

**BIOFILM PRODUCTION AND MULTIDRUG RESISTANT AMONG THE ISOLATES FROM ACUTE AND CHRONIC WOUND INFECTIONS****S.TAMARAISELVI¹, S.R.SWARNA², RADHA MADHAVAN³ AND S.GOMATHI⁴**

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ABSTRACT

Chronic wounds are major burden for the healthcare system due to the delayed wound healing. One important factor associated with the delayed wound healing is the bacterial biofilms that are polymicrobial and are mostly resistant to antibiotics. The present study was done to demonstrate the biofilm formation of the isolates from chronic and acute wound infection and its relation with multi-drug resistance. Pus swabs were collected from 34 patients with acute wound infection and 44 patients with chronic wound infection. Isolation and identification of the specimen was done using standard procedure followed by screening of drug resistance by antibiotic susceptibility testing. Biofilm formation was detected using microtitre plate method. 98.33% (59/60) of the isolates from chronic wound infection were biofilm producer as compared to 72.5% (29/40) isolates from acute wound infection. Significant difference was noted for the isolates from chronic wound infection in association with multi-drug resistance and biofilm formation.

KEYWORDS: Chronic wounds, acute wounds Biofilm, Diabetes mellitus, microtitre plate method

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INTRODUCTION

The increase in lifestyle diseases, such as obesity, diabetes and cardiovascular diseases are leading cause for occurrence of chronic wound throughout the world. In the developed countries, 1-2% of the population experiences a chronic wound in their lifetime¹. Chronic wounds, includes diabetic foot ulcers (DFU), pressure ulcers (PU), and venous leg ulcers (VLU), of which diabetic foot ulceration and infection are a major medical, social, economic problem leading to morbidity. In developing countries like India, 15% of all diabetes patients develop foot ulcer with infection which spreads rapidly leading to tissue destruction and subsequent amputation^{2,3}. In recent years, bacterial biofilms are gaining attention and it was reported that 80% of human infections are mediated by microbial biofilms. One best example is the chronic wounds in which the infections are polymicrobial in nature and known to form biofilms. Biofilms are bacterial aggregates enclosed in an extracellular polymeric matrix and cause delayed wound healing. Moreover, biofilm forming bacteria are resistant to antibiotics and escape host immune response. The present study was done to demonstrate the biofilm formation by the bacterial isolates from chronic and acute wound infection and its relation with multi-drug resistance.

MATERIALS AND METHODS

The prospective study was carried out in a tertiary care hospital near Chennai during the period of March 2011 to March 2012. Institutional ethical clearance was obtained. Informed consent was collected. Pus swabs were taken from patients with acute wound infection (n=34) which heals in less than 4 weeks' time and chronic wound infection (n=44) that takes more than 4 weeks to heal.

(i) Processing of specimens

Pus swabs were collected after cleaning the wound with sterile saline. Direct examination was made and inoculated on to blood agar, nutrient agar and Mac conkey agar and incubated at 37°C, overnight in aerobic atmosphere. Isolates were identified using standard procedure⁴.

(ii) Antibiotic susceptibility tests

Antibiotic susceptibility tests by disc diffusion method were performed as per the CLSI guidelines⁵. A microorganism was classified as multidrug resistant if it was found to be resistant to two or more classes of antimicrobials. The following antibiotic disks were employed : Cefoxitin (30µg), Cefuroxime(30µg), Cephalothin (30µg), Ampicillin(10µg), Ciprofloxacin (5µg), tetracycline (10µg), Chloramphenicol (30µg), Clindamycin(2µg), Co-trimoxazole(25µg), Erythromycin (15µg), Linezolid(30µg), Ofloxacin (5µg), Amikacin (30µg), Cefepime(30µg), Cefeperazone/Sulbactam (30/10µg), Ceftazidime/Clavulanate (30/10µg), Gentamicin (10µg), Imipenem (10µg), Netilmycin (30µg), Piperacillin/Tazobactam(100/10µg), and Tobramycin (10µg).

(iii) Biofilm detection by Microtitre plate method

The Biofilm formation was detected in plastic microtitre plates as described by Stepanovic et al⁶. On a sterile 96 well flat bottomed polystyrene microtitre plate (NUNC, Denmark), two hundred and thirty microlitre of Trypticase Soya Broth (TSB) was added. 20 µl of overnight bacterial culture was added into corresponding well (each strain in successive three wells). The negative control wells contained broth only. The plates were incubated aerobically for 24 hours at 35°C. The content of the plates were discarded and the wells washed three times with 300 µl of sterile distilled water. The bacteria adhering to the wells were fixed with 250 µl of methanol per well for 15 minutes. Then the microtitre plates were stained with 250 µl per well of crystal violet for 5 minutes. Excess stain was removed by washing with sterile distilled water and air dried. The dye bound adherent cells were dissolved with 250 µl of 33% (v/v) glacial acetic acid per well. The optical density (O.D.) of each well was measured at 490nm using an automated multiskan ELISA reader (Bio Rad, France). The tests were carried out in triplicate and the results were averaged. The cut-off O.D (O.D.c) was determined as three standard deviations above the mean O.D. of the negative control. Strains were classified as (i) no biofilm producer [O.D.≤ O.D.c]; Weak biofilm producer [O.D.c< O.D.≤(2xO.D.c)]; Moderate biofilm producer[(2xO.D.c)<O.D.≤ (4x O.D.c)]; Strong biofilm producer [(4xO.D.c)< O.D.]. Differences in the degree of biofilm formation were examined by the Friedman test, followed by the Wilcoxon signed ranks test. P<0.05 were considered significant.

RESULTS

1. Demographic data

In a total of 78 patients, 34 were with acute wound infections and 44 were with chronic wound infections. Among the 34 patients with acute wound, 71% (24/34) were males and 29% (10/34) were females where as 80% (35/44) were males and 20% (9/44) were females in chronic wound infections. Male patients were higher in both acute and chronic wound infections than females.

2. Isolation and identification

There was an equal distribution of Gram positive cocci (20/40; 50%) and Gram negative bacilli (20/40; 50%) among the bacterial isolates from acute wound infection. In chronic wound infections, Gram negative bacilli accounted for 63.93% (39/61) and Gram positive cocci for 36.06% (22/61). *Pseudomonas aeruginosa* was the most common isolate, accounting for 21.31% (13/39); followed by *Esch.coli*, *Staphylococcus aureus* and *Enterococcus spp* comprising of 19.67%(12/39), 16.39 (10/22) and 13.11% (8/22) respectively (Table I).

3. Antibiotic susceptibility testing

The antibiotic resistance pattern of the Gram positive cocci and Gram negative bacilli obtained from acute and chronic wound infection are shown by graphical representation (Figure I & Figure II). There was no significant difference between the antibiotic resistant pattern between GPC from acute and chronic wound infection. However, 83.87% (22/26) of *Staphylococcus*

aureus were cefoxitin resistant i.e., Methicillin resistant *S.aureus* (MRSA). All the *Staphylococcus aureus* isolates were sensitive to linezolid except for two

isolates, one each from acute and chronic wound infection. There was a significant difference in antibiotic

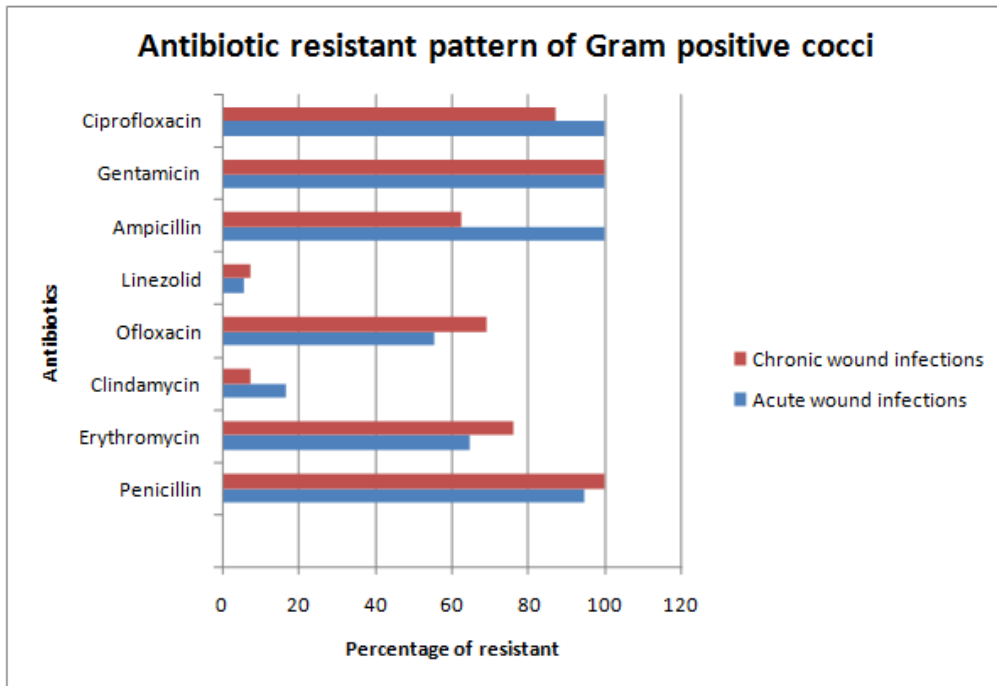
Table I
Aerobic bacterial profile of Acute and Chronic wound infections (n=101)

Total number of isolates	Isolates from	
	Acute wound infection (n=40)	Chronic wound infection (n=61)
Gram positive organisms		
<i>Staphylococcus aureus</i>	16(40%)	10(16.39%)
<i>Enterococcus spp</i>	2(5%)	8(13.11%)
CONS	2(5%)	3(4.91%)
<i>Streptococci</i>	-	1(1.64%)
Gram negative organisms		
<i>Pseudomonas spp</i>	8(20%)	13(21.31%)
<i>Esch. coli</i>	5(12.5%)	12(19.67%)
<i>Proteus spp</i>	2(5%)	5(8.21%)
<i>Klebsiella pneumoniae</i>	1(2.5%)	4(6.55%)
<i>Acinetobacter spp</i>	1(2.5%)	3(4.91%)
<i>Citrobacter spp</i>	3(7.5%)	1(1.64%)
<i>Enterobacter spp</i>	-	1(1.64%)
Total	40	61

resistance pattern between Gram negative isolates from acute and chronic wound infections for all the antibiotics used except for piperacillin/tazobactam and

ceftazidime/clavulnate. Among the multi-drug resistant Gram negative bacilli, 24% (14/59) were ESBL and 14.75% (9/59) were carbapenamase producers.

Figure I
Antibiotic resistant pattern of Gram Positive Cocci



4. Biofilm detection by microtitre plate method

40 isolates from acute wound infections (n=34) and 60 from chronic wound infections (n=44) were evaluated for biofilm production. Figure III represents the biofilm formation of the isolates from acute and chronic wound

infection by Microtiter plate method. 98.33% (59/60) of the isolates from chronic wound infection were biofilm producer as compared to 72.5% (29/40) isolates from acute wound infection (Table II).

Figure II
Antibiotic resistant pattern of Gram Negative Bacilli

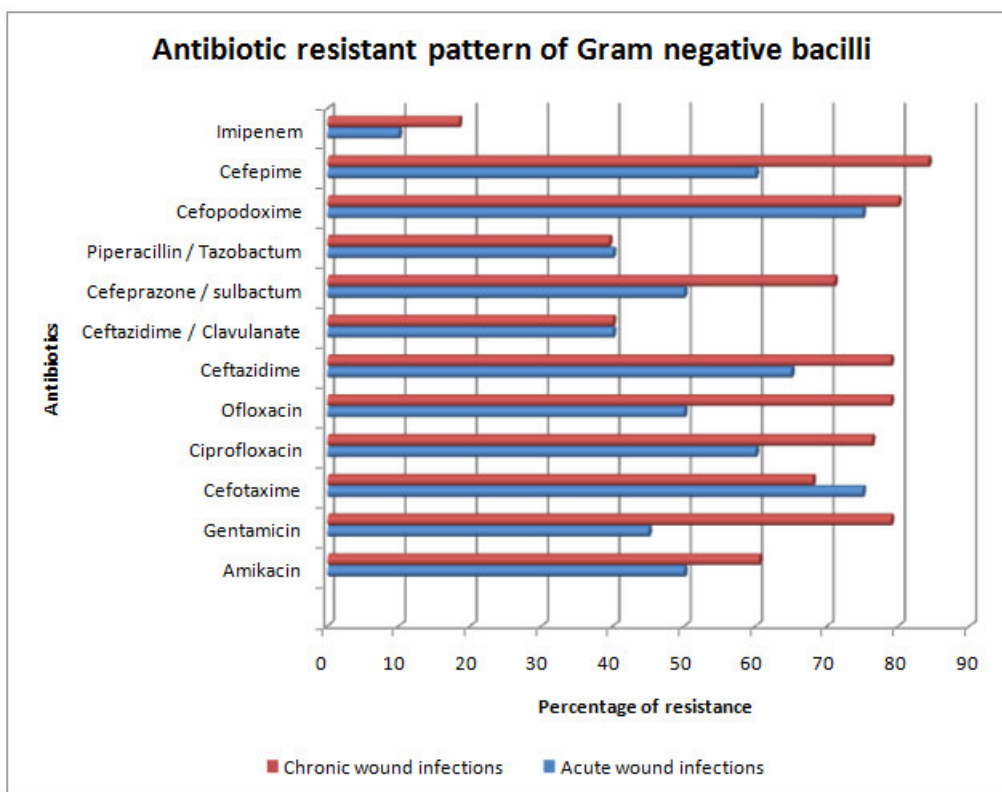


Figure III
Detection of biofilm formation by microtitre plate method

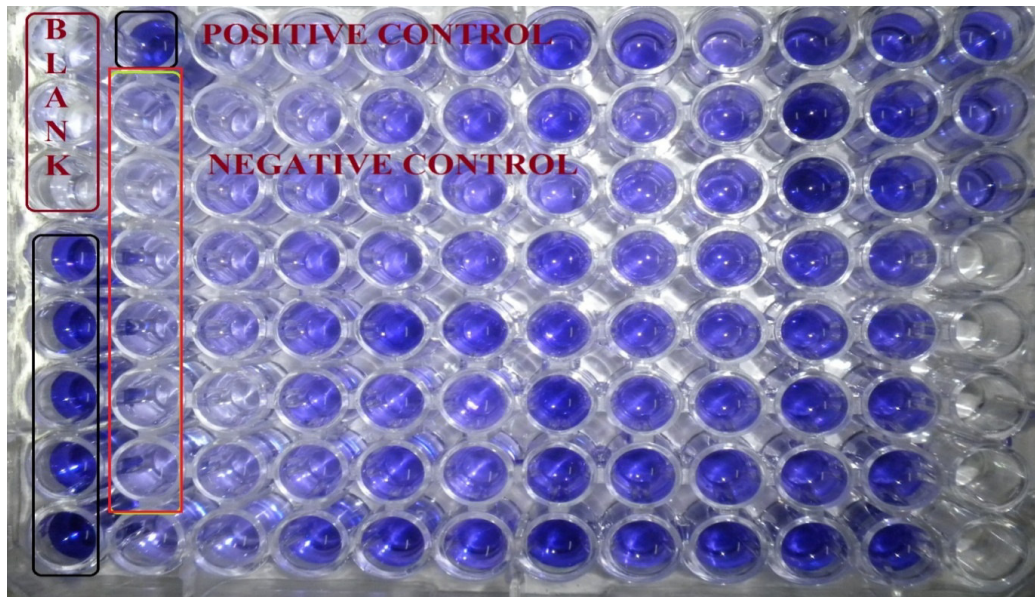


Table II
Biofilm production of isolates from acute and chronic wound infection

Biofilm Production	Acute wound infection	Chronic wound infection
Strong biofilm producers	n=19 <i>Staphylococcus aureus</i> (6), <i>Pseudomonas spp</i> (7), <i>Esch. coli</i> (1), <i>Klebsiella pneumoniae</i> (1), <i>Citrobacter spp</i> (3), <i>Proteus spp</i> (1)	n=28 <i>Staphylococcus aureus</i> (3), <i>Enterococcus spp</i> (4), <i>Pseudomonas spp</i> (10), <i>Acinetobacter spp</i> (2), <i>Esch. coli</i> (2), <i>Klebsiella pneumoniae</i> (1), <i>Citrobacter spp</i> (1), <i>Enterobacter spp</i> (1), <i>Proteus spp</i> (4).
Moderate biofilm producers	n=4 <i>Staphylococcus aureus</i> (1), <i>Pseudomonas spp</i> (1), <i>Acinetobacter spp</i> (1), <i>Esch. coli</i> (1)	n=19 <i>Staphylococcus aureus</i> (2), CONS (2), <i>Enterococcus spp</i> (3), <i>Pseudomonas spp</i> (3), <i>Acinetobacter spp</i> (1), <i>Esch. coli</i> (6), <i>Klebsiella pneumoniae</i> (1), <i>Proteus spp</i> (1).
Weak biofilm producers	n=6 <i>Staphylococcus aureus</i> (1), CONS (2), <i>Esch. coli</i> (2), <i>Proteus spp</i> (1).	n=12 <i>Staphylococcus aureus</i> (4), CONS (1), <i>Enterococcus spp</i> (1), <i>Esch. coli</i> (4), <i>Klebsiella pneumoniae</i> (2)

(Biofilm detection was done for all the isolates except *Streptococcus spp*)

Table III represents the association of biofilm forming isolates from acute and chronic wound infection with multi-drug resistant organisms. When chi square test was applied, statistically significant difference ($p <$

0.0001) was noted among the isolates of chronic wound infection for the correlation of biofilm formation and multi-drug resistance when compared with the isolates from acute wound infection.

Table III
Correlation of biofilm forming isolates with multi drug resistance

Biofilm formation by microtitre plate method	Acute wound infection (n=40)		Chronic wound infection (n=60) except <i>Streptococcus spp</i>	
	MDRO (n=31)	Non-MDRO (n=9)	MDRO (n=46)	Non-MDRO (n=14)
Biofilm production	67.74% (21/31)	88.88% (8/9)	97.82% (45/46)	100% (14/ 14)
Non- biofilm production	32.25% (10/31)	11.11% (1/9)	2.17% (1/46)	0

DISCUSSION

The characteristic feature of chronic wound infection is the presence of polymicrobial flora. This heterogenous

nature of the chronic wound infection is the major cause for longer hospital stay. The ability of the colonizing microflora in the chronic wound to establish, proliferate and encase themselves in an extracellular polymeric

matrix to form a biofilm constitute the key virulence factor for delayed wound healing. In the present study, except for *Streptococcus pyogenes* and *Enterobacter spp*, the bacterial profile of acute and chronic wound infection were same. Some studies on bacterial profile of chronic wound infection also shows similar findings of bacteria such as *Pseudomonas aeruginosa*, *Esch.coli*, *Staphylococcus aureus*, *Enterococcus spp*, *Proteus spp*, *Klebsiella spp*, coagulase negative *Staphylococci*, *Acinetobacter spp*, *Citrobacter spp* and *Enterobacter spp* as frequently encountered isolates^{7,8,9,10}. 83.87% of the *Staphylococcus aureus* were MRSA. This is very high when compared to other studies on diabetic foot infections which have reported only 10-65.5% MRSA^{2, 3, 10}. 80% (4/5) of CONS were also resistant to ceftoxitin. 1/10 (10%) isolates of *Enterococcus spp* were screen positive for vancomycin resistant by antibiotic susceptibility testing. However, confirmation was not done by MIC. One study has reported that 6.5% MRSA/VRE cocolonisation in the chronic wound infection¹¹. It was suggested that the emergence of vancomycin resistant *Staphylococci* was due to the increased use of it in the treatment of infection caused by Methicillin resistant *Staphylococci* and *Enterococci*^{12, 13}. In the present study, it was observed that 24% (14/58) of the members of Enterobacteriaceae were producing ESBL. Priyadharshini et al¹⁴ have reported ESBL production in 36.3% of bacterial isolates whereas Gadepalli et al³ have documented ESBL production in 44.7% of bacterial isolates. 14.75% (7/58) of the isolates were resistant to carbapenem in the present study. However, a study from Chennai has reported 45.4% isolates as carbapenem resistant¹⁴. Biofilm formation is a major virulence factor contributing to the chronicity of infections. In the present study, by microtitre plate method, biofilm formation was observed in 72.5% (29/40) isolates from acute wound infection and 98.33% (59/60) of isolates from chronic wound infection. Significant difference was observed between isolates from acute and chronic wound infection. 19/40 (47.5%) isolates of acute wound infection and 28/60 (46.66%) isolates from chronic wound infection were strong biofilm producers. Only 4/40 (10%) isolates from acute wound infection were moderate biofilm producers whereas 19/60 (31.66%) were from chronic wound infection. 6/40 (15%) of acute wound isolates and 12/60 (20%) of chronic wound isolates were weak biofilm producers. In our previous study, 70.73% of isolates from DFU were strong BF producer¹⁵. The results of our study correlates with the findings of a study which showed statistically significant difference (P < 0.001) in biofilm formation between the isolates from chronic and acute wound infection¹⁶. Srinivasa Rao et al¹⁷ have reported that 70.96% of *Acinetobacter baumannii* isolates obtained from wound infection were strong biofilm producers. Sanchez et al¹⁸ found that the clinical strains isolated from non-fluid sites such as superficial /

deep tissue, bone and respiratory tract could able to form higher proportion of biofilm than those of isolates from host fluids including blood or urine. They also suggested that the formation of biofilm reflects the adaptation favouring the survival of organisms in non-liquid physiologic environments like solid tissues thereby enabling difficulty in eradication and contributes to the chronicity of infections. Some other studies used technique other than semiquantitative microtitre plate method like ConA, Immunofluorescent staining with confocal scanning laser microscopy (CLSM), PNA-FISH and anti-alginate antibodies to demonstrate biofilm formation by *S.aureus* and *P.aeruginosa* from wound infections^{8, 19}. Biofilm mode of growth provides bacteria characteristics difference from those of their free or planktonic bacteria, such as protection against the activities of the host immune system and increased tolerance to antimicrobial treatment⁹. Therefore, bacterial isolates capable of forming biofilms are more often found to be a MDR phenotype and leads to an increased length of stay, with a higher mortality rate and cost. In the present study, the prevalence of MDRO was 77% (77/100). Other studies have reported MDRO in the range of 22% - 85%^{20, 21, 22}. Of the 40 isolates from acute wound infection, 67.74% were MDRO and biofilm producers whereas among the 60 isolates from chronic wound infection, 97.82% were MDRO and biofilm producers. A significant difference was noted between biofilm formed from MDRO isolates from that of Non – MDRO isolates of chronic wound infection. Similarly, few other studies have previously reported that there is a strong positive correlation between biofilm formation and presence of ESBL bla PER-1 among the *Acinetobacter* species and *P.aeruginosa*^{17, 23, 24}. In one study, biofilm formation was compared between ESBL producers and non-ESBL producers and suggested that ESBL producing isolates had increasing cell surface hydrophobicity as a function of bacterial physiology resulting in number of chromosomal rearrangement. Such expression of several virulence genes might be responsible for severity of infection and leads to higher mortality²⁵. Delissalde et al²⁶ have demonstrated that *P.aeruginosa* capable of forming biofilm after 24 hours emphasizing the correlation between biofilm formation and multi-drug resistance.

CONCLUSION

Current study suggests the significant association of biofilm formation and multi-drug resistant phenotype with the isolates obtained from chronic wound infection. Hence, proper screening of MDRO and detection of biofilm formation is essential to manage the situation by use of appropriate antibiotics. This might help to reduce the morbidity and also their length of stay in the hospital.

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