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RESEARCH ARTICLE

A STUDY OF BIOFILM FORMATION IN DIABETIC FOOT ULCER PATIENTS IN A TERTIARY CARE HOSPITAL

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ABSTRACT

Background: Diabetes mellitus is a widespread chronic illness with a steadily rising global prevalence. Among its most serious and financially burdensome complications are foot infections, which are often caused by pathogenic bacteria. These infections can be further complicated when the bacteria involved are resistant to antibiotics and equipped with multiple virulence factors, making effective treatment more difficult and reducing the chances of a successful recovery.These bacterial communities can be organized in polymicrobial communities, which may be responsible for diabetic foot ulcer chronicity. Therefore, this study was undertaken to identify the bacterial profile in infected foot ulcers, assess their patterns of antibiotic resistance, and evaluate their ability to form biofilms.

Aims:

- To identify the spectrum of bacteria causing diabetic wound infection and antimicrobial sensitivity pattern in our hospital.
- To detect the biofilm formation among these bacteria.

Methods: This was a prospective study carried out in a tertiary care medical facility. Samples were collected from 100 patients over the age of 18 with diabetic foot ulcers and they were processed using standard techniques for culture and sensitivity. Biofilm production was assessed using three different techniques: the Tissue Culture Plate (TCP) method, the Tube Method, and the Congo Red Agar (CRA) method. Data was analysed statistically.

Results: A total of 129 bacterial isolates were obtained from 100 patients with diabetic foot ulcers. The age group of these patients ranged from 28 to 86 years with maximum number in the age group 40-60 years. Klebsiella was the predominant organism (31.78%), followed by E.coli. Biofilm

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INTRODUCTION

India has the highest number of individuals living with diabetes globally, leading to its often-cited label as the "diabetes capital of the world"[1]. The country has a diabetic population of 7,60,429 in 2013, according to the IDF Diabetes Atlas (6th edition) with the 2014 statistics recording 8.63% prevalence. In diabetic patients, the commonest devastating complication is non-traumatic lower limb amputation mostly due to Diabetic Foot Ulcers (DFU) and Infections (DFI)[2]. Foot infections constitute 20% of diabetes related admissions[3].In India, 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. Bacterial infection, tissue ischaemia, and poor wound management can cause diabetic foot ulcers to heal slowly and to transform it to chronic wounds[4]. In individuals with diabetic foot, reduced blood flow compromises the ability of immune cells, such as phagocytes, to reach the site of infection, thereby facilitating microbial colonization. Diabetic Foot Infections (DFIs) are usually polymicrobial in nature, involving a mix of Gram-positive organisms like Staphylococcus ureus and Enterococcus species, as well as Gram-negative bacteria such as Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae, Proteus species, and various anaerobes. Many of these pathogens are classified as Multi-Drug Resistant Organisms (MDROs), making treatment particularly challenging[5]. These infections significantly raise the likelihood of limb amputation, prolong hospital stays, increase treatment costs, and contribute to both morbidity and mortality in diabetic patients[6].Biofilms represent a natural bacterial growth

mode. They are typically made up of diverse microbial communities that attach to surfaces and become embedded within a self-produced, hydrated matrix of extracellular polymeric substances. In clinical terms, a medical biofilm is most simply described as a community of microorganisms adhering to either living tissue or inert surfaces.Thus, most chronic infections, including bacteria that are associated with chronic wounds exist as biofilm communities[7]. Bacteria that form biofilms often exhibit distinct phenotypic traits compared to when the same strains are grown in free-floating (planktonic) conditions. 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[8]. So, physical removal of the biofilm is one of the most successful strategies for management of biofilm-related conditions, through frequent debridement of diabetic foot ulcers[9]. 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[10]. As, both systemic and topical antibiotics alone are unable to eradicate biofilm infections[11], there is an increasing interest in their etiological role. So, there is an increasing clinical need to identify biofilms in these wounds[12].

MATERIALS AND METHODS

This prospective study was carried out in the Department of Microbiology at a tertiary care teaching and research hospital affiliated with a Medical College and Research Institute for a period of two months.100 patients attending the surgery outpatient department of the hospitals were included in the study. Ethical approval was secured from the institutional review board, and written informed consent was obtained from all participants in a language they could understand. All patients above 18 years of age having chronic diabetic foot ulcer where ulcer duration is greater than 3 months were included in the study[13]. Children (<18years), pregnant women and patients with other co-morbid conditions like HIV infection, chronic venous insufficiency were excluded. The patients were assessed through detailed history and clinical examination[14]. The ulcers were assessed by the surgeons and after debridement, material for culture was collected with cotton tipped sterile swab from the deeper parts of the foot ulcer. The sample was promptly transferred to the microbiology department for culture analysis, sensitivity testing, and evaluation of biofilm formation. The received swab samples were streaked onto Blood agar and MacConkey agar plates, which were then incubated at 37°C overnight.Colonies obtained were identified by using standard techniques[15]. Antibiotic sensitivity was done using Kirby Bauer's disc diffusion technique as described in Clinical Laboratory Standard Institute (CLSI) guidelines 2012[16].

There are three methods for detection of biofilm

- Tube Method
- Congo Red Agar Method
- Tissue Culture Plate Method

Tube Method: Described by Christensen *et al*., this is a qualitative method for biofilm detection[17]. 10ml of Trypticase soy broth with 1% glucose was inoculated with a loopful of test organism from overnight culture on nutrient agar individually. Broths were incubated at 37°C for 24 hours. The cultures were poured off, and the tubes were rinsed with phosphate-buffered saline. The tubes were inverted and allowed to dry before being examined for evidence of biofilm development. Biofilm formation was deemed positive if a noticeable layer was present along the inner walls and bottom of the tube. The presence of a ring at the air-liquid interface alone was not considered a sign of biofilm production. The tubes were visually assessed, and biofilm formation was categorized using a scoring system: 0 for no biofilm, 1 for weak, 2 for moderate, and 3 for strong biofilm development.

Congo Red Agar Method: As described by Freeman *et al*[18], a specially prepared medium composed of Brain Heart Infusion (BHI) broth(37gms/L), sucrose(50gms/L), agar no.1(10gms/L) and congo red stain(0.8gms/L) was used. An aqueous solution of Congo red was prepared at high concentration and sterilized independently by autoclaving at 121°C for 15 minutes.After the agar medium had cooled to approximately 55°C, the sterile dye solution was incorporated.

The prepared plates were then inoculated and incubated under aerobic conditions at 37°C for 24 to 48 hours. Black colonies with a dry crystalline consistency indicated biofilm production (Figure 1). Weak producers remained pink, though occasional darkening at the centre of colonies were observed. Indeterminate outcomes were noted when colonies exhibited a dark colouration without displaying the characteristic dry, crystalline appearance. The tests were carried out in triplicate and repeated three times[19].

Figure 1. Black colonies with a dry crystalline consistency indicated biofilm production in Congo Red Agar (CRA).

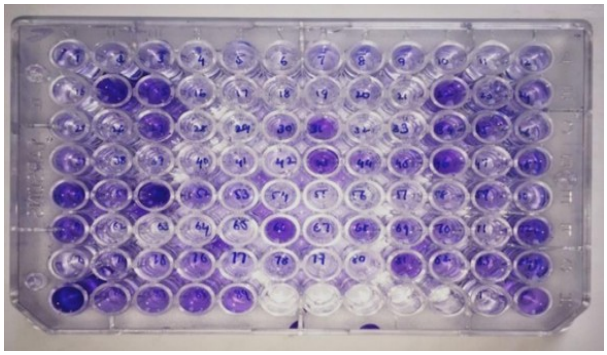

Figure 2. Tissue culture plate showing the result of biofilm assay.

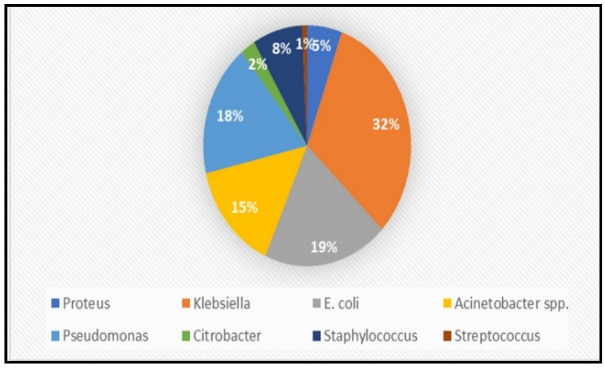

Figure 3. Percentage of bacterial isolates from infected diabetic foot ulcers.

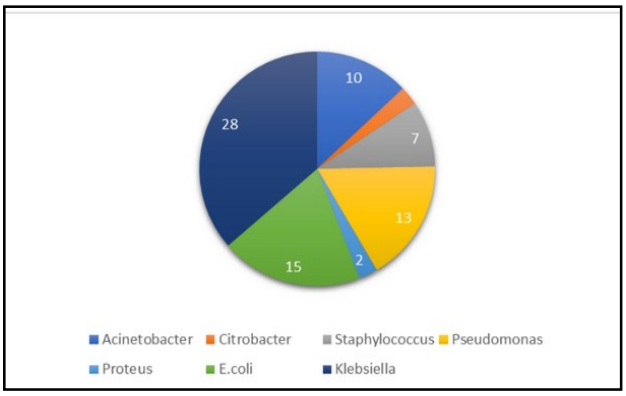

Figure 4. Isolate showing biofilm formation.

Tissue Culture Plate Method: Stepanovic *et al* described the Tissue Culture Plate (TCP) method in plastic microtitre plates[20]. In a sterile 96-well flat-bottom polystyrene microplate, 230 µL of Trypticase Soy Broth (TSB) was added to each well. To this, 20 µL of overnight bacterial culture was introduced into the corresponding wells, with each bacterial strain tested in triplicate. The negative control wells contained only broth. The plates were incubated aerobically at 35°C for 24 hours. After incubation, the contents of the wells were discarded, and the wells were washed three times with 300 µL of sterile distilled water.

To fix the bacteria adhering to the wells, 250 µL of methanol was added and left for 15 minutes. Subsequently, the wells were stained with 250 µL of a 1% crystal violet solution for 5 minutes. Any excess stain was removed by washing, and the wells were allowed to air dry. To dissolve the dye retained in the wells, 250 µL of 33% (v/v) glacial acetic acid was added. Optical density (OD) readings were taken at 490 nm for each well using an ELISA microplate reader (Figure 2). Each test was conducted in triplicate, and the mean OD values were computed. Standard deviation was also calculated. To correct for background absorbance, the average OD values from wells containing only sterile medium, fixative, and dye were subtracted from the readings of the test wells.The mean OD value obtained from media control well was deducted from all the test OD value.

Classification of bacterial adherence

For the purpose of data collection, we used classification (Table A) based on OD values obtained for individual organisms[21]. Data was compiled and descriptive statistics was applied using Microsoft Excel 2010 edition.

RESULTS

The study included 100 patients with diabetic foot ulcers, consisting of 61 males and 39 females. The age ranged from 28 to 86 years with mean age being 57.1 years. Diabetic foot ulcers were observed more frequently in the age group of 40-60 years, followed by 61-80 years. The bacterial growth patterns of the culture positive cases are represented in table B. Out of 100 specimens, 94 specimens showed bacterial growth in which 129 organisms were isolated while 6 specimens did not show any growth. It represents an average of 1.29 organisms per case. Figure 3 illustrates the bacterial isolates. Among the aerobic bacterial isolates, gram negative comprised of 91.47% and gram positive accounted for 8.53%. Klebsiella species was the most common isolate, followed by Escherichia coli. The antibiotic susceptibility profile of Staphylococcus aureus revealed a 62.5% resistance rate to cefoxitin. All isolates were completely sensitive to tetracycline, doxycycline, and linezolid, while showing complete resistance to penicillin.They exhibited 75% and 62.5% resistance to erythromycin and cotrimoxazole respectively. Klebsiella showed a high level of resistance to amoxicillin+clavulanic acid (95.12%), ceftriaxone (87.80%), ceftazidime (85.37%), cefepime (80.49%), cotrimoxazole (78.05%), ertapenem and piperacillin+tazobactam (both 75.61%). The organism was most sensitive to tigecycline (56.1%). All isolates showed complete resistance to ampicillin (100%). In E.coli majority of strains were resistant to amoxicillin+clavulanic acid (92%), ceftazidime, ciprofloxacin, ampicillin (each 84%) and ceftriaxone (80%). It was most sensitive to amikacin (88%).

Table A. Classification of bacterial adherence by Tissue Culture Plate (TCP) method.

MEAN OD VALUE	ADHERENCE	BIOFILM FORMATION
<0.180	NON	NON/WEAK
0.180-0.360	MODERATELY	MODERATE

Table B. Growth pattern in culture of foot ulcer samples of 100 patients.

CULTURE REPORTS	NUMBER OF CASES
POSITIVE CULTURE	94
PURE BACTERIAL GROWTH	60
MIXED GROWTH	34
NO GROWTH	6

Table C. Screening of isolates for biofilm formation by Tissue Culture Plate (TCP), Tube Method (TM)and Congo Red Agar (CRA) methods.

NO. OF ISOLATES	BIOFILM FORMATION	TCP	TM	CRA
129	STRONG	11(8.53%)	14(16.85%)	7(5.43%)
	MODERATE	66(51.16%)	37(28.68%)	45(34.88%)
	WEAK/NONE	52(40.31%)	78(60.47%)	77(59.69%)

Among 129 isolates, TCP, the standard method, detected 11 as strong, 66 as moderate and 52 as weak/ non biofilm producers. Klebsiella was the predominant biofilm former, with 28(36.36%) of the isolates testing positive for biofilm formation. The second highest biofilm formation was by E.coli (19.48%), followed by Pseudomonas aeruginosa (16.88%), Acinetobacterspp. (12.99%), Staphylococcus aureus (9.09%), Proteus sp. (2.6%) and Citrobactersp. (2.6%). This is represented in figure 4. Streptococcus did not form biofilm. By Tube method, the number of strong biofilm producers were 14,

moderate were 37 and weak or non-biofilm producers were 78. Whereas in Congo Red Agar(CRA) method, only seven isolates showed black colonies with crystalline appearance (Table C).TCP method was considered the gold standard for this study as various researchers proved this method superior[19].

DISCUSSION

This study presents a comprehensive clinical and microbiological profile of infected diabetic foot ulcers in hospitalized patients with special reference to the study of biofilm production in the bacterial isolates. Despite all preventive measures, it is well known that patients with Diabetes Mellitus (DM) complicating with foot ulceration and these infections if left untreated results in the need for distal limb amputation[22]. Some studies report that diabetic foot infections contribute to approximately 20% of hospitalisations[5]. India has the highest population of individuals living with diabetes. As higher resistance is a growing problem, effort was made to study the association of different study characteristics with the presence of resistant organisms. In our hospital, 100 DFI patients were included during the period of the study from 25% of inpatient cases of General Surgery Department. The prevalence of diabetic foot ulcers among male subjects was found to be 61% against 39% in female i.e, a ratio of 1.56:1 which may be due to higher level of outdoor activity among males compared to females[23]. In present study, we found polymicrobial etiology in 34/100(34%) and monomicrobial in 60/100(60%) patients. Studies numbered [23], [24], and [25] reported polymicrobial infections at rates of 33%, 66%, and 83%, and monomicrobial infections at 56.6%, 23%, and 16.2%, respectively. The results of our study align with those of Zubair *et al*.[23], who also observed a 33% incidence of polymicrobial infections. Typically, infections begin as monomicrobial and tend to become polymicrobial as they advance over time. And ulcers that are deeper and that have a higher degree of necrosis tend to be polymicrobial (as per Wegener classification). In our study, it could also be attributed to the fact that some of the patients were on antimicrobial treatment during sampling and only the multi-drug resistant organisms not responding to the treatment would have been cultured. 91.47% of the isolates were Gram negative while 8.53% were Gram positive in our study. The predominance of Gram-negative organisms has been noted in several studies[5,6]. Al Benwan, *et al*.[26] also reported Gram-negatives were more prevalent, but predominant organisms isolated were members of Enterobacteriaceae. However, this was very different from the findings of Rani *et al*[27] in which 46.1% of the microbes were Gram negatives and 53.9% were Gram positive.

In our study, Klebsiella species (31.78%) was the most commonly isolated organisms followed by Escherichia coli (19.38%). These results were similar to those obtained by Sivaraman *et al*[28] where Klebsiella was the predominant organism (20.5%). But earlier studies have documented Gram-positive bacteria as the predominant organisms associated with diabetic foot infections[29,30]. Therefore, there seems to be a changing trend in the organisms causing diabetic foot infections, with Gram-negative bacteria replacing Gram-positive bacteria as the commonest agents. This study revealed multi drug-resistant organisms are very common in hospitalized patients with diabetic foot ulcers. This is in accordance with the reports of Hartemann-Heurtier *et al*., 2004 and Zubair *et al*., 2010 a, b, c[23,31]. The present study reveals a high incidence of Klebsiella species in the pus samples and their tendency towards antibiotic resistance. In our study, Klebsiella species are 56.1% sensitive to tigecycline. They showed a high resistance to commonly used antibiotics like amoxicillin+clavulanic acid (95.12%), ceftriaxone (87.80%), ceftazidime (85.37%), cefepime (80.49%), cotrimoxazole (78.05%), ertapenem and piperacillin+tazobactam (both 75.61%). These findings could be related with the findings of Asati Rakesh Kumar[32] where 88.8% resistance to cotrimoxazole and more than 80% resistance to third generation cephalosporins were noted.

With reference to other Gram-negative bacteria except Klebsiella, they were most sensitive to gentamicin (63.7%) amikacin (61.9%) and piperacillin+tazobactam (52.3%). They showed a high level of resistance to ampicillin (93.8%), amoxicillin+clavulanicacid (91.3%), ceftriaxone (76.1%), and ciprofloxacin (74.2%). In an earlier Indian study, all members of Enterobacteriaceae were found to be uniformly sensitive to gentamicin[33]. In a study by SM Sekhar *et al*, all Gram-negative isolates except Acinetobacter were highly sensitive to amikacin. The high degree of drug resistance to the other antibiotics can be due to excessive usage of broad-spectrum antibiotics leading to selective survival advantage of pathogen. This is often seen in tertiary care centres[34].

Amongst the Gram-positive organisms, Staphylococcus were 10(7.75%) in number. They exhibited high-level resistance to penicillin G (100%), erythromycin (75%), cotrimoxazole (62.5%) and cefoxitin (62.5%). There was a lower level of resistance to other antibiotics that were tested against. They were completely sensitive to tetracycline, doxycycline and linezolid. The prevalence of Staphylococcus in our study was significantly lower as compared to Nadeem Sajjad Raja study in which it was 44%[35]. Studies have shown that biofilm associated microorganisms can be up to 1000 times more resistant to antibiotics than free floating planktonic bacteria[36].In the present study, 81 isolates (62.79%) were multi-drug resistant, out of the which 77 (59.69%) also exhibited biofilm formation.Swarna *et al* reported that majority of the MDRO were biofilm formers[2].

Reason for this high antimicrobial resistance among the biofilm producers appears to be due to the close cell-cell contact that permits bacteria to more effectively transfer plasmids to one another than in the planktonic state. These plasmids can encode for resistance to several different antimicrobial agents[37]. Another factor contributing to resistance is quorum sensing, which through the processes described above can force bacteria into a slow-growing state when placed in an environment with adverse growth conditions; when in this state of intermission, bacteria are less susceptible to antimicrobial attack[38].The biofilm offers physical protection to bacteria, as antimicrobial agents struggle to penetrate it. This reduces the concentration of the agents reaching the bacterial cells within the biofilm, consequently diminishing their effectiveness[37]. In addition to the resistance to antimicrobials, biofilms also appear to have an antiphagocytic property within the biofilm, which renders leukocytes present within the matrix ineffective. Additionally, there is also an element within the matrix that disables both complement and host antibodies[39]. In our study, 59.7% of the isolates showed biofilm formation. This result was similar to findings by James *et al* which recorded a rate of 60% in

chronic wounds However this was unusual, as compared to prior studies in which it ranged from 73%-77.1%[2,34]. Such a deviation from the norm could be due to effective debridement procedures[34] or shorter duration of ulcer in patients. Klebsiella species was the predominant biofilm former, with 36.36% of the isolates testing positive for biofilm formation. This is the expected result, with prior literature supporting the biofilm forming nature of Klebsiella[40]. The limitation of this study was the inability to isolate anaerobes. Numerous studies report anaerobes as comprising a majority of the isolated organisms[24].

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Conflict of Interest: None declared.

Key points

- Diabetic foot ulcers are a significant burden on patients and healthcare systems, and biofilm formation can contribute to chronicity and infectious complications.
- Bacteria that produce biofilms, like Klebsiella and E.coli, tend to exhibit resistance to antibiotics and disinfectants, highlighting the importance of accurate detection and effective management.
- The study found a 59.7% prevalence of biofilm formation in diabetic foot patients, with Gram-negative bacilli (Klebsiella and E.coli) being predominant.
- Liberal debridement combined with appropriate antibiotics can help manage diabetic foot infections and reduce the emergence of multi-drug resistant organisms.
- Routine screening for biofilm formation in diabetic foot ulcers can aid in effective management and reduce morbidity and mortality.

Glossary of Abbreviations

BHI- Brain Heart Infusion.
CLSI- Clinical Laboratory Standard Institute.
CRA- Congo Red Agar
DFU- Diabetic Foot Ulcers.
DFI- Diabetic Foot Infections.
DM- Diabetes Mellitus
MDROs- Multi-Drug Resistant Organisms.
OD- Optical Density.
TCP- Tissue Culture Plate.
TM- Tube Method.
TSB- Trypticase Soy Broth.

REFERENCES

1. Mohan V, Sandeep S, Deepa R, Shah B, Varghese C. Epidemiology of type 2 diabetes: Indian scenario. Indian J Med Res 2007;125:217-230.
2. Swarna SR, Radha Madhavan, S.Gomathi, Devaraj, S. Thamaraiselvi. A study of Biofilm on Diabetic Foot Ulcer. International Journal of Research in Pharmaceutical and Biomedical Sciences Dec 2012;3(4):1809-1814.
3. Reginald A, Benjamin R, Blessed W, D Nathaniel, Samson D, Clarence S, Jacob J, Venkata R, Mohan, Jasmine Helan P, K S Jacob. Risk Factors for Foot Ulcers in Patients with Diabetes Mellitus- A short report from Vellore, South India. Indian J Community Med 2010 January; 35(1):183-185.
4. Frykberg R. An evidence-based approach to diabetic foot infections. Am J Surg 2003; 186/5A:44S-54S.
5. Shankar E M, Mohan V, Premalatha G, Srinivasan R S, Usha A R. Bacterial etiology of diabetic foot infections in South India. Eur J InternMed 2005; 16:567-570.
6. Gadepalli R, Dhawan B, Srinivas V, Kapil A, Ammini AC and Chaudhry R. A Clinico-microbiological study of diabetic foot ulcers in an Indian tertiary care hospital. Diabetes Care 2006; 29:1727-1732.
7. James G A, Swogger E, Wolcott R, Pulcini E, Secor P, Sestrich J, Costerton J W and Stewart P S. Biofilms in chronic wounds. Wound Repair Regen 2006; 16:37-44.
8. Fey P D. Modality of bacterial growth presents unique targets: how do we treat biofilm-mediated infections? Current opinion in Microbiology 2010; 13:610-615.
9. Dowd S E, Sun Y, Secor P R, Rhoads D D, Wolcott B M, James G A and Wolcott R D. Survey of Bacterial Diversity in chronic wounds using Pyrosequencing, DGGE, and Full Ribosome Shotgun Sequencing. BMC Microbiology 2008; 8:43-58, ISSN 1471-2180(online).
10. Bjarnsholt T, Kirketerp-Moller K, Jensen P O, Madsen K G, *et al*. Why chronic wounds will not heal: a novel hypothesis. Wound Repair and Regeneration 2008; 16(1):2-10.
11. Arciola C.R, Baldassari L, Montanaro I. Presence of ica A and ica D genes and slime production in a collection of Staphylococcal strains from catheter-associated infections. J Clin Microbial 2001; 39:2151-56.
12. Angela O, Frank L, Andrew J. The Visualization of Biofilms in Chronic Diabetic Foot Wounds Using Routine Microscopy Methods. Journal of Diabetes Research 2014. Article 1D; 153: 586-8.
13. Lauren C, Samina S. Diagnosis and Treatment of venous ulcers. Am Fam Physician 2010 Apr 15; 81(8):989-996.
14. Sriram Bhat M. Diabetic foot and Diabetic gangrene. SRB's Manual of Surgery 2016; 1:206-207.
15. Collee J G, Fraser A G, Marmion BP, Simmons A. Mackie and McCartney Practical Medical Microbiology. 14th ed.Newyork Churchill Livingstone 2006.
16. CLSI. Performance standards for Antimicrobial Susceptibility testing; Twenty First International Supplement. CLSI document M100-S21. Wayne, PA:Clinical and Laboratory Standards Institute 2012.
17. Christensen GD, Simpson WA, Bisno AL, Beachey EH. Adherence of slime producing strains of Staphylococcus epidermidis to smooth surfaces. Infect Immun 1982; 37:318-26.
18. Freeman D J, Falkiner FR, Keane CT. New method for detecting slime production by coagulase negative Staphylococci. J ClinPathol 1989; 42:872-4.
19. Mathur T, Singhal S, Khan S, Upadhyay DJ, Fatma T, Rattan A. Detection of biofilm formation among the clinical isolates of Staphylococci: An evaluation of three different screening methods. Indian Journal of Medical Microbiology 2006; 24(1):25-29.
20. Stepanovic S, Vukovic D, Dakic L, Savic B, Svabic-Vlahovic M. A modified microtiter-plate for

quantification of staphylococcal biofilm formation. J Microbial Methods 2000; 40(2): 175-179.
21. Christensen GD, Simpson WA, Younger JA, Baddour LM, Barrett FF, Melton DM, *et al*. Adherence of coagulase negative Staphylococci to plastic tissue cultures: a quantitative model for this adherence of staphylocci to medical devices. J ClinMicrobiol 1985; 22:996-100.
22. Lipsky BA, BerendtAR, Embil J, *et al*. Diagnosing and treating diabetic foot infections. Diabetes Metab Res Rev 2004;20(suppl 1):56-64.
23. Zubair, M., Abida, M. and Jamal, A. Clinico bacteriology and risk factors for the diabetic foot infection with multidrug resistant microorganisms in North India. Biology and Medicine 2010.2(4), 22-34. European Journal of Internal Medicine. 16, 567-570.
24. Citron DM, Goldstein EJC, Merriam V C and Lipsky BA. Bacteriology of moderate to severe diabetic foot infections and in vitro activity of antimicrobial agents. J Clin Microbial 2011; 45(9), 2819-2828.
25. Zubair M, Malik A, and Ahmad J. Clinicomicrobiological study and antimicrobial drug resistance profile of diabetic foot infections in North India 2011. Foot(Ednib); 21(1), 6-14.
26. Al Benwan K, Al Mulla A, Rotimi VO. A study of the microbiology of diabetic foot infections in a teaching hospital in Kuwait. J Infect Public Health 2012;5:1-8.
27. Rani V, Nithyalakshmi J. A comparative study of Diabetic and Non-diabetic wound infections with special reference to MRSA and ESBL. Int J. Curr. Microbiol. App. Sci 2014;3(12):546-554.
28. Sivaraman Umadevi, Shailesh Kumar, NoyalMariya Joseph, Joshy M Easow, G Kandhakumari, Sreenivasan Srirangaraj, Sruthi Raj, Selvaraj Stephen. Microbiological study of diabetic foot infections. Indian Journal of Medical Specialities 2011;2(1):12-17.
29. Mantey I, Hill RL, Foster AV, Wilson S, Wade JJ, Edmonds ME. Infection of foot ulcers with Staphylococcus aureus associated with increased mortality in diabetic patients. Commun Dis Public Health 2000;3:288-90.
30. Dang CN, Prasad YD, Boulton AJ, Jude EB. Methicillin-resistant Staphylococcus aureus in the diabetic foot clinic: a worsening problem. Diabet Med 2003;20:159-61.
31. Hartemann-Heurtier A, Robert J, Jacqueminet S, Ha Van G, Golmard J L, JarlierV,*et al*. Diabetic foot ulcer and multidrug-resistant organisms: risk factors and impact. Diabet Med 2004;21:710-715.
32. Asati Rakesh Kumar. Antimicrobial sensitivity pattern of Klebsiella pneumonia isolated from pus from tertiary care hospital and issues related to the rational selection of antimicrobials. Journal of Chemical and Pharmaceutical Research 2013; 5(11):326-331.
33. Anandi C, Alaguraja D, Natarajan V, Ramanathan M, Subramaniam CS, Thulasiram M, *et al*. Bacteriology of diabetic foot lesions. Indian J Med Microbial 2004; 22:175-8.
34. Zubair M, Malik A, Ahmad J, Rizvi M, Farooqui KJ, Rizvi MW. A study of biofilm production by gram-negative organisms isolated from diabetic foot ulcer patients. Biology and Medicine 2011; 147-157.
35. Nadeem Sajjad Raja. Microbiology of diabetic foot infections in a teaching hospital in Malaysia: a retrospective study of 194 cases. J Microbial Immunol Infect 2007; 40:39-44.
36. Ashok D, *et al*. Why Diabetic Foot Ulcers do not heal? JIMSA October-December 2011; 24(4):205.
37. Mah T, O'Toole G. Mechanisms of biofilm resistance to antimicrobial agents. Trends in Microbiology 2001; 9:34-39.
38. Mertz P. Cutaneous biofilms: friend or foe? Wounds 2003; 15:129-132.
39. Percival L, Bowler P. Biofilms and their potential role in wound healing. Wounds 2004; 16:234-240.
40. KimiaSeifi, Hossein Kazemian, *et al*. Evaluation of Biofilm Formation among Klebsiella pneumoniae isolates and Molecular characterization by ERIC-PCR. Jundishapur Journal of Microbiology 2016;9(1).

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