ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 9, Issue, 08, pp.55767-55777, August, 2017

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

MICROBIOLOGICAL PROFILE OF SKIN GRAFT INFECTIONS WITH SPECIAL REFERENCE TO BIOFILM FORMATION

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ARTICLE INFO

Article History:
Received 19th May, 2017
Received in revised form
10th June, 2017
Accepted 15th July, 2017
Published online 31st August, 2017

Key words:

Skin graft infection,
Biofilm.

ABSTRACT

Background: Infection of the skin graft is the common complication leading to failure of the graft. Successful skin graft "TAKE" is less likely to occur on experimental or clinical wounds that contain more than 10^5 viable bacteria per gram of tissue. Skin graft infection with organisms forming biofilm puts a great financial burden on patients and could lead to morbidity and mortality. Appropriate pre and post operative wound care, adequate antibiotic therapy and surgical strategies must be described to eradicate infection. For the above reason this study was done.

Methods: It is a prospective cross sectional study where 100 patients having skin graft infection were included in the study from Feb 2014 – July 2015; The study was conducted in the department of Microbiology B&LCH, samples were taken from patients attending plastic surgery department.

Results: Skin grafting done for burn wounds was the one most commonly affected
both in males and females .*Pseudomonas aueriginosa* was the organism most commonly isolated and also the organism causing biofilm followed by *Methicillin resistant staphylococcus aureus* and *Klebsiella* spp. No anaerobes and fungal species were isolated. Unhygienic practices and Diabetes Mellitus were found to be important risk factors. Biofilm forming organisms were commonly associated with antibiotic resistance, so even aggressive antibiotic therapy was also inadequate to eliminate infection. Conservative surgical treatment (debridement) was necessary.

Conclusion: The gram negative bacteria are more predominant as causative agents of skin graft infections than the gram positive bacteria. The commonest organism causing skin graft infection and forming biofilm is pseudomonas species. Appropriate pre and post operative wound care ,adequate antibiotic therapy, and surgical strategies must be described to eradicate infection.

Citation: Dr. Manasa, S. and Dr. Asima Banu 2017. "Microbiological profile of skin graft infections with special reference to Biofilm formation", *International Journal of Current Research*, 9, (08), 55767-55777.

INTRODUCTION

Skin is the largest organ of the human body and has a number of essential functions. It forms a protective barrier against pathogens and the internal and external environment. It acts as a water resistant barrier so that essential nutrients are not washed out of the body. It provides a dry and semi-permeable barrier to fluid loss. Langerhan cells in the skin are part of the adaptive immune system. The skin plays an important role in sensation and contains a number of nerve endings that respond to heat and cold, vibration, pressure, touch and pain. Thermoregulation is another essential function of the skin. Finally, the skin also plays a vital role in the synthesis of Vitamin D. It is imperative that skin cover is preserved in humans for all the reasons mentioned above. Skin graft is one of the most indispensable techniques in plastic surgery and dermatology. Since Reverdin first performed skin auto transplantation in 1869 (Reverdin, 1871), many pioneers have tried to improve the results of grafting (Blair and Brown, 1929). In 1929, Brown *et al*. established their technique of split-thickness skin grafting, and they differentiated between full-thickness, intermediate thickness, and epidermal (Thiersch) grafts, pointing out the advantages and disadvantages of each. These fundamental principles of skin grafting still hold true today. Skin grafts are used in a variety of clinical situations, such as traumatic wounds, defects after oncologic resection, burn reconstruction, scar contracture release, congenital skin deficiencies, hair restoration, vitiligo, and nipple-areola reconstruction 7. Skin grafts are generally avoided in the management of more complex wounds. Conditions with deep spaces and exposed bones normally require the use of skin flaps or muscle flaps. The clinical outcome of skin grafting depends on a variety of factors, some more substantiated than others. Only a limited number of publications on skin graft loss due to infection exist and they are mostly related to the management of burn wounds. A number of the studies have

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focused on the importance of quantitative rather than qualitative bacteriology others vice versa. From the literature it can be deducted that a successful skin graft ''take'' is less likely to occur on experimental or clinical wounds that contain more than 10^5 viable bacteria per gram of tissue (Krizek *et al*., 1967). The majority of publications discussing qualitative bacteriology point out haemolytic streptococci in particular Streptococcus pyogenes as the predominant species leading to graft lysis (Bang *et al*., 1999). Graft lysis due to non-group A beta-haemolytic streptococci has also been reported (Wilson *et al*., 1988) together with Staphylococcus aureus (Gilliland *et al*., 1988) and Pseudomonas Aeruginosa (McGregor and McGregor, 2000). Skin graft failure due to P. aeruginosa is not a novel hypothesis. It was proposed in 1951. Since then, there have been only few articles published concerning this issue. The hypothesis was confirmed by Gilliland *et al*. (1988) more than 20 years ago, stating the isolation of Pseudomonas from an ulcer prior to skin grafting significantly impairs skin graft take. Despite this knowledge it still seems to be a problem of high relevance. The major pathogenic factor of organisms causing skin graft infection is the ability to form biofilm on polymeric surfaces. Microorganisms growing in a biofilm are intrinsically more resistant to antimicrobial agents than planktonic cells. High antimicrobial concentrations are required to inactivate organisms growing in a biofilm, as antibiotic resistance can increase 1,000 fold. According to a publication by the National Institutes of Health, more than 80% of all infections involve biofilms. Skin graft infection with organisms forming biofilm puts a great financial burden on patients and hospital resources and could lead to morbidity and mortality. Appropriate pre and post operative wound care, adequate antibiotic therapy and surgical strategies must be described to eradicate infection. For the above reason this study was done.

MATERIALS AND METHODS

Source of data

It is a prospective cross sectional study where 100 patients having skin graft infection shall be included in the study from feb2014 to july 2015.The study will be conducted in the department of Microbiology B&LCH, samples are taken from patients attending plastic surgery department of all teaching hospitals attached to Bangalore Medical College & Research Institute, Bangalore.

Inclusion criteria

All patients having skin graft infection are included for the study.

Exclusion criteria

- Patients co infected with HIV, HBSAg
- Patients having malignancy

MATERIALS AND METHODS

Samples for microbiological examination will be collected from secretions adjacent to the infected graft by sterile cotton swabs i.e 3 swabs are collected from each patient and immediately transferred to the microbiology lab. The specimens will be processed immediately first by inoculating onto media culture and then direct smear examination by Gram's Stain to avoid contamination.

The samples will be processed by inoculating onto the following media:

i. 5% sheep blood agar and mackonkey agar, incubated at 37^0 C aerobically for 24 hrs.
ii Chocolate agar, incubated at 37^0 C in the presence of 5-10% of CO_2 in a candle jar for 24hrs.
iii SDA incubated one at 37^0 C and other at 28^0C aerobically For 30 days.
iv Brain heart infusion broth 5-10 ml, incubated at 37^0 C aerobically
v Anaerobic culture will be done using Robertson's cooked meat media and incubated at 37^0 C.

The culture on blood agar, chocolate agar will be incubated for 48 hours and if there is no growth, these media will be incubated for 8 more days to allow the growth of slow growing or fastidious organisms. The cultures for anaerobic bacteria will be incubated for upto 2 weeks for any evidence of growth. The control strains used were Escherichia Coli ATCC 25922, Pseudomonas aeruginosa, ATCC 27853 and Staphylococcus aureus ATCC 25923. Controls were put up as recommended. Reference strain of positive biofilm producer *Staphylococcus epidermidis* ATCC 35984, *Staphylococcus aureus* ATCC 35556, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 35218 and *Staphylococcus epidermidis* ATCC 12228 (non-slime producer) were used as control.

Direct smear

Two smears were prepared on a sterile glass slides and air dried. The air dried smears were fixed in 95% methanol prior to staining. Gram's stain was done and smears were examined for the presence of pus cells and organisms.

Criteria for laboratory confirmed growth

1) Growth on a single media correlating with direct microscopy findings.
2) Growth of the bacteria from the swabs on two or more of the inoculated media.
3) Repeated isolation of the same organisms from two or more specimens of the patients.
4) Semi-confluent growth on one or more solid media at the inoculation site.
5) Any growth in anerobic media.

Based on the gram stain of the culture the isolates were identified by the following tests using standard techniques.

For gram positive cocci in clusters

i. Catalase test
ii. Slide and tube coagulase
iii. Mannitol fermentation
iv. Novobiocin susceptibility
v. Phosphatase test
vi. Pigment production on milk agar.

Identification for gram positive diplococci with lanceolate shape:

i. Colony morphology
ii. Bile solubility

iii. Optochin sensitivity

Gram positive cocci in short chains and pairs indentified by:-

i. Colony morphology and haemolysis on blood agar
ii. Catalase test
iii. Bacitracin sensitivity testing
iv. Aesculin hydrolysis on bile esculin agar
v. Salt tolerance test
vi. Heat tolerance test
vii. Mannitol fermentation
viii. Growth on crystal violet blood agar

Gram positive bacilli with endospores indentified by:

i. Gram stain
ii. Colony morphology
iii. Presence of haemolysis on sheep blood agar
iv. Hanging drop preparation
v. Gelatin hydrolysis
vi. Lecithinase activity
vii. Growth on penicillin agar

Gram positive filamentous bacilli were identified by:

i. Gram stain
ii. Colony morphology
iii. Modified acid –fast stain
iv. Growth in L J medium
v. Hydrolysis of urea
vi. Nitrate reduction
vii. Growth at 45 degree.

Gram positive short bacilli were identified by:

i. Motility testing by hanging drop
ii. Catalase test
iii. Oxidase test
iv. Growth on potassium tellurite agar
v. Sugar fermentation tests

The gram negative bacilli were identified by:

i. Motility testing by hanging drop
ii. Catalase test
iii. Oxidase test
iv. Indole test
v. Urease test
vi. T S I test
vii. Nitrate reduction test
viii. Of test
ix. Sugar fermentation

All the cultures was subjected for antimicrobial susceptibility by means of agar disc diffusion method of Kirby Bauer according to the guidelines of clinical and laboratory standards institute (2014). All the strains of Staphylococcus aureus that were resistant to Ampicillin and Cephalosporins were tested for methicillin resistance on Mueller Hinton agar with 4% NaCl using oxacillin (1μg) discs. The plates were incubated at 30^0 C and reading taken after 24 hours. The antibiotics used are listed in Table 1.

Detection of biofilm formation

Tissue Culture Plate Method

Mathur *et al*. (2006) Isolates from fresh agar plates were inoculated in TSBGlu and incubated for 18 hours at 37^0 C in stationary condition and diluted 1 in 100 with fresh medium. Individual wells of sterile, polystyrene, 96 well – flat bottom tissue culture plates wells were filled with 0. 2 ml aliquots of the diluted cultures and only broth served as control to check sterility and non- specific binding of media. The tissue culture plates were incubated for 18 hours and 24 hours at 37°C.

Table 1. Antibiotics used for antibiotic susceptibility test

Gram positive organisms		Gram negative organisms	
Amikacin	30 mcg	Amikacin	30mcg
Amoxiclav	30 mcg	amoxiclav	30mcg
Cefoxitin	30mcg	ampicillin /sulbactam	10/10mcg
Clindamycin	2mcg	aztreonam	30mcg
Cefuroxime	30 mcg	cefuroxime	30mcg
Cotrimoxazole	25mcg	ceftazidime	30mcg
Chloramphenicol	30mcg	ceftazidime/clavulanic acid	30/10mcg
Ciprofloxacin	5mcg	cefepime	30mcg
Erythromycin	15mcg	chloramphenicol	30mcg
Gentamycin	10mcg	ciprofloxacin	5mcg
Linezolid	30mcg	cotrimoxazole	25mcg
Vancomycin	30mcg	gentamycin	10mcg
Pencillin G	10units	Imipenam	10mcg
Tetracycline		Pipericillin/ tazobactum	100/10mcg

After incubation content of each well was gently removed by tapping the plates. The wells were washed four times with 0. 2mL of phosphat e buffer saline (PBS pH 7. 2) to remove free-floating 'planktonic' bacteria. Biofilms formed by adherent 'sessile' organisms in plate were fixed with sodium acetate (2%) and stained with crystal violet (0. 1% w/v). Excess stain was rinsed off by thorough washing with deionized water and plates were kept for drying. Adherent cells usually formed biofilm on all side wells and were uniformly stained with crystal violet. Optical density (OD)of stained adherent bacteria was determined with a micro ELISA auto reader (model 680,Biorad) at wavelength of 570 nm (OD 570nm). These OD values were considered as an index of bacteria adhering to surface and forming biofilms. To compensate for background absorbance, OD readings from sterile medium, fixative and dye were averaged and subt racted from all test values. For the purpose of data calculation, Table 2 shows the classification based on OD values.

Table 2. Classification of biofilms by Tissue culture plate method

Mean O.D value	Adherence	Biofilm formation
< 0.120	NON	NON / WEAK
0.120 – 0.240	MODERATE	MODERATE
>0.240	STRONG	HIGH

Tube method (TM): Mathur *et al*. (2006)

TSBg lu (10 mL) was inoculated with loopful of micro organism from overnight culture plates and incubated for 24 hours at 37°C. The tubes were decanted and washed with PBS (pH 7. 3) and dr ied. Dried tubes were stained with crystal violet (0. 1%). Excess stain was removed and tubes were washed with deionized water. Tubes were then dried in inverted position and observed for biofilm formation. Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Ring formation at the liquid interface was not indicative of biofilm formation. Tubes were examined and the amount of biofilm formation was scored as Table 3 Experiments were performed in triplicate and repeated three times.

Table 3. Classification of biofilms by Tube Method

0	Absent
1	Weak
2	Moderate
3	Strong

Congored agar method: Freeman *et al* (1989) had described an alternative method of screening biofilm formation by *Staphylococcus* isolates; which requires the use of a specially prepared solid medium -brain heart infusion broth (BHI) supplemented with 5% sucrose and Congo red. The medium was composed of BHI (37 gms/L), sucrose (50 gms/L), agar no.1 (10 gms/L) and congo red stain (0.8 gms/L). Congo red was prepared as concentrated aqueous solution and autoclaved at 121°C for 15 minutes, separately from other medium constituents and was then added when the agar had cooled to 55°C. Plates were inoculated and incubated aerobically for 24 to 48 hours at 37°C. Positive result was indicated by black colonies with a dry crystalline consistency. Weak slime producers usually remained pink, though occasional darkening at the centers of colonies was observed. A darkening of the colonies with the absence of a dry crystalline colonial morphology indicated an indeterminate result. The experiment was performed in triplicate and repeated three times.

RESULTS

From a total of 100 patients who were investigated in the present study 95 (95%) were culture positives and 5 (5%) were culture negatives. Among the culture positives only one organism was isolated and there were no mixed growth of more than one organism. The study which we conducted also showed a male preponderance compared to females. Males were 69 (69%) and females were 31 (31%).The male to female ratio was 2.22 : 1.This is represented in Fig 1.

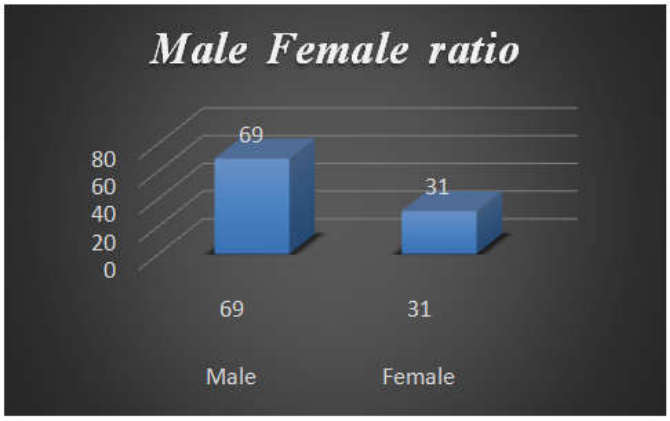


Fig. 1. Male and female ratio of skin graft infection

The age and sex distribution of the study group is shown in Table 4.

The age of patients ranged from 10yrs to 78yrs. The highest number of cases of people who underwent skin grafting and of which were infected belonged to an age group of 40-50 years of age. Table 5 shows the percentage of culture positive results and Table 6 and Fig 2 shows the indications for skin grafting

Table 4. The age and sex distribution of the study group

Age in yrs.	Total no. of cases	% of age	Males in that age group	% of age	females in that age group	% of age
0-10	0	-	0	-	0	-
10-20	05	05%	03	60%	2	40%
20-30	15	15%	10	66.6%	5	33.%
30-40	22	22%	16	72.7%	6	27.2%
40-50	26	26%	21	80.7%	5	19.2%
50-60	16	16%	09	56.2%	7	43.7%
60-70	13	13%	07	53.8%	6	46.1%
70-80	03	03%	03	100%	-	-
Total		100%	69	71%	31	29%

Table 5. The percentage of culture positive results

Culture	No. of cases	% of age
Positive	95	95%
Negative	05	05%
Total	100	100%

Table 6. Indications for skin grafting

Indication for skin grafting	No. of cases	Percentage
Burns	75	75%
Chronic venous leg ulcer	12	12%
Trauma	08	08%
Diabetic wounds	05	05%
TOTAL	100	100%

In our study the most common cause for which skin grafting was done and which were infected was for burns 75(75%) followed by chronic venous leg ulcers 12 (12%), trauma 8 (8%) and diabetic wounds 5(5%).

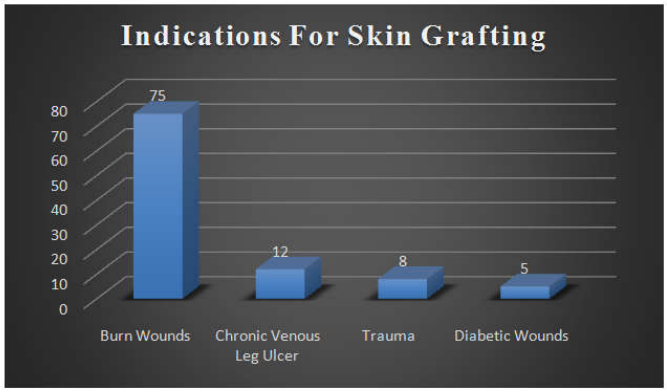


Fig. 2. Indications for skin grafting

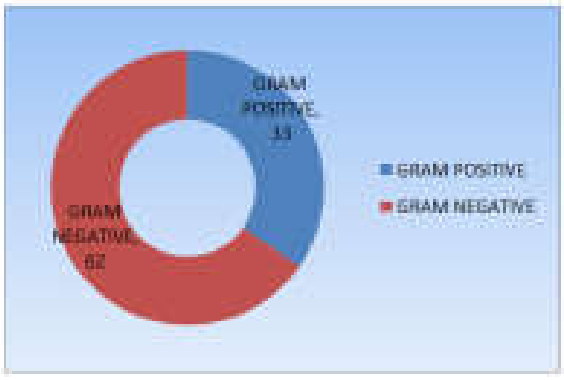


Fig. 3. Distribution of gram positive and gram negative isolates

In our study gram negative organisms 62 (65.2%) are more common when compared to gram positive organisms 33 (34.7%). The commonest gram positive bacteria isolated was MRSA 20 (21%) and the commonest gram negative bacilli isolated were pseudomonas aeruginosa 37 (38.9%) followed by klebsiella species 12(12.6%). Fig 3 shows distribution of gram positive and gram negative isolates

Fig 4 shows MacConkey Agar Showing Lactose Fermenting E.Coli And Non Lactose Fermenting Pseudomonas Species and Fig. 5 shows Various Reactions of Pseudomonas Aeruginosa.

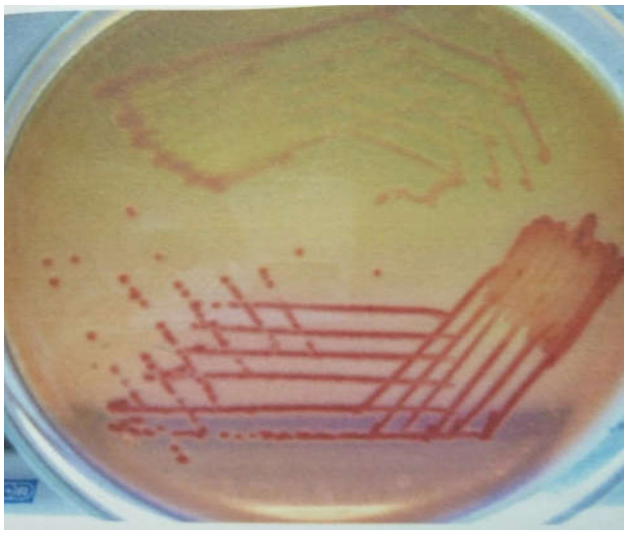


Fig. 4. MacConkey Agar Showing Lactose Fermenting E.Coli and Non Lactose Fermenting Pseudomonas Species

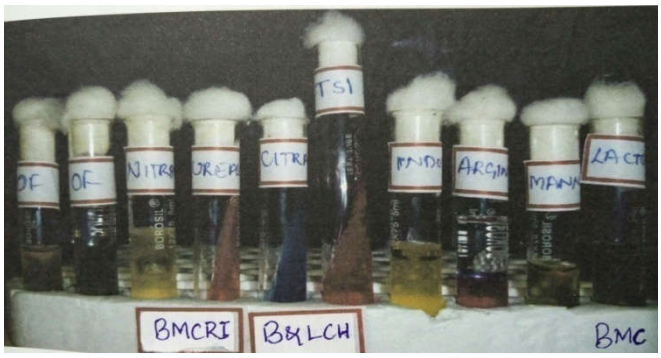


Fig. 5. Various reactions of pseudomonas aeruginosa

Micro-organisms isolated

Only one organism was isolated from each sample there were no poly microbial infection in our study. From total of 95 isolates which were obtained from 100 patients, the most common organism isolated was pseudomonas species 37 (38.9%). The second most common organism isolated was MRSA 20 (21%) followed by MSSA 10 (10.5%), klebsiella species 12 (12.6%) Proteus species 8 (8%) and E.coli 5 (5%).Table 7 shows total number of organisms isolated in the present study and Fig 6 shows various organisms isolated in skin graft infections. Among the organisms isolated Pseudomonas auriginosa is most commonly seen in skin grafting done for burns 25(26.3%) followed by chronic venous leg ulcers 7(7.3%) and traumatic wounds 5(5.2%). Pseudomonas aureginosa was not isolated from skin grafts done for diabetic wounds. MRSA was seen most commonly in grafts of burns 18(18.9) followed by chronic venous leg ulcers 2(2.1%). MSSA was seen only in skin graft done for burns 10(10.5%). Klebsiella was isolated from skin grafting done for burns 9(9.4%) and traumatic wounds. Table 8 shows distribution of isolates among the indication for skin grafting.

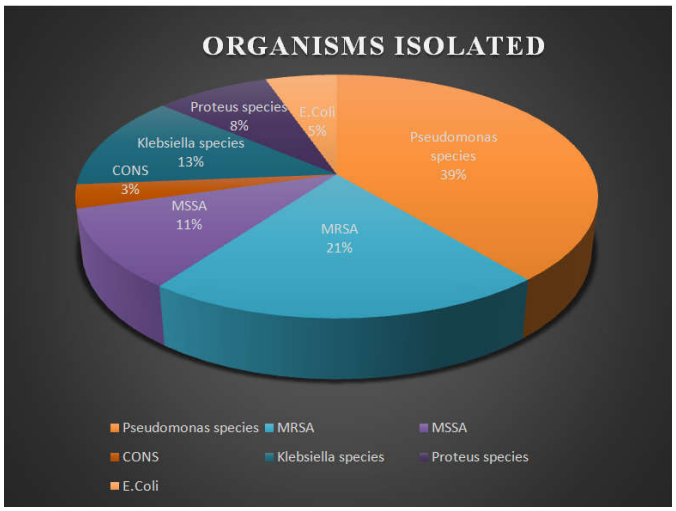


Fig. 6. Organisms isolated from skin graft infection

Table 7. Total number of organisms isolated in the present study

S.No.	Organism isolated	No. of isolates	Percentage
1	Pseudomonas aeruginosa	37	38.9%
2	MRSA	20	21%
3	MSSA	10	10.5%
4	Klebsiella species	12	3.1%
5	Proteus	08	12.6%
6	E.coli	05	8.4%
7	CONS	03	3.1%
	Total	95	100%

Antibiogram

The results of the susceptibility tests of the gram negative bacterial isolates to the commonly used antibiotics such as amikacin, amoxiclave, ampicillin sulbactam, aztreonam, cephalothin, ceftazidime, ceftazidime/clavulanic acid, cefepime, chloramphenicol, ciprofloxacin, cotrimoxazole, gentamycin, imipenam and piperacillin/ tazobactam are charted. Fig 8: Antibiotic susceptibility pattern of Pseudomonas species.

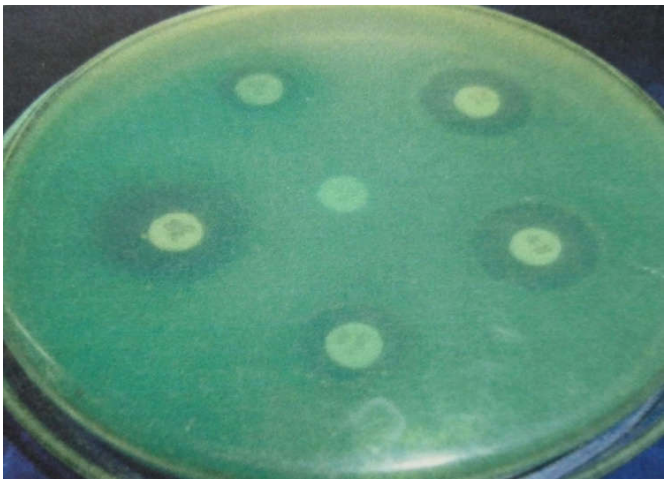
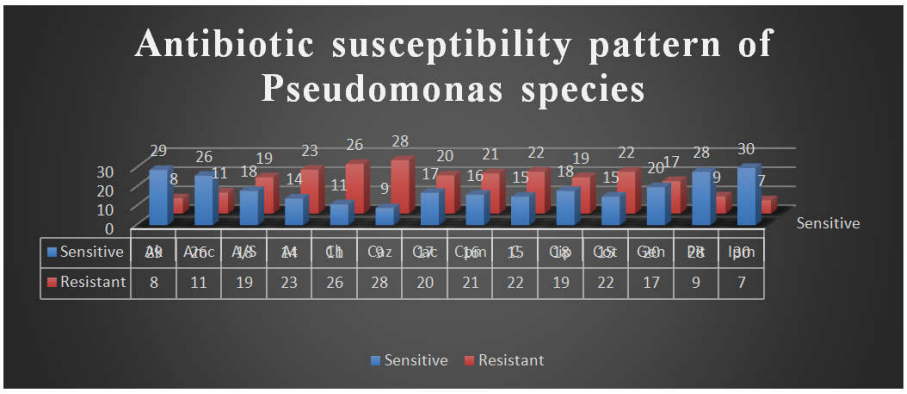
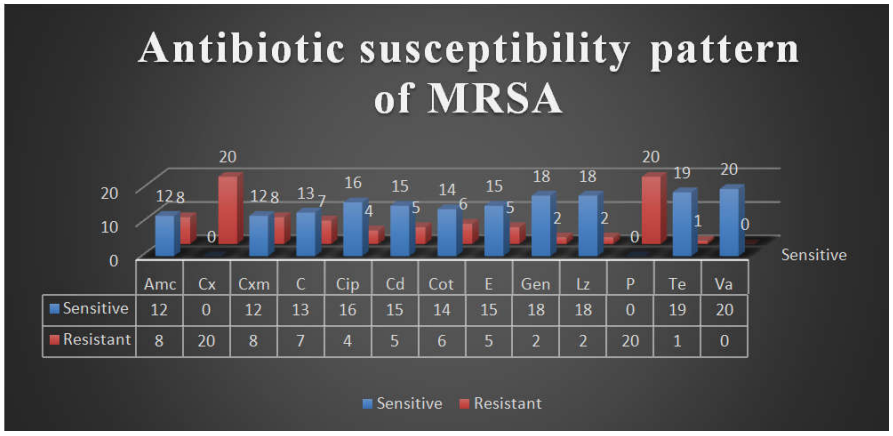


Fig.7. Antibiotic susceptibility test by disc diffusion method



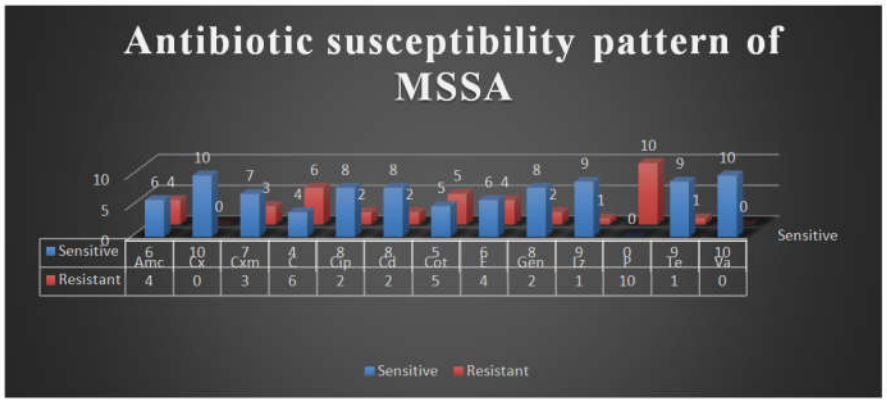
	Ak	Amc	A/S	At	Ch	Caz	Cac	Cpm	C	Cip	Cot	Gen	Pit	Ipm
Sensitive	29	26	19	23	26	28	20	21	22	19	22	17	28	30
Resistant	8	11	19	23	26	28	20	21	22	19	22	17	9	7

Fig. 8. Antibiotic susceptibility pattern of Pseudomonas species



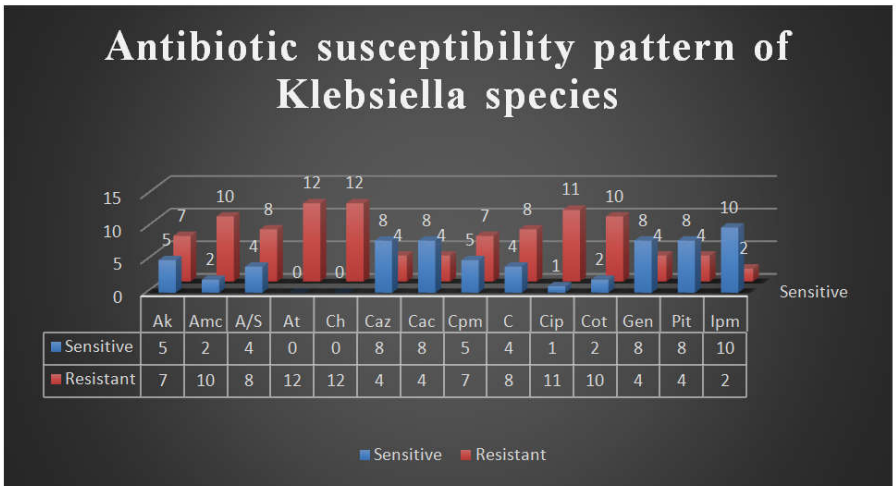
	Amc	Cx	Cxm	C	Cip	Cd	Cot	E	Gen	Lz	P	Te	Va
Sensitive	12	0	12	13	16	15	14	15	18	18	0	19	20
Resistant	8	20	8	7	4	5	6	5	2	2	20	1	0

Fig. 9. Antibiotic Susceptibility Pattern of MRSA



	Amc	Cx	Cxm	C	Cip	Cd	Cot	E	Gen	Lz	P	Te	Va
Sensitive	6	10	7	4	8	8	5	6	8	9	0	9	10
Resistant	4	0	3	6	2	2	5	4	2	1	10	1	0

Fig. 10. Antibiotic susceptibility pattern of MSSA



	Ak	Amc	A/S	At	Ch	Caz	Cac	Cpm	C	Cip	Cot	Gen	Pit	Ipm
Sensitive	5	2	4	0	0	8	8	5	4	1	2	8	8	10
Resistant	7	10	8	12	12	4	4	7	8	11	10	4	4	2

Fig. 11. Antibiotic Susceptibility Pattern of Klebsiella Species

Table 8. Distribution of isolates among the indication for skin grafting

S.No.	Organisms isolated	Total	Burns	Chronic venous ulcers	Trauma	Diabetic wound
1	Pseudomonas aueriginosa	37(38.9%)	25(26.3%)	7(7.3%)	5(5.2%)	-
2	MRSA	20(21%)	18(18.9%)	2(2.1)		-
3	MSSA	10(10.5%)	10(10.5%)	-		-
4	Klebsiella species	12(12.6%)	9(3.1%)	-	3(3.1%)	-
5	Proteus species	8(8.4%)	-	3(3.1%)		5(5.2%)
6	E.coli	5(5.2%)	5(5.2%)	-		-
7.	CONS	3(3.1%)	3(3.1%)	-		-
	TOTAL	95	70(73.6%)	12(12.6%)	08(8.4%)	05(5.2%)

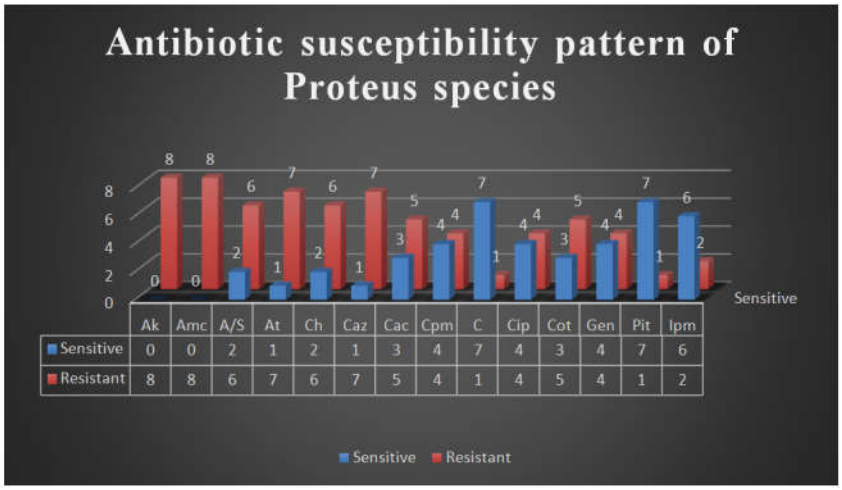


Fig. 12. Antibiotic susceptibility pattern of Proteus species

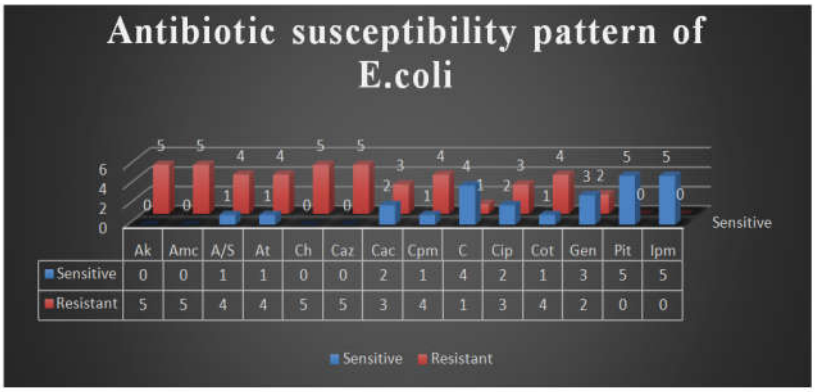


Fig. 13. Antibiotic susceptibility pattern of E. Coli

Pseudomonas represented 37(38.9%) isolates among 95 culture positives out of which 29(78.3%) were sensitive to amikacin, 26(70.2%) to amoxiclave, 18 (48.6%)to ampicillin sulbactam, 14 (37.8%) to aztreonam, 11 (29.7%)to cephalothin,9 (24.3%) to ceftazidime, 17 (45.9%) to ceftazidime/clavulanic acid,16 (43.2%) to cefepime, 15 (40.4%)to chloramphenicol, 18(48.6%) to ciprofloxacin,15(40.5%) to cotrimoxazole, 20(54%) to gentamycin, 30(81%) imipenam and 28 (75.6%)to piperacillin/ tazobactam. For gram positive isolates the drugs used for antibiotic susceptibility testing was amoxiclav, cefoxitin, cefuroxime, chloramphenicol, ciprofloxacin, clindamycin, cotrimoxazole, erythromycin, gentamycin, linezolid, pencillin G, tetracycline and vancomycin and the results are charted. MRSA represented 20(21%) among 95 isolates, out of which all were sensitive to 20 (100%) vancomycin and all were resistant to cefoxitin and pencillin G. Sensitivity of Rest of the drugs is as follows i.e 12(60%) sensitive to amoxiclav, 12 (60%)to cefuroxime, 13 (65%) to chloramphenicol, 16(80%) to ciprofloxacin, 15 (75%) to clindamycin, 14(70%) to cotrimoxazole, 15(75%) to erythromycin,18 (90%) to gentamycin, 18(90%) to linezolid and 19 (95%) to tetracycline. Fig 9 shows Antibiotic susceptibility pattern of MRSA. MSSA represented 10 (10.5%) among 95 isolates, out of which all were sensitive to 10 (100%) vancomycin and cefoxitin. All the isolates were resistant to penicillin G. Sensitivity of Rest of the drugs is as follows i.e 6(60%) sensitive to amoxiclav, 7 (70%)to cefuroxime, 4(40%) to chloramphenicol, 8(80%) to ciprofloxacin, 8 (80%) to clindamycin, 5(50%) to cotrimoxazole, 6(60%) to erythromycin, 8 (80%) to gentamycin, 9(90%) to linezolid and 9(90%) to tetracycline. Fig 10 shows Antibiotic susceptibility pattern of MSSA. Klebsiella species represented 12(12.6%) isolates among 95 culture positives out of which all were resistant to aztreonam and cephalothin. 5(41.6%) were sensitive to amikacin, 2(16.6%) to amoxiclave, 4 (33.3%) to ampicillin sulbactam, 8 (66.6%)to ceftazidime, 8 (66.6%) to ceftazidime/clavulanic acid,5 (41.6%)to cefepime, 4 (33.3%) to chloramphenicol,

1(8.3%) to ciprofloxacin, 2(16.6%) to cotrimoxazole, 8(66.6%) to gentamycin, 10(83.3%) imipenam and 8 (66.6%)to piperacillin/ tazobactam. Fig 11 shows Antibiotic susceptibility pattern of Klebsiella species.

Proteus species represented 8(8.4%) isolates among 95 culture positives out of which all were resistant to amikacin and amoxiclave. 1(12.5%) were sensitive to aztreonam,2(25%) to cephalothin, 2 (25%)to ampicillin sulbactam, 1 (12.5%)to ceftazidime, 3 (37.5%) to ceftazidime/clavulanic acid,4 (50%) to cefepime, 7 (87.5%) to chloramphenicol, 4(50%) to ciprofloxacin, 3(37.5%) to cotrimoxazole, 4(50%) to gentamycin, 6(75%) imipenam and 7 (87.5%) to piperacillin/ tazobactam. Fig 12 shows Antibiotic susceptibility pattern of Proteus species. E.coli represented 5(5.2%) isolates among 95 culture positives out of which all were resistant to amikacin, amoxiclave, cephalothin and ceftazidime. 1(20%) were sensitive to aztreonam, 1 (20%)to ampicillin sulbactam, 2 (40%) to ceftazidime/clavulanic acid,1 (20%)to cefepime, 4 (80%) to chloramphenicol, 2(40%) to ciprofloxacin, 1(20%) to cotrimoxazole, 3(60%) to gentamycin, 5(100%) imipenam and 5 (100%) to piperacillin/ tazobactam. Fig 13 shows Antibiotic susceptibility pattern of E. coli.

Out of 95 isolates which were isolated 42 (44.2%) were biofilm positive and 53 (55.7%) Were biofilm negative. Pseudomonas species is also the common organism producing biofilm 22 (52.3%) followed by MRSA 10(23.8%), kelbsiella species 3 (7.1%)

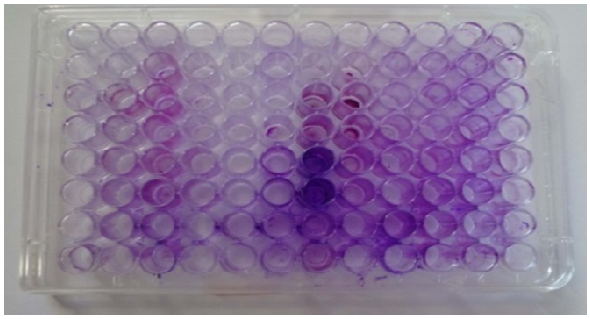


Fig. 14. Biofilm detection by tissue culture plate

In TCP method ,from the total number of 95 isolates tested for biofilm formation, strong biofilm producers were 36 (37.8 %), 6 (6.3%) were moderate and 53 (55. 7%) isolates were considered as non or weak biofilm producers. Fig 14 shows biofilm formation by TCP method, Fig 15 shows the Tube method and Fig 16 shows the detection of biofilm by congored agar method.

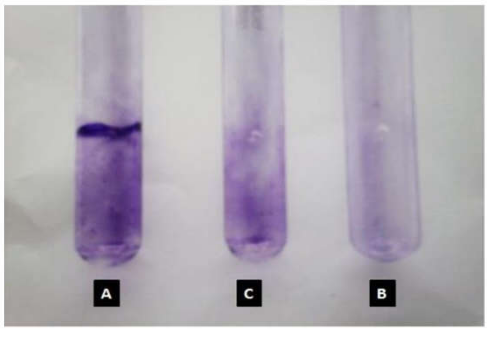


Fig. 15. Biofilm detection by Tube method

In tube method, from the total number of 95 isolates tested for biofilm formation, strong biofilm producers were 30 (31.5 %), 10 (10.5%) were moderate and 60 (63.1%) isolates were considered as non or weak biofilm producers. This was in concordance with the tissue culture plate method of biofilm detection. In congo red agar method, from the total number of 95 isolates tested for biofilm formation, 20 displayed black colonies but no dry crystalline morphology, and 22 displayed black dry crystalline morphology and the rest 53 (55. 7%) isolates were considered as non biofilm producers as they did not displayed black , dry and crystalline colonies.

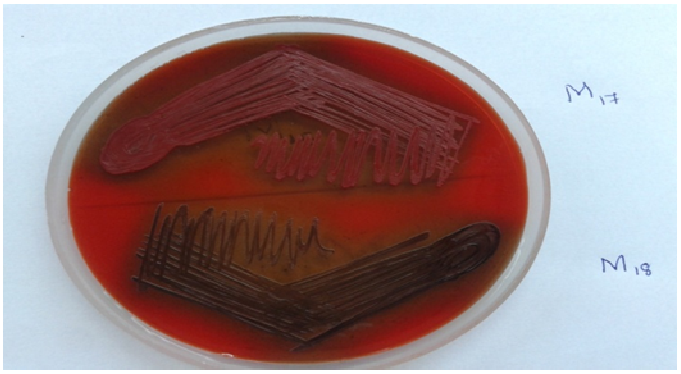


Fig. 16. Biofilm detection by congo red agar method

DISCUSSION

Skin graft infections continue to pose a problem for plastic surgeon. Diagnosis and treatment of skin graft infections is complicated by the formation of a bacterial biofilm when bacteria have changed their phenotype to an extremely sessile form of life. The surge of multidrug resistant bacteria that easily adhere to surgical materials stresses the value of adequate diagnosis leading to proper therapy of these patients. However, organisms which have adhered to the skin grafts are occasionally impossible to detect by common bacterial culture. Various sampling techniques including direct swabs, tissue biopsy from the margins of the skin grafting is also done to detect the organisms causing infections. No unifying hypothesis can explain the loss of skin transplants. A wide range of things are believed to adversely influence skin graft take; haematoma or shearing movements (McGregor and McGregor, 2000), inadequate compliance, deficient blood supply, presence of micro thrombi in the dermal blood vessels (Freeman *et al*., 1989; Gilliland *et al*., 1988), local fibrin deficiency in the wound bed and former thrombophlebitis in relation to primary deep vein incompetence (Schmeller *et al*., 2000) are examples. Skin graft loss due to infection make up only a minor part in the literature with very few publications on deterioration of skin grafts due to P. aeruginosa (McGregor and McGregor, 2000), particularly in the field of chronic lower limb ulcerations. These papers do not reach an agreement on the severity of the role of P. aeruginosa. They all concentrate on microbiology assessed immediately prior to grafting or post grafting. To our knowledge, the only paper with regards to leg ulcers is more than 20 years old (Gilliland *et al*., 1988). In this paper Gilliland *et al* reported that the initial swab results (at admission) were not related to the outcome of skin grafting, it was only the presence of bacteria (Pseudomonas and S. aureus) in the immediate preoperative or postoperative periods that played a role. Surgical debridement was only executed if necessary, but to what extent and details concerning antibiotics

were undocumented. Likewise, Pseudomonas was not subdivided into species and the group of patients was heterogeneous by taking all leg ulcers into account despite aetiology. Unal *et al*. (2005) found that P. aeruginosa was an equally prominent danger as S. pyogenes in skin graft survival in routine plastic surgery practice. They only focused on bacteriological cultures assessed postoperatively and only obtained samples from the defected grafts, thereby fail to elucidate what came first; P. aeruginosa or the defects? Mc Gregor, (McGregor and McGregor, 2000) in contrast, claims that infection with P.aeruginosa reduces graft take but not to an extent comparable with S. pyogenes. He also stated that its presence is a nuisance rather than a disaster and it may reduce graft takes by 5–10% at most. The lack of successful antibiotic treatment is probably explained by ability of colonizing bacteria to establish themselves and proliferate in a biofilm. A biofilm is a multi cellular aggregate encased in a extracellular matrix of polysaccharides, protein, DNA etc, compared to single free swimming bacteria termed planktonic cells (Stewart and Costerton, 2001). The clinical implications of bacterial biofilms are particularly pronounced in chronic infections (Davies, 2003). In addition to being highly tolerant to antibiotics, biofilms are also impervious to the body's natural immune defence system (Boutli-Kasapidou *et al*., 2006). P. aeruginosa and S.aureus are well recognised for forming chronic biofilm-based infections in their hosts. Normally radical debridement of the infected area is the treatment of choice in case of biofilm infections. In this study a thorough debridement was performed down to viable and visually non-infected tissue. Despite this, detection of P. aeruginosa prior to surgery, reduced graft take significantly. This indicates that P. aeruginosa resides deep down in the tissue, and is probably protected from antibiotics and the immune system due to biofilm formation. This is in accordance with a study by Fazli *et al*. (2009) showing a non-random distribution of P. aeruginosa and S. aureus where P. aeruginosa is found deeper into the tissue than S. aureus. The sampling for microbiology of this study was performed by surface swab or by. Several researchers have previously reported that surface swabs are considered equivalent to biopsy cultures (Unal *et al*., 2005) and that it reflects the microflora of deeper tissues (Bowler and Davies, 1999). However, this is up for debate. Bjarnsholt *et al*. (Bjarnsholt *et al*., 2008) indicated that bacteria are assembled in microcolony based structures found in bacterial biofilms and are far from evenly distributed within the wound, thereby implicating that cultures from a biopsy or swab are not likely to be representative for the total bacteriological load in the wound.In our study there was 95% of culture positivity with a single organism, the high percentage of positive bacterial cultures of the wound swab may be attributed to the fact that the burn wound has a much higher incidence of infections compared with other forms of trauma because of extensive skin barrier disruption as well as alteration of cellular and humoral immune responses.

Sex distribution

In our study males 69(69%) were more affected than the females 31(31%) and the male female ratio is 2.22: 1 which is in concordance to the study done by Krizek *et al*. (1967) in which males were 77.3 % and females were 22.7% . Our study is in contrast to the study done by Trine hogsberg *et al* in their study they had 82 patients out of which 45 (54.9%) were women and 37 (45.1%) were men and also another study done by Gilliland *et al* (1988) where males are 21(23.9)% and females are 67(76.1%). There is a male preponderance in our study. This may be due to high frequency of skin grafting done for non healing venous ulcers and diabetic ulcers which are more common in male than in female.

Age distribution

In our study the age of patients ranged from 10yrs to 78yrs. The highest number of cases of people who underwent skin grafting and of which were infected belonged to an age group of 40-50 years of age i.e 26 (26%) The other age group that made a good number of representation was between 30-40 year, i.e., 21 (22%) cases. This may be because people are more prone to vascular ulcers and non healing diabetic ulcers after the age of 40yrs and also they are more prone to get accidental burn injuries, may be due to work or pleasure. Skin grafting done for trauma wounds is also more common in the elderly people who are economically productive and employed individuals, i.e., occupational injuries.

Distribution of cases

In our study the most common cause for which skin grafting was done and which were infected was for burns 75(75%) followed by chronic venous leg ulcers 12 (12%), trauma 8 (8%) and diabetic wounds 5(5%) i.e the major cases included in the study was skin grafting done for burn wounds, this was in contrast with many studies done where the major cases included in the study was vascular ulcers. In a study done by Unal S, Ersoz *et al* vascular ulcers was (9.2%), burns was (14.5%), traumatic tissue defects (36.6%), and flap donor site defects were (39.7%). Table 10 shows the distribution of cases of skin grafting in various studies.

Author	Males (% Age)	Females (% Age)
Trine hogsberg *et al*. (1999)	45.1%	54.9%
Krizek *et al*. (1967)	77.3%	22.7%
Gilliland *et al*. (1988)	23.9%	76.1%
Present Study	69%	31%

Table 10. Distribution of cases of skin grafting in various studies

Author	Burns	Venous ulcers	Traumatic wounds	Diabetic wounds	Doner site defects
Unal *et al.* (2005)	14.5%	9.2%	36.6%	_	39.7%
Trine hogsberg *et al.* (1999)	17.5%	64.2%	_	18.3%	_
Present study	75%	12%	8%	5%	_

The most common organism isolated in our study was Pseudomonas aureginosa followed by staphylococcus aureus, klebsiella species, proteus and E.coli. There is a predominance of gram negative bacteria i.e Pseudomonas species compared to gram positive organisms which is in concordance with Unal *et al*. (2005). In their study they revealed that the graft loss secondary to infection was due to Pseudomonas aeruginosa in 58.1% of cases followed by Staphyloccocus aureus, Enterobacter, Enterococci and Acinetobacter and 58.3% of grafts in vascular ulcers 47.4% of grafts in burns 16.7% of grafts in traumatic tissue defects and 13.5% of grafts in donor site defects were lost due to infection and also a study by Trine Hogsberg *et al*. (1999) from Denmark in their study showed that success rate of split thickness skin grafting of chronic venous leg ulcers depend on the presence of Pseudomonas

aeruginosa. Biofilms are microbial communities encased within polysaccharide rich extracellular matrix on surfaces of wounds. They are associated with drastically enhanced resistance against most antimicrobial agents leading to treatment failures. In our study 42 isolates showed biofil m production by Tissue Culture Plate method. Most common organism producing biofilm was Pseudomonas species followed by Staphylococcus *aureus*; this was in correlation with Seth *et al* whose study evaluated the effect of clinical strategies against biofilm infected wounds in quantitative in vivo models and showed that Pseudomonal biofilm markedly impairs wound healing. Biofilm producing organisms were associated with therapeutic failure and infection was resolved only on surgical debridement. In TCP method, from the total number of 95 isolates tested for biofilm formation, strong biofilm producers were 36 (37.8 %), 6 (6.3%) were moderate and 53 (55. 7%) isolates were considered as non or weak biofilm producers. In tube method, from the total number of 95 isolates tested for biofilm formation, strong biofilm producers were 30 (31.5 %), 10 (10.5%) were moderate and 60 (63.1%) isolates were considered as non or weak biofilm producers. This was in concordance with the tissue culture plate method of biofilm detection. In congo red agar method, from the total number of 95 isolat es tested for biofilm formation, 20 displayed black colonies but no dry crystalline morphology and 22 displayed black dry crystalline morphology and the rest 53 (55. 7%) isolates were considered as non biofilm producers as they did not displayed black, dry and crystalline colonies.

In another study, Ruzicka *et al*. (2004) noted that out of 147 isolates of *S. epidermidis*, TM detected biofilm formation in 79 (53.7%) and CRA detected in 64 (43.5%) isolates. They showed that TM is better for biofilm detection than CRA. Baqai *et al*. (2008) tested TM to detect biofilm formation among uropathogens. According to their results, 75% of the isolates exhibited biofilm formation. (2008) With the CRA method, 11 were found to be biofilm producing bacteria and 99 as non-biofilm producers. The CRA method showed very little correlation with the other methods and parameters of sensitivity (11%), specificity (92%) and accuracy (41%) were very low. By this method, three isolates were found to be false positive and 62 were false negative. Knobloch *et al*. (2002) did not recommend the CRA method for biofilm detection in their study. Out of 128 isolates of *S. aureus*, CRA detected only 3.8% as biofilm producers as compared to TCP which detected 57.1% as biofilm producing bacteria. (Knobloch *et al*., 2002) The tube test correlates well with the TCP test for strongly biofilm producing isolates but it was difficult to discriminated between weak and biofilm negative isolates due to the variability in observed results by different observers. Consequently, high variability was observed and classification in biofilm positive and negative was difficult by tube method. In agreement with the previous reports, tube test cannot be recommended as general screening test to identify biofilm-producing isolate (Mathur *et al*., 2006). Our data indicated that TCP method was an accurate and a reproducible method which could be used for biofilm detection and that this technique could serve as a reliable quantitative tool for determining biofilm formation.

Conclusion

Appropriate pre and post operative wound care for dirty wounds especially when skin grafting is done for burn wounds and in patients with poor condition should be done with more caution. Meticulous preoperative skin decontamination and perfect sterilization of operative and dressing instruments are mandatory. Bacteria might also be transmitted from one patient to another by imperfectly sterilized instruments, or could be introduced into the surgical wound through faulty dressing technique. The obtained data confirm the necessity of describing an effective management scheme including debridement, irrigation, and antibiotic administration to prevent skin graft loss. Due to the high risk of biofilm production by organisms causing ODRIs an appropriate antibiotic policy must be put in place in order to eradicate infection. Studies in India and other developing countries are few. The strain on the health services and economy of the society necessitates further studies to determine causative micro organisms, their antibiotic susceptibility, and associated risk factors, in order to institute timely and effective preventive measures or appropriate and aggressive treatment, reducing the cost and improving quality of life. Biofim formation is a two stage biological process controlled by surface adhesins and cell-to-cell communication pathways. Aggregated bacterial cells protected and/or coated by extracellular matrix are insensitive to both nutritional stimulation and hostile attacks. In the human body, biofilms may trigger persistent infections with chronic inflammation. After a positive detection of biofilm related medical conditions, both surgical debridement and medicinal treatment should be considered. Ideally, an effective remedy for biofilm associated conditions should contain antibiotics, anti-inflammatories, and anti-biofilm activities (3A remedies). The road from molecular mechanisms of biofilm formation to anti-biofilm products is promising, but long. Non-invasive and/or minimally invasive detection methods and standard biofilm assays that mimic clinical conditions are opening the door for new, biofilm oriented solutions. A large number of biofilm inhibitors are currently under comprehensive investigation. If clinicians are made more aware of the importance of bacterial biofilm formation and their associated diseases, more translational research will be designed and new therapeutic approaches may be developed.

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