern in gram negative organisms.

Organism / Antibiotic		*E. coli* N = 9		*Klebsiella spp.* N = 13		*Pseudomonas spp.* N=14		*Enterobacter spp.* N= 2		*Proteus spp.* N=3	
		N	%	N	%	N	%	N	%	N	%
Imipenem	R	7	77.78	12	92.31	12	85.71	0	0	2	66.67
Meropenem	R	5	55.56	11	84.62	11	78.57	0	0	1	33.33
Amikacin	R	5	55.56	9	69.23	10	71.43	0	0	1	33.33
Ceftazidime	R	7	77.78	13	100	13	92.86	1	50	2	66.67
Ciprofloxacin	R	8	88.89	13	100	12	85.71	1	50	0	0
Aztreonam	R	9	100	11	84.62	12	85.71	1	50	1	33.33
Ampicillin/Sulbactam	R	9	100	11	84.62	-	-	-	-	1	33.33
piperacillin/Tazobactam	R	6	66.67	12	92.31	11	78.57	0	0	1	33.33
Gentamycin	R	7	77.78	4	30.77	11	78.57	0	0	0	0
Cefotaxime	R	5	55.56	12	92.31	-	-	1	50	1	33.33
Cefoxitin	R	7	77.78	11	84.62	-	-	-	-	1	33.33
Cefipime	R	7	77.78	12	92.31	11	78.57	1	50	1	33.33
Levofloxacin	R	8	88.89	13	100	12	85.71	1	50	0	0
Colistin	R	4	44.44	1	7.69	5	35.71	0	0	-	-
Tobramycin	R	6	66.67	10	76.92	12	85.71	1	50	2	66.67

As shown in table (5), all isolates of *Staph. aureus* showed resistance (100%) to penicillin, oxacillin and cefoxitin, 71.43% were resistant to teichoplanin, 57.14%were resistant to erythromycin, 28.57% were resistant to ciprofloxacin and gentamycin, 42.86% of *Staph.aureus* isolates showed resistance to vancomycin and fusidic acid, 14.29 % of *Staph. aureus* isolates showed resistance to linezolid and doxycycline. 66.67% of *Enterococci* showed resistance to penicillin G and erythromycin, 33.33% showed resistance to the remaining antibiotics.

Table 5: Antibiotic resistance pattern in gram positive organisms.

Organism / Antibiotic		*Staphylococcus aureus* N=7		*Enterococcus spp.* N=3	
		N	%	N	%
Penicillin G	R	7	100	2	66.67
Oxacillin	R	7	100	-	-
Vancomycin	R	3	42.86	1	33.33
Linezolid	R	1	14.29	1	33.33
Cefoxitin	R	7	100	-	-
Doxycycline	R	1	14.29	1	33.33
Erythromycin	R	4	57.14	2	66.67
Gentamycin	R	2	28.57	1	33.33
Ciprofloxacin	R	2	28.57	1	33.33
Teicoplanin	R	5	71.43	1	33.33
Fusidic acid	R	3	42.86	1	33.33

As regard the biofilm forming assay in our study, it was found that 58.5 % out of all isolates were biofilm former. *Pseudomonas spp.* was the most frequently biofilm forming organism followed by *E. coli*, *Klebsiella spp*., *Staph. aureus*. *Proteus spp.* and *Enterococcus spp.* were the least frequently biofilm forming organisms (33.3% each), *Candida spp.* and *Enterobacter spp.* haven't produced biofilm (Table 6).

Table 6: Biofilm formation assay of all isolated pathogenic organisms:

	Biofilm formation assay		
	Biofilm producers (%)	**Non biofilm producers (%)**	**total**
E. coli	6(67%)	3(33%)	9
Klebsiella spp.	8(61.5%)	5(38.5%)	13
Pseudomonas spp.	10(71.4%)	4(28.6%)	14
Proteus spp.	1(33.3%)	2(66.7%)	3
Candida spp.	0 (0%)	2 (100%)	2
Staph.aureus	3(42.9%)	4(57.1%)	7
Enterococcus spp.	1(33.3%)	2(66.7%)	3
Enterobacter spp.	0 (0%)	2 (100%)	2
Total	31(58.5%)	22(41.5%)	53

DISCUSSION

This study was carried out on 50 samples. Regarding age and sex distribution in this study, it was found that the age range was 10-75 years with the mean age 44.04 ± 13.23, 26 were females (52%) and 24 were males (48%), this is more or less similar to a study performed by Upreti et al., [13], Janssen et al., [14]. On the other hand, a study in Ethiopia by Mama et al., [15] showed that, out of 150 specimens obtained from wound infections, there were 107 (71.3%) males and 43 (28.7%) females. The ages of the patients ranged from 6 months to 90 years with mean age of 31.68 ± 17.12.

Regarding the type of growth in the current study, it was more or less like other studies by Mama et al., [15], Asres et al., [16], and Upreti et al., [13]. On the other hand, in another study by Janssen et al.,[14] it was reported that, of the 67 wound swab samples, infection was monomicrobial in 17 (25.4%) and polymicrobial in 50 (74.6%). The variances in age, sex, and type of growth between studies could be related to differences in sample collection locations, predominant patient type, patient characteristics, numbers of specimens collected, and environmental circumstances. Concerning the type of the isolates, our results were more or less consistent with another study performed by Elnahal et al., [17]. On the contrary, it was reported in another Egyptian study by Hosny et al.,[18] that, of the 150 tested wound isolates, the most isolated organism was *Staphylococcus aureus*, followed by *Klebsiella pneumoniae*.

Regarding the antimicrobial susceptibility results of all isolates in this study, it was found that *Pseudomonas*

spp., showed the least resistance to colistin, amikacin, gentamycin, meropenem, cefepime and piperacillin-tazobactam. This is quite like a study by Santella et al.,[19]. In case of *Klebsiella spp*., our work showed that the most efficient antimicrobial agents with the least resistance rate were colistin, gentamycin and amikacin. In case of *E. coli*, colistin, amikacin, meropenem and cefotaxime were the most effective antimicrobial agents. Our results were in accordance with the reports of Alfouzan et al.[20] and Sader et al.[21]. In the present study, it was found that *proteus spp*. revealed the most sensitivity to levofloxacin, ciprofloxacin, gentamycin, ampicillin-sulbactam, cefoxitin, cefepime, cefotaxime, piperacillin- tazobactam, aztreonam, amikacin and meropenem which agree with the study by Bahçeci[22] .In this study it was found that *Enterobacter spp*., showed the least resistance to imipenem, meropenem, piperacillin- tazobactam, colistin, gentamycin and amikacin, this is more or less similar to previous reports [23,24] .

As regard gram positive isolates in our research, *Staph. aureus* showed the least resistance to linezolid and doxycycline followed by ciprofloxacin and gentamycin then vancomycin, fusidic acid and erythromycin which is quite similar to a study performed by Abdelghafar et al., [25]. On the other hand, a relatively higher sensitivity of the *Staph. aureus* isolates were detected to vancomycin, chloramphenicol in a study performed by Abdeen et al., [26] in Egypt. In our study, 66.67% of *Enterococcus spp*. isolates revealed resistance to penicillin G and erythromycin, 33.33% showed resistance to vancomycin, linezolid, doxycycline, gentamycin, ciprofloxacin, fusidic acid and teicoplanin. In another study done by Esmail et al., [27], all isolates of *Enterococcus spp*. were completely resistant to cefepime, ampicillin, and tetracycline, 53.8% of isolates were resistant to vancomycin, and 23.1% to linezolid. In another study by Alam et al., [28] *Enterococcus spp*. showed the least resistance to vancomycin (0%), linezolid (3.3%).

The evolution of this high rate of resistance can be attributed to the uncontrolled extensive use of antibiotics in hospitals and community, and the difference in the sensitivity pattern among the above-mentioned studies can be related to different antibiotic policies, emergence of resistant strains due to empirical use of antimicrobial therapy, the immune status of the patient, different infection control measures, frequent hospitalization, and the transfer of resistance genes by transport means[29].

As regard the biofilm forming assay in our study, it was found that 58.5 % out of all isolates were biofilm former. *Pseudomonas spp.* was the most frequently biofilm forming organism followed by *E. coli*, *Klebsiella spp*., *Staph. aureus*, which differs from a study by Banu et al.,[30] in which among 82 isolates, 46.3% of the isolates showed biofilm formation. *Staph. aureus* was the predominant biofilm former, followed by *Pseudomonas aeruginosa*, *Citrobacter spp. E. coli*, *Proteus spp.* and *Klebsiella spp*. Another study conducted by Hashem et al., [31], informed that biofilm production was higher among *Klebsiella spp. (*72%), *Pseudomonas* (65%) and *E. coli* strains (58%).

In the current study, 71.4% of *Pseudomonas spp.* were biofilm former, this is similar to several studies conducted by Rossi et al., [32], Perez et al., [33]. Zaranza et al., [34]. Vatan et al.,[8]. Our study demonstrated that 67% of *E. coli* isolates formed biofilm, and this is similar to another study by Gawad et al., [35]. In our study, 61.5% of *Klebsiella spp.* isolates were biofilm formers. However, Karimi et al.[36] and Vatan et al.[8] found that 74.5% and 40% *K. pneumoniae* isolates were biofilm former respectively. In the current study, 42.9% of *Staph. aureus* isolates produced biofilm. Which is quite similar to the study performed by Thiran et al.,[37]. In another research performed in Egypt, it was noticed that about all *Staph. aureus* isolates produced biofilms[38]. As regard *Candida spp.*, it showed no biofilm formation in our study, which is quite similar to Gültekin et al.[39] who reported that only two of the *Candida albicans* isolates produced biofilm. On the contrary, Paiva et al.[40] reported that *Candida tropicalis* produced high levels of biofilm.

These discrepancies might be attributed to different sample size, for example in our study there were none biofilm producers among *Candida spp.,* and *Enterobacter spp*., this can be explained by the very small number of isolated organisms, also this may be related to the immune and health status of patients, virulence factors and resistance pattern of isolated organisms, different environments and different types of specimens.

CONCLUSION

Gram negative organisms were the most frequently isolated organisms from chronic ulcerative lesions. Most of the isolated pathogens showed high rate of resistance to most of the tested antimicrobial agents, with high rate of biofilm formation among most of isolates.

This manuscript has not been previously published and is not under consideration in the same or substantially similar form in any other reviewed media. I have contributed sufficiently to the project to be included as author. To the best of my knowledge, no conflict of interest, financial or others exist. All authors have participated in the concept and design, analysis, and interpretation of data, drafting and revising of the manuscript, and that they have approved the manuscript as submitted.

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