Training Report

BACTERIOLOGY OF DIABETIC FOOT INFECTIONS AND ANTIMICROBIAL SUSCEPTIBILITY

Internship Training Report

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Master of Science in Clinical Microbiology

Submitted by:
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DECLARATION

I hereby declare that the work embodied in this internship report was carried by me under the supervision of Mr. Naresh Kumar (Internal supervisor), Lovely Professional University and Dr. Anish Mohan MBBS, MD (Pathology), Ahalia Central Laboratory and Research Centre. This work has not been submitted in part or in full in any other university for any degree or diploma.

Name: Joslin T J

Date: …….

Place: …….

CERTIFICATE

This is to certify that **Mr./*Ms.*** ……Jostin T.J…….. bearing **Registration Number** 11401307…. has completed his/her Master of Science in Clinical .Microbiology internship under our guidance and supervision. This report is record of the candidate own work carried out by him/her under my supervision. I certify that the matter embodied in this report is original and has been not submitted anywhere for the reward of any other degree.

DR. ANISH MOHAN A

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01/05/2016

ABSTRACT

The purpose of this Clinico- microbiological investigation was to evaluate the diversity of bacterial pathogens in diabetic foot infections and to compare them with their degree of antimicrobial susceptibility. The objectives of this study were to identify predominant bacteria in diabetic foot infections and to know their degree of antimicrobial susceptibility. In this prospective experimental study, hundred positive culture samples were taken from a study population of 60 patients during four months period, from a tertiary care hospital in central Kerala. The hypothesis was that there is significant association between frequently isolated bacteria and the antibiotic resistance. The bacteria were identified using conventional culture methods, and by biochemical parameters. Among hundred samples 108 bacterial pathogens were isolated, with eight poly microbial infections. Among these isolates, 90 strains were drug resistant (83.3%). Gram negative bacilli were predominant with Escherichia coli having the highest prevalence (26%). *Staphylococcus aureus* was also common (18%). Among the isolated bacteria, 67 isolates among a total of 108 were Multi Drug Resistant (62%), 19 isolates were Extensively Drug Resistant (17.6%) and 4 isolates were Pan Drug Resistant (3.7%). There was significant association between frequently isolated Gram Positive Cocci and Gram Negative Bacilli, and their antibiotic resistance (at Chi square value of 0.537 at 0.05 level of significance, the critical value of chi square being 3.841).The results of the study shows that both GNBs and GPCs are prevalent in Diabetic Foot Infections. The study concludes that frequently occurring bacteria had more number of drug resistant strains.

PREFACE

The study titled "**Bacteriology of diabetic foot infections and antimicrobial susceptibility"** focuses mainly on antibiotic resistance that DFI bacteria possess. It was conducted as a requirement for partial fulfilment of Masters Degree in Clinical microbiology from Lovely Professional University, Punjab. When selecting the topic for the study, the commonness of the topic was a serious concern, but through in- depth reading it was understood that a Clinico-microbiological study about antimicrobial susceptibility is never outdated, the main reason being rapid evolution of bacteria. The reason for undertaking this study at a hospital where I pursued my internship was mainly its usefulness. As a diabetic speciality hospital, most patients visiting this place are the ones with diabetic foot ulcers. This project was aimed at identifying bacterial pathogens prevalent in this locality in central Kerala and to study their antimicrobial susceptibility.

The research problem was selected and formulated together with my supervisor and pathologist of the hospital, Dr. Anish Mohan, who has been a source of motivation throughout the study. The research was little difficult, but with the help of my supervisor, Dr. Anish Mohan and my co-supervisor Mr. Naresh Kumar, lecturer at Lovely Professional University, who guided me in selecting and in conducting my research, I was able to complete the process without much chaos. I was fortunate to have supervisors who were available and willing to give answers for my questions. I would like to thank my supervisors for helping me and providing me with resources to complete this research.

I wish to thank the patients from whom the samples were collected, without whom there is no scope for this research. I thank Ahalia Diabetes Hospital, Palakkad, for giving me a chance to conduct research in their organization. To all my colleagues at Ahalia Central Laboratory and Research Centre (ACLRC), and lab in-charge Mr. Paul Raj, I extend my deep felt gratitude for all the support and co operation you have provided, and for understanding and responding to my requirements. I specially thank Dr.Aparna, all microbiologists and staff of microbiology department, who taught me, guided me and supported me in carrying out my study. I extend my sincere gratitude to Dr. Koushik, Podiatry surgeon of Ahalia Diabetes Hospital, for his guidance and for answering my queries. I thank Dr. Hisham, statistician who helped me with suggestions for the statistical analysis between his busy schedules. I thank Dr. Anish Mohan who has suggested me to seek help from right persons.

I thank all my friends and family for being there for me, to support and motivate if I ever lost interest. It was your wonderful cooperation and ideas which guided me. I wish to thank my colleague, Ms. Fazeela of Podiatry department, for providing me with information and resources I needed and for the motivation. My mother deserves a special note of thanks; your motivation, wise counsel, and kind words have served me well. I would also like to thank my family for all the support during my study. I also extend my gratitude to all the staff of Ahalia Diabetes Hospital, and all those who helped me directly or indirectly, to complete my study.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

TERMINOLOGY

DFI -Diabetic foot infection- Foot infections in diabetic patients, seen below the malleoli.

DFU-Diabetic foot ulcer which may be infected or uninfected

DFO-Diabetic foot Osteomyelitis - infection of bone

Drug Resistance- the ability of a pathogen to resist the action of one or more antibiotics

Antimicrobial susceptibility- the antibiotic resistance or sensitivity shown by the bacteria against one or more antibiotics

MDR- multi drug resistant bacteria are bacteria resistant to more than one drug in all categories of antibiotics as given by CLSI performance guidelines.

XDR- Extensively drug resistant bacteria are those which are resistant to all except two categories of antibiotics.

PDR-Pan drug resistant bacteria are those which are resistant to all categories of antibiotics given.

CLSI- Clinical Laboratory Standards Institution

IDSA-Infectious Diseases Society of America

ATCC- American Type Culture Collection

GPC- Gram Positive Cocci

GNB- Gram Negative Bacilli

MSSA- Methicillin Sensitive *Staphylococcus aureus*

MRSA- Methicillin Resistant *Staphylococcus aureus*

ESBL- Extended Spectrum Beta Lactamases, rendering increased multidrug resistance to bacteria

I/V- Intravenous route of drug administration.

PO- '*Per Os'*- oral route of drug administration

CDC - Centres for Disease Control and prevention

ECDC- European Centre for Disease Control and prevention

MBL- Metallo Beta Lactamases

Inter species interactions- The interactions between two or more bacterial species in a polymicrobial wound

Poly microbial infection – infection with more than one micro-organism

CHAPTER – I

INTRODUCTION

CHAPTER-I

INTRODUCTION

Evolution is the driving force of the universe, which can be good, bad or both. This is the change that enabled us to stand erect, and is the same change which may make us fall.

Evolution is also the property which enables bacteria to tolerate or resist antibiotics, and by this perspective it is always a thought provoking idea which must be explored in-depth. Bacteria are single celled, primitive organisms and one of the smallest forms of life on earth. Still they don't fail to fascinate, if not to threaten human race. This is because the higher rate of multiplication and evolution that they possess. The abilities like antibiotic resistance result from these and this phenomenon has been under study in different parts world. Different techniques are being used to study this, ranging from conventional laboratory culture and sensitivity methods to Morbidostat, a device tracking real time antibiotic resistance evolution in bacteria.(1)

Originated from a Greek word meaning honey urine, Diabetes Mellitus is still no sweet word to hear. There is a wide range of health hazards related to diabetes, known and unknown. Among these, one that doesn't fail to attract attention is Diabetic Foot Infections (DFIs), due to its association with an alarming rate of increasing lower leg amputations. Hence study of the most important agents of pathogenesis of DFIs is necessary; the bacteria. Different bacteria may be prevalent in different regions, and so is the antibiotic resistance. While knowing prevalence of particular bacteria helps in specialized treatment, understanding the degree of antimicrobial susceptibility helps to know the seriousness of issue and adds to future implications (2)

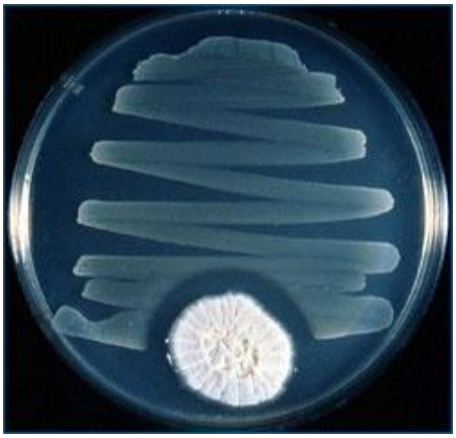

Figure 1:1: In this dish we see a *Penicillium* fungus (White blob at the bottom of the image) preventing the growth of bacteria (the horizontal smear)[a].

[a] *Penicillium* image by Christine L. Case/Skyline College, retrieved from http://evolution.berkeley.edu/evolibrary/article/side_0_0/turboevolution_01

Every thirty seconds, a leg is lost to diabetes somewhere in the world. Diabetic foot infections are the single most common cause of non traumatic below knee amputation worldwide. Currently, more than 65 million people are diabetics globally and 70% of all lower extremity amputations are due to diabetes. This life threatening complication most often start as a small wound in leg which progresses to what we call a Diabetic Foot Infection (DFI).(3)
Diabetes has taken the centre stage in 2016 and for the first time ever, diabetes was the theme selected for the world health day 2016 by World Health Organization (WHO)(4). Diabetes is a chronic, metabolic disease characterized by elevated levels of blood glucose (or blood sugar), which leads over time to serious damage to the heart, blood vessels, eyes, kidneys, and nerves. It is one of the most ancient disorders to be described in Egyptian manuscripts, as well as in ancient Indian and Greek writings. The ancient Indian physicians Charaka and Sushrutha had described the type1 and type2 diabetes very early in 500 BC, and even now we are unable to handle this disorder and prevent its complications(5).

Nerve damage in diabetes can be more pronounced in legs, especially in feet due to pressure applied on that area. This frequently leads to Diabetic Foot ulcer following an unnoticed injury and then leads to infection. The Diabetic Foot Infection (DFI) is defined as soft tissue or bone infection below the malleoli, is the most common complication of diabetes leading to hospitalization and most frequent cause of non traumatic lower extremity amputation. The clinical diagnosis of DFI is done based on the presence of at least two classic findings of inflammation or purulence. These signs include swelling, redness, pain, heat and loss of function at the site of injury (2,5).

WHY IS DIABETIC FOOT INFECTION SO THREATENING?

Diabetic infections usually tend to be chronic infections and are difficult to manage. They heal slowly and if not taken care of, can lead to systemic infection. This is due to:

1. Most of the diabetic wounds are painless due to peripheral nerve disorder in diabetes. So patients keep walking and this lead to worsening of ulcers.
2. Since Diabetes leads to impaired immunity, the body's defence mechanisms cannot handle the invasion of germs. This leads to spread of infection to blood, bone and muscle.
3. The blood supply to the legs is greatly reduced in most diabetics due to vasculopathy or disease of blood vessels. This will delay wound healing even if the patient is on antibiotics, because the antibiotics would not reach the site of infection due to reduced blood circulation.
4. Poor nutrition, kidney and liver diseases also decrease wound healing.
5. Chronic wounds may have underlying bone infection and this increases infection and delays healing.

Most infections occur on skin and 85% of all lower extremity amputations in diabetic patients are preceded by an ulcer. Frequently DFIs are poly microbial that is more than one microbe

causing the infection. Osteomyelitis, the infection of bone underlying the tissue is one of the threatening complications of DFI, which increases the rate of amputations in diabetic patients (5).

ROLE OF MICROBES IN DFIs

The microbes which are broadly divided into two categories, Gram positive and Gram negative bacteria are the cause of DFIs. Globally, the most frequent pathogens in DFIs are aerobic Gram positive cocci, mainly staphylococcal species. This can vary from place to place and person to person. Gram negative bacteria can be the agent in chronic or long-term ulcers (2).

The impaired immunity in diabetes favours the invasion of microbes and spread of infection. Antibiotics are available for most of the pathogens but many bacteria are now becoming multi drug resistant and these are difficult to treat. Multidrug resistance is a property that some microbes acquire in the course of time and they can transfer this property to same species or different species of micro organisms. Mostly the pathogens in DFIs are bacteria and their antibiotic susceptibility is important to determine treatment (6). Here the role of a clinical microbiologist becomes crucial in identification and management of DFIs. A multi disciplinary approach is always preferred in management of diabetic foot infections (7).

CLASSIFICATION OF DIABETIC FOOT INFECTIONS

According to Infectious Diseases Society of America (IDSA) and International Working Group on the Diabetic Foot classified DFIs into categories mild, moderate and severe (7).

Table 1: 1 classification of DFIs

Sl. No.	Parameters	Category
1.	Local infection- invades only skin and subcutaneous tissue without signs of systemic inflammatory response.	Mild DFI
2.	Local inflammation with erythema (redness) of more than 2 cm around wound and involves skin, subcutaneous tissue, fascia and bone, or joint without systemic inflammatory response.	Moderate DFI
3.	Local infection with signs of systemic inflammatory response.	Severe DFI

Diabetic foot infections usually turn chronic due to immune suppression together with bacterial drug resistance. One million limb amputations occur yearly in persons with diabetes. This number can be significantly reduced by means of effective foot care and prevention of infections. The chance of occurrence of antibiotic resistant bacterial strains will be high, due to chronic infections. Here the study about antimicrobial resistance in DFIs becomes important (8)

BACKGROUND OF STUDY

Diabetes is a slow and silent killer and DFIs are one of the most frequent complications of diabetes. Among 422 million diabetics worldwide, 62 million are Indians and this reveals the burden of diabetes in India which is in 2nd position in diabetic patients. Foot infections are the commonest reason for both diabetes related hospitalization and lower extremity amputation. Optimal treatment of diabetic foot ulcers require correct recognition of infected diabetic foot ulcers. For many years, the DFIs were confirmed by positive culture results and it is followed now too. But recently, methods of molecular biology and many other newer techniques are being introduced for the definitive diagnosis and treatment of DFIs. People with diabetes have about a 25% chance of developing a foot ulcer in their lifetime (9).

IMMUNOLOGY OF DFIS

The pathophysiology of foot infections in persons with diabetes is quite complex, but their prevalence and severity are mostly a consequence of host-related disturbances like vasculopathy and neuropathy. Usually such a deformed foot develops an ulcer when some form of trauma disrupts the protective skin envelope. This leads to invasion of subcutaneous tissue with pathogens. The clinical infection is defined usually by bacterial overgrowth. This usually starts as a local injury and progresses to produce inflammation and tissue destruction. At this stage the ulcers can be chronic and slow healing, usually with poly microbial etiology. Micro organisms with drug resistance can also contribute to the outcome of infection. This scenario makes it necessary to have knowledge about predominant pathogens in DFIs and antibiotic resistant strains of bacteria, for better management of DFIs. Moreover, as diabetes is an immunocompromised state, the progression of the infection is more likely. Weakened leukocyte phagocytosis has been found in both Type 1 and Type 2 DM and this explains diminished bacterial clearance of the infecting bacteria during hyperglycemia. There was 50% reduction in phagocytosis by the leukocytes in diabetic mice infected by *Staphylococcus aureus (10).* The main factor of neutrophil dysfunction is the hyperglycemia, which alters neutrophil chemotaxis, phagocytic action and intracellular killing of the bacteria. Alterations in levels of complement fractions can also be found. Though, there is still controversy concerning the effect of Diabetes Mellitus on the function of human lymphocytes (11). Humoral immunity in diabetic patients can be affected as well, which was shown by a shorter duration of the protective antibodies after tetanus vaccination and lower levels of tetanus antitoxin compared with those in non diabetic patients (12). Along with skin infections, diabetics are more prone to other kinds of infections too. The presence of frequent asymptomatic bacteriuria in diabetic patients is a considerable health issue and long duration of Diabetes Mellitus treatment especially with insulin, and retinopathy has been associated with elevated risks for recurrent UTIs. Diabetic patients are at an increased risk for severe periodontitis, lower respiratory tract infections (LRTIs) as well as cellulitis, Osteomyelitis, peritonitis and sepsis. This shows that how likely are diabetics in

acquiring infections, even during the hospital stay. Skin infections are common in diabetics, and patients exhibit dermal accumulation of advanced glycation end products, which is associated with severity and delayed healing of the diabetic wounds. Interestingly, ciprofloxacin, which is used to treat diabetic skin ulcers, penetrates well in the tissues, modulates the response of monocytes by elevated prostaglandin E2 production and suppresses adhesion molecule expression on human monocytes (13). But ciprofloxacin resistance is shown by a variety of GNBs and GPCs. Also stress and infections in diabetic patients can lead to hyperglycemia and diabetic ketoacidosis. Hyperglycemia is due to augmented gluconeogenesis, increased glycogenolysis and poor utilization of glucose in the tissues. Diminished insulin levels and increased concentrations of cortisol, catecholamine and glucagon can contribute to both hyperglycemia and ketoacidosis (12)

MICROBIOLOGY OF DFIs

Microbiology of DFIs varies in mono microbial and poly microbial. Mostly Gram positives and Gram negatives prevail, among these *Pseudomonas species*, *Escherichia coli*, and *Staphylococcus aureus* are more common in DFIs. The pattern of microbial infection in patients with diabetic foot infections is usually inconsistent and, therefore evaluation of microbial characteristics and their antibiotic sensitivity is necessary for the selection of appropriate antibiotics for management of diabetic foot infection. The following tables show the global prevalence of bacteria in DFI and DFO (14).

Table 2:1 common bacteria in DFIs

Aerobic Gram positive	**Aerobic Gram negative**	**Anaerobes, facultative anaerobes**
Staphylococcus aureus	*Enterobacteriaceae*	*B. fragilis*
Streptococcal spp.	*Pseudomonas aeruginosa*	*Peptococcus and peptostreptococcus*
Enterococcus spp.		
Coagulase-negative staphylococci (from deep tissue)		

Table 2:2 common bacteria in Diabetic Foot Osteomyelitis

Aerobic Gram positive bacteria	Anaerobic and Gram negative bacteria
Staphylococcus aureus Group A and B Streptococcus spp. Enterococcus spp. Coagulase-negative staphylococci	*Enterobacteriaceae* *Pseudomonas aeruginosa* *Escherichia coli* *Serratia marcescens* *Haemophilus influenza*

Superficial infections (cellulitis, cellulitis with blisters, and shallow ulcers) are typically caused by *S. aureus* or beta-hemolytic streptococci. Infections of ulcers that are chronic or previously treated with antibiotics may be caused by aerobic Gram-negative bacilli, *S. aureus* or Streptococci. Deep soft tissue infections, Osteomyelitis, and gangrene are more often poly microbial, including aerobic gram-negative bacilli and anaerobes; anaerobic streptococci, *Bacteroides fragilis* group, Clostridium species, but *Staphylococcus aureus* is also common in mono bacterial DFIs (14).

ANTIBIOTIC THERAPY AND ANTI MICROBIAL RESISTANCE IN DFIs

As an initial treatment, physicians usually prefer empiric antibiotic therapy, which uses a non specific usually, broad spectrum antibiotic for the treatment of DFIs. After identifying the specific pathogen and the anti microbial susceptibility by culture and sensitivity or other methods, this approach is replaced by definitive antibiotic therapy which is specific to pathogen/pathogens. For evaluation of infection, curettage from the base of an appropriately debrided ulcer is most preferred. Deep tissue obtained by biopsy is also preferable. The tissue sample is then usually sent for microbiological culture and sensitivity to a clinical microbiology laboratory. For the diagnosis of Diabetic Foot Osteomyelitis (DFO), bone biopsy with histopathological examination or bone culture is used, along with clinical radiographic findings to assess the extent of infection. This approach gives more accurate results in the treatment of DFOs (15).

The commonly used antibiotics in treating DFIs and their classes are categorized below:

Table 2:3Suggested antibiotics in DFIs (15)

Pathogen / grade of infection	**Antibiotic**	**Class**
1. Mild infection GPC with or without MRSA	Amoxicillin/clavunate	Beta lactams
	Cefdinir	3rd gen cephalosporins(cephems)
	Cefalexin	5th gen cephem
	Clindamycin	Aminoglycosides
	Dicloxacillin	Beta lactams
	Doxycycline	Aminoglycosides
	Levofloxacin	Flouroquinolons
	Linezolid	Aminoglycosides
	Minocycline	Aminoglycosides
	Trimethoprim	Sulfonamides
2. Moderate to severe infection GPC, GNB, anaerobes with or without Multi drug resistance (MRSA, ESBL strains and Vancomycin resistant Enterococci)	Ampicillin/sulbactam	Beta lactams
	Cefoxitin	2nd gen cephem
	Ceftriaxone	3rd gen cephem
	Clindamycin	Amino glycosides
	Daptomycin	Polymyxins
	Etrapenem	Beta lactams
	Linezolid	Aminiglycosides
	Moxifloxacin	Flouroquinalones
	Piperacillin- Tazobactam	Beta lactams
	Ticarcillin	Beta lactams
	Vancomycin	Beta lactams

Table 2: 1 suggested antibiotics in DFI treatment

Though, recent studies by the International working group on diabetic foot and infectious diseases society of America showed that there is no single superior regimen of antibiotics for the treatment of DFIs. The choice of antibiotics should be guided by the nature of antibiotic, extent of infection, culture results, clinical response and local antibiotic resistance patterns, extent of immunosuppression and co-morbidities.

Among these antibiotics, beta lactams aim to inhibit cell wall synthesis of bacteria, amino glycosides inhibit protein synthesis, Polymyxins inhibit membrane function, and Quinolones inhibit nucleic acid synthesis in bacterial cell. Sulphonamides are anti metabolites and Cephalosporin is a subgroup of beta lactams, has same action but resist the beta lactamases.

Bacteria have achieved fair degree of resistance to almost all of these classes of antibiotics, ranging from MRSA to ESBL production (Extended Spectrum Beta Lactamases). Multi-drug resistant Gram-negative organisms described in DFI are especially ESBL, but most resistant organisms were reported from India and other warmer climates. Antibiotic resistance is the most developing threat posed by the bacteria currently. Realizing the risk of antimicrobial resistance, the United States white house recently announced a national strategy for combating antibiotic resistance. The strategy focuses on slowing down the development of resistant strains of bacteria, strengthening surveillance, innovative and rapid diagnostic techniques, and improving international collaboration (16).

As bacteria are primitive yet highly evolving species on earth, antimicrobial resistance is a global health care issue that is to be taken care of. It is by unnecessary use of antibiotics, the bacterial strains with resistance grow fast. Bacteria are capable of high rate of evolution and hence competent methods should be implemented to combat antimicrobial resistance. This will only be possible with proper investigation about microbiology of infections, and their antimicrobial susceptibility. This study aims at studying bacteriology of DFI and investigating the degree of antimicrobial resistance of those bacteria, in a specific locality, which can improve the knowledge about pathogens in DFIs and hence improve the standards of health care (16).

OBJECTIVES OF THE STUDY

The topic for the present study is Bacteriology of diabetic foot infections and antimicrobial susceptibility. This is a Clinico-microbiological study investigating the prevalence of different bacteria in DFIs and the occurrence of antibiotic resistant strains among them. The study was planned under following objectives.

1. To identify predominant bacteria in diabetic foot infections in hundred selected samples in Ahalia Diabetes Hospital, Palakkad, Kerala.
2. To understand the antimicrobial susceptibility of bacteria isolated from diabetic foot infections.
3. To assess the degree of Drug Resistance shown by each bacterium in diabetic foot infections.

HYPOTHESES

- **H^0**- There will be significant association between frequency of Gram positive cocci and Gram negative bacilli isolated, and their antibiotic resistance in Diabetic foot infections.

- **H^1**- There will not be significant association between frequency of Gram positive cocci and Gram negative bacilli isolated, and their antibiotic resistance in Diabetic foot infections.

CHAPTER –II

REVIEW OF LITERATURE

CHAPTER-II

REVIEW OF LITERATURE

Studies on diabetic foot infections mainly focus on scientific assessment of the severity, risks, complications, and effectiveness of management, and future prospects. The studies reviewed here are categorized under two headings; studies on diabetic foot infection and antimicrobial susceptibility of pathogens in DFIs.

DIABETIC FOOT INFECTIONS

Diabetic foot infections (DFIs) can be diagnosed primarily by any two signs of inflammation and it is confirmed usually by positive culture results. The sample for microbial culture can be a wound or pus swab or, of tissue and or bone. DFIs can be poly microbial, although early-stage DFIs are generally mono bacterial due to *Staphylococcus aureus*, more advanced DFIs, especially those associated with vascular insufficiency, usually involve aerobic Gram-negative rods and anaerobes.

E.Senneville et al. (2009) assessed the diagnostic value of wound swab culture and percutaneous bone biopsy in diabetic foot infections in seventy six patients. The data showed that *Staphylococcus aureus* was the predominant organism in positive cultures and study concluded that superficial wound swabs do not reliably identify bone bacteria, and bone biopsy is more useful. However, a positive bone culture is used by many to confirm Osteomyelitis (17). Similarly, Gardner et al. (2009) evaluated the diagnostic value of clinical signs of infection in diabetic foot infections. The study included 64 subjects and assessed the microbial load in DFIs. The results shown that 39% of all DFIs were having high microbial load and that no individual clinical sign predicted high microbial load (18).

Shakil S et al. (2010) conducted a Clinico- bio informative study to assess the risk of Multi Drug Resistant Gram Negative Bacilli (MDR GNB) - diabetic foot infection among male and female patients. The researchers also assessed the ESBL (Extended spectrum beta lactamases, rendering multidrug resistance to bacteria which is seen in GNB) producers in DFIs. Among all *E.coli* isolates, 27.5% were ESBL producers. The study found that male patients had poor diabetes control and showed higher mortality rates compared to their female counterparts (19).

Coagulase negative Staphylococci are a sub-category of Staphylococci which until recently were considered as normal flora and as common laboratory contaminants. But studies show that *Staphylococcus epidermidis*, a coagulase negative staphylococcus is a real pathogen in diabetic foot infections. Sanchez et al. (2010) stated that *Staphylococcus epidermidis* is a serious pathogen in diabetic foot Osteomyelitis. In the present study, among 134 subjects eleven cases had only this bacterium as pathogen (20).

Uncontrolled diabetes is an immuno-compromised condition which predisposes patient to infections. Researchers also assessed the immunological perspective of diabetic foot infections. ZMH Khairalla et al. (2012) studied the association of complement C3 and IL1beta levels with diabetic foot infections and their bacteriological profile. Study included fifty subjects and showed that DFI patients had abnormal C3 and IL1 beta levels. The study is concluded with an emphasis on Zinc therapy to reduce IL1beta levels, and to thereby treat diabetic foot infections (21).

Many studies have been conducted to assess the microbial load in diabetic foot infections and attempted to identify specific pathogens in DFIs, by conventional and novel methods. Demetriou et al. (2013) examined the determinants of microbial load in diabetic foot ulcers (DFUs) of 63 patients. The researchers used the Texas classification, PEDIS (perfusion, extent, depth, infection and sensation.) grading, and number of isolates from DFIs for assessing this. The conclusion was high microbial load is related to more number of isolates. This can be simply stated as poly microbial infections are more serious (22).

Similar study by Sue.E.Gardner et al. (2015) stated that in the absence of clinical signs of infection, bacterial cultures of tissue samples were not useful in predicting outcome of DFIs. This shows that if no clinical infection present and bacterial culture gives positive results, it is likely that the foot ulcer to heal within short time. Their findings are in agreement with the guidelines provided by Infectious Diseases Society of America (IDSA) (23).

Another study examined the concordance of nasal colonization and diabetic foot ulcer (DFU) colonization by *Staphylococcus aureus.* The study in 79 subjects assessed MRSA also. But results had shown no concordance with nasal and the DFU colonization. So it can be concluded that endogenous spread of *Staphylococcus aureus* from nasal site to DFU is not likely (Ambar Haleem et al., 2013). *Staphylococcus aureus* had been the main focus of study for many researchers, as it is the predominant pathogen in DFUs globally (24). Albert sotto et al. (2013) evaluated the use of miniature Oligonucleotide arrays to discriminate S. aureus in DFIs. The study concluded that this technique is a promising method to diagnose and to predict outcome of DFIs (25).

Bacteriophages are viruses that can kill bacteria. As a therapy for bacterial infections lytic bacteriophages can be used. This was studied by Sanjay Chhibber et al. (2013) in diabetic mice with hind paw infection by *Staphylococcus aureus*. The result pointed out the effectiveness of single administration of phage was equal to that of Linezolid, an antibiotic used in Staphylococcal infections. However, the study concludes that combination therapy using both arrested the entire infectious process. This method can be promising to treat MRSA infections and to control antibiotic resistance (26).

Immunological aspects of staphylococcal antigens have also been studied. Super antigens (SAg) are those antigens which can bind to two types of immunological cells; the T-cell Receptors and

on MHC of antigen presenting cells and lead to a heightened immune response. Study by Bao G. Vu et al. (2014) shows that SAg of *Staphylococcus aureus* causes many cases of severe DFIs. The presence of MRSA strains was also assessed. The results shown that most of the strains were Methicillin sensitive and that SAgs of *Staphylococcus aureus* play a role in facilitating DFIs. The study concludes by suggesting the need of therapies to neutralize or reduce the SAg to treat *Staphylococcus aureus* infections (27).

When grown in-vitro, many pathogens show variations from the classical type. *Staphylococcus aureus* is one of them and the variants of this bacteria shows different colony morphology. Estrella Servantes-Gracia et al. (2015) studied the Small Colony Variants (SCVs) of *Staphylococcus aureus* from DFIs. This prospective clinical research included 120 DFI cases and found that common antibiotics used for Staphylococci were not useful in MRSA-SCVs. The study concludes by stating that the intracellular location of SCV *Staphylococcus aureus* protects them from immune system. This can be vital information for drug development (28).

Poly microbial infections are also common in diabetic foot ulcers. These are difficult to diagnose as well as to treat. Can Imirzalioglu et al. (2014) did a case report on distinct poly microbial populations in a chronic DFI. The bacteria were difficult to isolate and the infection, difficult to treat. In this study the superficial biopsy revealed a mixture of *Staphylococcus aureus*, *Proteus vulgaris,* and *Fusobacterium nucleatum,* whereas the tissue-deep biopsy harboured a mixture of four different bacterial species, namely *Gemella morbillorum, Porphyromonas asaccharolytica, Bacteroides fragilis,* and *Arcanobacterium haemolyticum*. The study conclusion is that the poly microbial infections should be treated with a microbe- adapted antibiotic therapy, but complete wound closure is difficult to achieve in such tissues due to extensive tissue damage (29).

Many researchers aimed at revealing the incidence, severity, complications and assessing micro biome of DFIs. Lower limb amputation is one of the most feared complications of diabetic foot, which can significantly reduce the quality of life for the affected person. Limb salvage means to avoid the amputation by Dane K. Wukich et al. (2013) argues that the severity of DFI and rate of limb salvage are related. The study was conducted with 100 subjects, and according to data 55% of severe DFI had undergone amputation. Patients with moderate DFI had higher rate of limb salvage (30). This shows that apt management can curb the progress of infection and reduce complications. Khalid Al-Rubeaan et al. (2015) a similar kind of retrospective study to assess the complications and risk factors of diabetic foot ulcers. In this cross sectional study, the results reveal that risk factors for DFIs are high, and so the risk of complications. The study recommends proper foot care and vascularisation of diabetic foot ulcer patients, in order to reduce the risk of DFIs (31).

Thimothy.C et al. (2014) conducted a different study to assess the micro biome of infected foot ulcers and antibiotic treatment among diabetic and non diabetic patients. The results show that there was no predominance of Gram negative organisms in diabetics, but diabetics usually tend

to receive broad spectrum Gram negative therapy. This kind of treatment was proved unnecessary by the study (32).

ANTIMICROBIAL SUSCEPTIBILITY

Antibiotic treatment is an unavoidable step in the management of DFIs. This can be empirical, which is guesswork by the physician about which antibiotics to prescribe. Most physicians prefer a broad spectrum antibiotic against Gram negative bacteria in the beginning, before getting a culture and sensitivity report of the sample. After identification of pathogen, the antibiotic regime is changed to definitive, which target the identified organism (s) specifically. The empiric antibiotics in treating diabetes should be reviewed from time to time, because they always don't show effectiveness. For this reason, it is necessary to identify common pathogens in that particular area or hospital, and their antimicrobial susceptibility.

Antimicrobial susceptibility testing measures the ability of a specific organism to grow in the presence of a particular drug in vitro and it is performed using guidelines established by the Clinical and Laboratory Standards Institute. Many studies have been conducted to identify common bacteria in DFIs and their antibiotic susceptibility. Researchers studied the effect of one particular agent as well as group of antibiotics. Reviewing such studies give an idea about which antibiotics are effective and which are not.

Ellie JC Goldstein et al. (May 2006) studied in vitro activities of Dalbavancin and twelve other anti microbial agents against 329 Gram positive bacteria found in DFI. The other antimicrobials in the study were Vancomycin, Linezolid, Daptomycin, Meropenem, Imipenem, Piperacillin-Tazobactam, Penicillin, Amoxicillin-Clavunate, Levofloxacin, Clindamycin, Cefotetan and Oxacillin. There were 209 aerobes and 120 anaerobes. Among all the above mentioned antibiotics, Dalbavancin appeared most effective against all Gram positive bacteria including MRSA and MSSA (33). In another study (Sept.2006), these researchers assessed the effectiveness of Ceftobiprol (a broad spectrum Cephem used against *S. Aureus*.) against 443 anaerobes in DFIs. The study included Gram positive and Gram negative bacteria and among these 90% of bacteria were inhibited by Ceftobiprol. The activity of Ceftobiprole was effective against MRSA, MSSA, *Pseudomonas* species and Enterobacteriaceae (34).

Another study conducted by the same researchers (2008) showed that Doripenem was the most active Carbapenem drug against *Pseudomonas* and *Proteus species*. Among 423 bacteria isolated from DFIs, GPCs except *Corynebacterium spp.* showed sensitivity to Doripenem. The study concluded that Doripenem was more effective than Meropenem and Imipenem (35).

Interspecies interactions are a cause of expanding antimicrobial resistance. This is mostly the aetiology of non- healing wounds in poly microbial DFIs. Trevor Dalton et al. (2011) prepared a wound biofilm model in mice, to study this phenomenon. The experimental animal showed delayed wound healing and, or antibiotic resistance when the wounds were infected with four bacteria simultaneously, using a biofilm. The biofilm was prepared using *Pseudomonas*

aeruginosa, *Enterococcus faecalis*, *Finegoldia magna* and *Staphylococcus aureus*. Microscopy of Hematoxylin- Eosine stained sections showed different bacteria staying in close proximity to each other and *Pseudomonas aeruginosa* showed more growth than other species. It was also observed that poly microbial infections had increased anti microbial tolerance with Gentamicin and Biocide (36). This study revealed that DFIs can be better studied using wound poly microbial biofilm infection model.

Similar kind of study was carried out in- vitro and in-vivo to check synergistic interactions between *Pseudomonas aeruginosa* and *Staphylococcus aureus* in a wound model (Stephanie et al. 2014). The study was done using in -vitro wound-like model as well as on mouse wound model, where pathogens were introduced into surgical excisions of mice. It was observed that when grown together, *Pseudomonas aeruginosa* and *Staphylococcus aureus* had the ability to survive antibiotic treatment. Conclusion of the study was that data shows synergistic interactions between the two species increase antibiotic resistance in both and leads to slow healing wound in diabetic mice (37). This is also an example for interspecies interaction through which one bacterium can pass their antibiotic resistance, tolerance and or, other properties to another.

Many antibiotics are available currently to treat DFIs with Gram positive and Gram negative bacteria. Though, no single antibiotic is effective against all bacteria, in all cases. In a study by NC Schapper et al. (2012) the safety and efficacy of two antibiotics, Moxifloxacin and Piperacillin- Tazobactam in DFI of different grades was compared. The initial I/V treatment with either of these antibiotics was followed by Amoxycillin- Clavunate oral regime. The efficacy of both antibiotics showed no differences, but based on the results Moxifloxacin was safer compared to Pipiracillin- Tazobactam due to less adverse effects (38). On the other hand, Piperacillin-Tazobactam is commonly used for the treatment of chronic DFIs.

Multidrug resistance is a challenge faced by health sector globally. In case of DFIs this can be more serious when the infection is poly microbial. Priya Shanmugham et al. (2013) assessed the antimicrobial resistance of bacteria found in DFIs. The data shows that Pseudomonas aeruginosa was most prevalent in DFIs. From the bacteria isolated from DFIs, 35.7% of GNB were ESBL producers, 31% were Carbapenamase producers among 83% ploy microbial infections (39). This reveals that most GNB has one or another kind of anti microbial resistance.

Shailesh K et al. (2013) argues that Escherichia coli detected from DFI shows 100% sensitivity to Piperacillin- Tazobactam where the bacteria were found to have ESBL and Carbapenamase activity (which contributes to antimicrobial resistance), detected through Multiplex PCR. The researchers detected the genes for antibiotic resistance by molecular docking of these genes using Piperacillin- Tazobactam and Clindamycin. This was done to study the molecular basis of antibiotic resistance. The results show that use of these antibiotics resulted in incomplete protein production by specific genes and drugs had significant interactions with active site residues in the gene and prevented resultant protein (40). Studying molecular basis of anti microbial resistance may provide more insight about how to overcome antimicrobial resistance.

To combat with antimicrobial resistance, novel antibiotics are being introduced currently. Bacteriocin is a peptide obtained from a bacteria *Bacillus subtilis.* Study of the bacteriocin on DFI bacteria showed that it has antimicrobial property (Baby Joseph et al. 2013). The antimicrobial activity of Bacteriocin was significant against 99% of bacterial pathogens in DFIs. The study also reveals that Bacteriocin had a high level of activity against *Klebsiella* species, a predominant MDR pathogen in DFIs (41). Such new compounds can help in defending the menace of antimicrobial resistance in present scenario.

Another study (Robert K et al. 2015) reveals that Pexiganan a topical antimicrobial agent currently under clinical trials, is effective against Gram negative and Gram positive bacteria. The drug was more effective when applied topically, and was not affected by antibiotic resistance. The study recommended the use of Pexiganan as a topical agent for the management of DFU and DFIs (42).

The reasons causing antibiotic resistance are many. These can range from previous infections to unnecessary use of broad spectrum antibiotics. Lawrence.A et al. (2014) studied the risk factors for MRSA in DFI. This was a Retrospective Cohort study and the main reasons for MRSA in DFI were previous infection with MRSA, nasal colonisation with MRSA and poly microbial infections with drug resistant bacteria. Though, the study concludes that the nasal colonisation with MRSA doesn't predict infecting agent in DFI. At the same time, negative nasal swabs rule out the chance of MRSA in DFIs with 90% accuracy (43).

The bacteria in diabetic foot infections may be different in different localities and based on the degrees of severity the bacteria can change. In case of chronic infections Gram negative bacteria are more common whereas in acute conditions Gram positive bacteria prevail. Many infections can be poly microbial with both Gram positive and Gram negative bacteria. Sometimes the pathogenic bacteria found in DFIs and DFOs can be the normal flora of the person's skin (Sanchez. et al.2010) (44). Bacterial aetiology and antibiotic susceptibility pattern was assessed by Mohammed T et al. (2015) in Tabriz, Iran. From sixty samples 92 bacterial strains were isolated and among this the predominant bacterium was *Staphylococcus aureus*. This was followed by Enterobacteriaceae including *Escherichia coli*. Most of the DFIs were poly microbial and most GPCs were sensitive to Linezolid, where most GNBs were susceptible to Imipenem. The study concludes that poly microbial infections would require combined anti microbial therapy, for initial management (45).

There had been always confusion about when to stop antibiotic therapy in DFIs; after disappearance of signs and symptoms or after a negative culture report. Physicians decide on this issue differently. Youjie Chu et al. (2005) investigated, whether we can stop antibiotics when the signs and symptoms have resolved, in DFIs. The randomized controlled trials were carried out with experimental group on which antibiotic therapy was stopped after disappearance of signs and symptoms, and control group which received continued antibiotic therapy. The researchers found that in DFIs with severe Peripheral Arterial Disease (PAD), continuing antibiotic therapy

was preferable. Though, in patients without PAD there was no significant difference between experimental and control groups. Study concludes that continuing antibiotic therapy would improve clinical outcome in patients with moderate to severe DFIs (46).

CONCLUSION

DFIs are an area of focus which is extensively studied for past decades. DFIs cannot be attributed only to developed, developing or underdeveloped countries. Its incidence is pandemic and so is that of DFIs. However, better diagnosis and systematic management can limit the disease. The bio burden of DFIs can be managed by newer technologies and drugs to limit its progression. The various researches done on DFIs and their causative organisms throw light on how to manage DFIs with better precision and newer methods. Study of the microbiology of DFIs is not a new approach; it has helped to improve the health care for patients, with precision and accuracy. Immunological studies on DFIs have also contributed to newer, effective treatment methods for DFIs. Together with better knowledge of DFI and proper health care, innovations and discoveries can lead to a better future in DFI management.

CHAPTER- III

RESEARCH METHODOLOGY

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RESEARCH METHODOLOGY

3.1: EQUIPMENTS:

Autoclave, Hot air oven, bio-safety cabinet, anaerobic glass jar, the anaerogas pack with indicator, weighing machine, incubator all these equipment were availed from the Ahalia Central Laboratory and Research Centre (ACLRC), Palakkad, Kerala.

3.2: MATERIALS:

Glass wares, inoculating loops and straight wires, forceps, glass slides, microscope, chemicals and reagents used in this project work were supplied from the Ahalia Central Laboratory and Research Centre (ACLRC), Palakkad, Kerala.

3.3: EXPERIMENTAL SETUP:

For the present study experimental set up was arranged at Clinical Microbiology Laboratory, ACLRC, Palakkad, Kerala. The setup was free of contamination and was following CLSI guidelines 2014-2015 for microbial culture and antimicrobial susceptibility. All the procedures were carried out aseptically in the bio-safety cabinet, taking universal precautions.

The study area was Ahalia Diabetes Hospital, Palakkad. The clinical microbiology lab of Ahalia Central Laboratory and Research Centre was the setting of study. The study was conducted during the period January 2016 to April 2016.

3:4: SAMPLES

One hundred and one samples were taken from 60 subjects. The samples were collected during the period January 2016-April 2016. Only positive bacterial cultures of diabetic foot infections (DFIs) were selected.

The samples were collected after minor debridement of the DFIs, or during surgery. The wound/ pus swabs were taken from deep ulcers with sterile swabs. The samples were transported to the laboratory in sterile containers.

3:5: THE BACTERIAL CULTURES:

Culture media:

The culture media used for bacterial culture and isolation were 5% Sheep blood agar, Mac Conkey agar, and occasionally CLED (Calcium Lactose Electrolyte Deficient) agar, in some cases of Poly microbial infections.

1. Blood Agar

The blood agar used for this study was Biomeriux Columbia blood agar. It contains Columbia agar with 5%sheep blood, which helps better distinguishing *Staphylococcus spp.*, *Streptococcus spp.* and fastidious organisms

Table 3:1 Media composition of blood agar

Ingredients	**Gms / Litre**
Peptone, special	23.000
Corn starch	1.000
Sodium chloride	5.000
Agar	15.000
Sheep blood	5 %
Final pH (at 25°C)	7.3±0.2

2. Mac Conkey agar

The Mac Conkey Agar was prepared manually in the laboratory under sterile conditions.

Table 3: 2 Media composition of Mac Conkey agar

Ingredients	**Grams/litre**
Peptone (Pancreatic digest of gelatin)	17 gm
Proteose peptone (meat and casein)	3 gm
Lactose monohydrate	10 gm
Bile salts	1.5 gm
Sodium chloride	5 gm
Neutral red	0.03 gm
Crystal Violet	0.001 g
Agar	13.5 gm
Distilled Water	Add to make 1 Liter

Final pH -7.1 +/- 0.2 at 25 degrees C

Inoculation, cultivation and isolation:

The bacterial culture was isolated from the tissue and bone samples from diabetic foot ulcers of 60 patients. Two types of bacterial cultures were performed, aerobic culture and anaerobic culture. Culture was carried out by inoculating the specimen on blood agar and Mac Conkey agar plates using sterile inoculating loop by streaking. The inoculated plates were incubated for 24-48 hrs for aerobic cultures.

The anaerobic culture was done on blood agar plates, and incubated for 72 hrs for anaerobic culture. HiMedia Anaero gas Pack (1.5 litre) Code No. : LE002F was used for anaerobic culture. Facultative anaerobes like Streptococci, Staphylococci, Enterobacter species and Pseudomonas species were isolated by anaerobic culture. Most anaerobic organisms were isolated from poly microbial infections.

Figure 3: 1 anaerobic jar with anaerogas pack

3:6: IDENTIFICATION

Identification of bacteria was carried out by studying **macroscopic study** (colony morphology) and comparing them with ATCC subculture plates, **microscopic study** after performing Gram staining to differentiate Gram positive and Gram negative bacteria and specific biochemical tests for genus and species identification.

Macroscopic Study:

The bacterial cultures were grown on blood agar and Mac Conkey agar and their macroscopical characteristics were analysed. This included colony morphology, amount of growth, presence or absence of haemolysis and Lactose fermentation.

The colony morphology was also compared with ATCC strains of *Escherichia coli (ATCC no. 25922), Staphylococcus aureus (ATCC no. 12600), Pseudomonas aeruginosa (ATCC no. 25619), and Klebsiella pneumonia (ATCC no. 10031),* which are commonly found in DFIs. The culture plates of these bacteria taken from the current work and their culture characteristics are following.

Culture characteristics OF *Escherichia coli*

Escherichia coli were grown on blood agar, Mac Conkey agar and CLED agar (Calcium Lactose Electrolyte deficient medium). The CLED agar was occasionally used for differential study. The colony characteristics of *Escherichia coli* on CLED agar were yellow, opaque, with center slightly deeper yellow and shows luxuriant growth on this differential media.

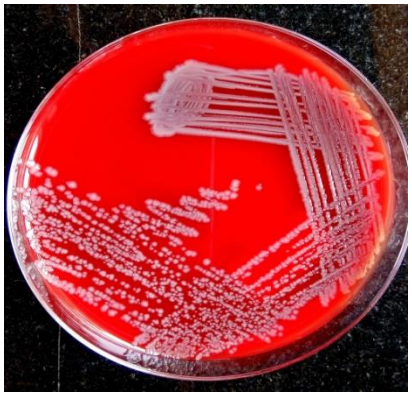


Figure 3:2

Escherichia coli on blood agar

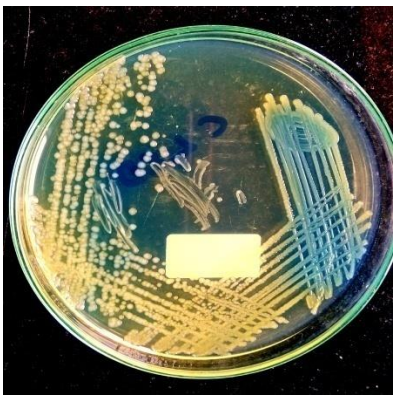


Figure 3:3

Esherichia coli on CLED agar

On blood agar, the bacterium appeared as grayish white, shiny, non hemolytic colonies. However, hemolytic strains occurred rarely. On Mac Conkey agar, the bacterium showed lactose fermentation in deep pink color.

Culture characteristics of Staphylococcus species

Staphylococcus species showed Lactose fermentation on Mac Conkey agar, with light pink, small colonies. On blood agar, the characteristics varied according to different species.

Figure 3:4

Staphylococcus aureus on blood agar

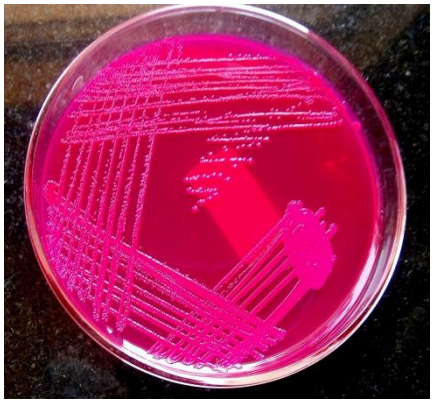


Figure 3:5

Staphylococcus aureus on Mac Conkey agar

On blood agar, the bacteria showed white or sometimes yellowish white colonies as shown in fig.6:3. The yellowishor Golden colur was produced by some strains due to the pigment, Cerotenoides.This species were detected by the beta haemolysis produced on blood agar. *Staphylococcus epidermidis* showed white colonies, where *Staphylococcus saprophyticus* were slightly creamy white, mucoid, colonies. Both species showed non hemolytic colonies.

Culture characteristics of *Pseudomonas aeruginosa*

Pseudomonas showed grey or grey white colonies on blood agar with greenish tint and complete (beta) hemolysis. On Mac Conkey agar the organism showed pink-orange lactose fermentation, which turns plates into a yello orange color on prolonged incubation. It also showed a chacteristic muddy smell or grape wine smell on two days incubation.

Figure 3:6

Pseudomonas aeruginosa on blood agar

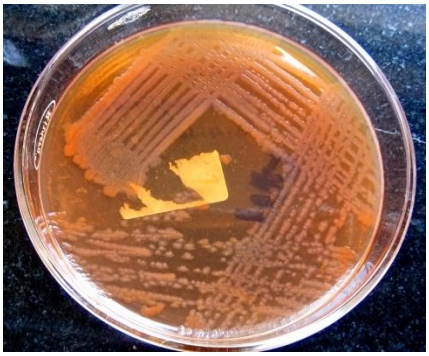


Figure 3:7

Pseudomonas aeruginosa on Mac Conkey agar

Culture characteristics of streptococcus species

Streptococci typically appear as tiny or pin point colonies, with or without haemolysis. Streptococcus viridans showed alpha hemolysis on blood agar plates, and lactose fermentation on Mac Conkey agar. Streptococcus agalactiae and Streptococcus pyogens showed beta (complete) hemolysis on blood agar. One isolate of Enterococcus was among the isolates and this bacterium was non haemolytic.

Figure 3: 8 Alpha hemolysis (green) shown by *Streptococcus viridians* (arrow marked) on blood agar along with beta hemolysis of staphylococcus aureus in a mixed growth plate

Culture characteristics of *Klebsiella pneumoniae*

Klebsiella showed no hemolysis but showe deep pink lactose fermentation on Mac Conkey agar. Differentially on CLED agar these showed yellow to bluish colonies. Klebsiella typically produce mucoid colonies on both blood and Mac Conkey agar.

Figure 7:9

Klebsiella pneumoniae on blood agar

Figure 7:10

Klebsiella pneumoniae on Mac Conkey agar

Culture characteristics of other bacteria

The other bacteria isolated from DFIs include: *Proteus* species and *Enterobacter* species. Proteus species showed swarming growth on blood agar. These are Urease positive. Both the Proteus species: *Proteus vulgaris and Proteus mirabilis* showed swarming growth and produced fishy smell.

The *Enterobacter* species were lactose fermenting pink colonies on Mac Conkey agar, and on blood agar they were non haemolytic. These were VP and citrate positive. The other colony characteristics resembled coli forms of Enterobacteriaceae family.

MICROSCOPIC EXAMINATION:

The selected bacterial isolate was microscopically identified after staining it with Gram stain. The air dried slides after staining are next observed microscopically under 10X, 40X and 100X (oil immersion) of compound light microscope.

GRAM STAINING

Gram staining is one of the most important and widely used differential staining techniques in diagnostic microbiology. The Gram staining procedure was developed by Christian Gram in 1883, a Danish physician who was working as a pathologist in Municipal Hospital IN Berlin.

Principle:

Gram-positive bacterial cells have a thick peptidoglycan cell wall that is able to retain the crystal violet-iodine complex that occurs during staining, while Gram-negative cells have only a thin layer of peptidoglycan. Thus Gram-positive cells do not decolorize with ethanol, and Gram-negative cells do decolorize. This allows the Gram-negative cells to accept the counter stain safranin. Gram-positive cells will appear blue to purple, while Gram-negative cells will appear pink to red.

Reagents:

The Gram stain has four different reagents:

- Primary stain (crystal violets)
- Mordant (iodine)
- Decolouriser (Alcohol)
- Counter stain (Safranin)

Procedure:

1. Fix the smear of specimen (sputum, fluid or culture) either by heating or alcohol fixation.
2. Cover the fixed smear with crystal violet stain for 30 seconds and then wash off with water.
3. Cover the smear with gram iodine for 30 seconds and wash it off with water.
4. Decolorize by pouring acetone (5 seconds) and rapidly wash off.
5. Now cover the slide with Carbol fuchsin for 30 seconds and wash it off with water.
6. Allow the slide to air dry, observe under oil immersion lens.

Interpretation:

Violet color: Gram positive bacteria

Red/Pink color: Gram negative bacteria

Microscopic examination of slides reveal whether the bacteria are cocci, bacilli, or coccobacilli and Gram positiove or Gram negative. It also provides information about how the bacteria are arranged; as dipplococci, chains or clusters. The microscopic view of Gram stained slides of some common bacteria of DFIs, taken during current project are shown in the following page.

MICROSCOPIC VIEW OF BACTERIA

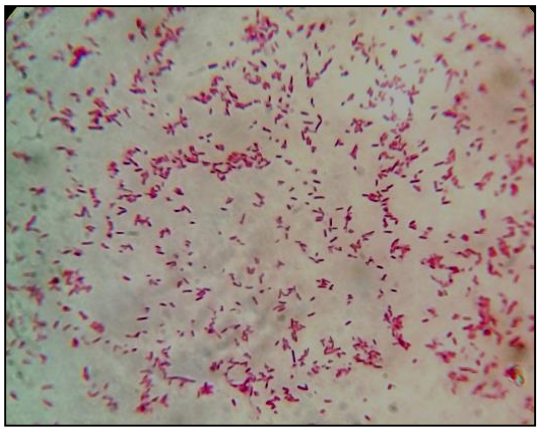


Figure 3:11 Escherichia coli (GNB) under 100X power

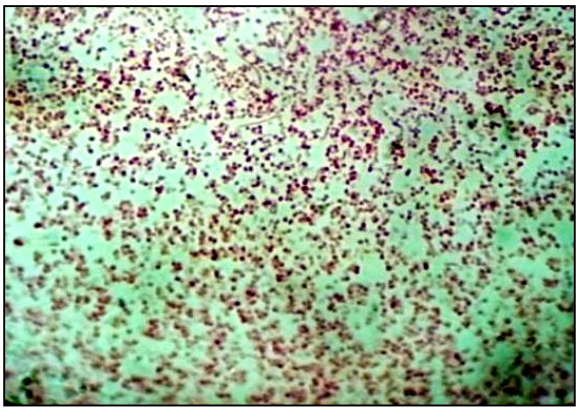


Figure 3:14 Klebsiella pneumoniae under 100X power

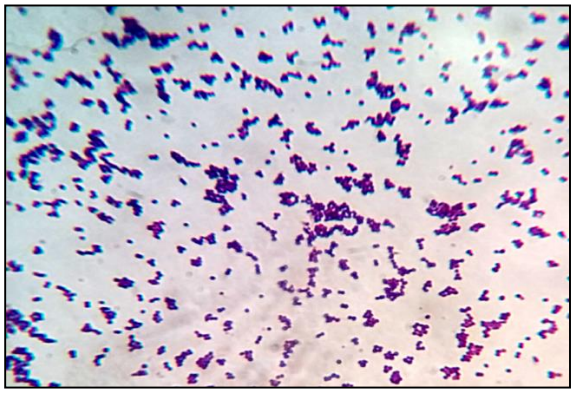


Figure 3:12 Staphylococcus aureus under 100X power

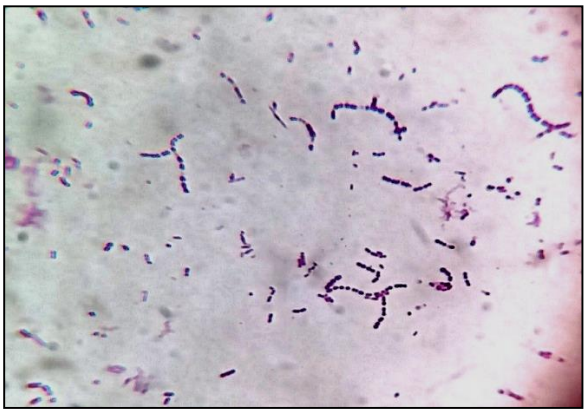


Figure 3:15 Streptococci in chains under 100X power

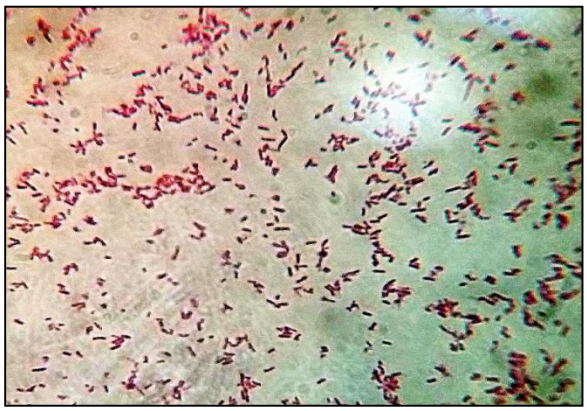


Figure 3:13 Pseudomonas aeruginosa under 100X power

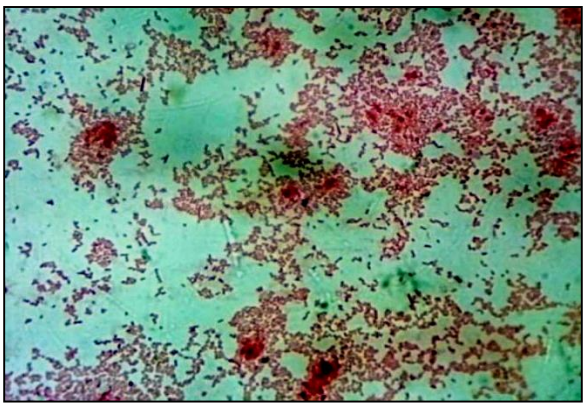


Figure 3:16 Proteus species under 100X power

SPECIFIC BIOCHEMICAL TESTS FOR SPECIES IDENTIFICATION

The specific biochemical tests used in the current study were; Catalase test, Oxidase test, Urease test, IMViC tests (Indole test, Methyl red test, Vogues- Proskauer test and Citrate utilization test) and TSI agar slants (Triple Sugar Iron agar slants- Lactose, Sucrose and Glucose utilization, with ferrous sulfate for indicating H_2S production, phenol red as indicator of acid production and slant also shows gas production as cracks/ bubble) for identification of specific bacteria.

OXIDASE TEST:

Purpose:

To determine the presences of an enzyme, Cytochrome oxidase which catalyses oxidation of reduced cytochrome by molecular oxygen.

Principle:

This test depends on the presence of cytochrome oxidase in bacteria that will catalyze the transport of electrons between electron donors and redox dye. Tetramethyl-p-phenylene diamine dihydrochloride in the reagent is reduced to deep purple color. This test is used for the screening of Pseudomonas, Vibrio, Neisseria, Brucella and Pasteurella, which give positive test. Enterobacteriaceae are oxidase negative.

Reagents:

Oxidase reagent is specially prepared as 10g/l or 1% solution of Tetramethyl-p-phenylene diamine dihydrochloride. Oxidase discs are available commercially.

Procedure:

Oxidase disc Method

Place an oxidase disc in an empty Petri dish and using a sterile straight wire, or sterile toothpick remove a colony of test organisms from a culture plate and smear it on the disc.

Interpretation:

Oxidase positive organisms give blue color within 5-10 seconds, and in oxidase negative organisms, color does not change.

CATALASE TEST

Principle:

Catalase is an enzyme that breaks down hydrogen peroxide (H_2O_2) into H_2O and O_2. A catalase is a way to get rid of the peroxide in a cell. The test is performed by placing a small portion of bacterial colony on a microscope slide and adding a few drops of H_2O_2 onto the smear. A positive result is bubbling due to a rapid evolution of O_2. A negative result is no bubbles or scattered bubbles.

Procedure:

- Place a small amount of growth from the culture onto a clean microscope slide. If using colonies from a blood agar plate, be very careful not to scrape up any of the blood agar—blood cells are catalase positive and any contaminating agar could give a false positive.
- Add a few drops of H_2O_2 onto the smear. If needed, mix with a toothpick. DO NOT use a metal loop or needle with H_2O_2. It will give a false positive and degrade the metal.
- A positive result is the rapid evolution of O_2 evidenced by bubbling.
- A negative result is no bubbles or only a few scattered bubbles.
- Dispose of your slide in the biohazard glass disposal container. Dispose of any
- Toothpicks in the Pipette Keeper.

SUGAR FERMENTATION TEST:

Purpose:

To differentiate aerobic gram negative bacteria based on their ability to ferment sugar with production of acid and gas.

Principle:

The test is done in peptone water containing 1 % of any carbohydrate (glucose, lactose, manitol , Xylose and sucrose) to differentiate aerobic gram negative bacteria based on their ability to ferment sugar with production of acid and gas.

Andrade's reagent is used as an indicator. The sugar media is a colorless liquid media and containing an inverted Durham's tube.

Ingredients:

Peptic digest of casein	: 10 g
Test carbohydrate	: 10 g
NaCl	: 5 g
Andrade's indicater	: 10 g
Distilled water	: 1000 ml
Final pH	: 7.4

Preparation:

- Mix the basal ingredients, heat to boiling, and sterilize at 121^0c for 15 minutes.
- Cool to 50° C; add pre-sterilized solutions of carbohydrates to achieve a final concentration of 1 %.
- Alternatively, as listed above the carbohydrates may be added directly to the medium. Carbohydrate –impregnated discs are also commercially available.

Procedure:

- Inoculate the test organism to the sugar media, and inoculates at 35^0c for 24-48 hours.

Interpretation:

The sugar fermentation is indicated by the change in color of the media.

IMViC REACTIONS:

IMViC reactions are a set of four reactions that are commonly employed in the identification of members of family Enterobacteriaceae. The four reactions are: Indole test, Methyl Red test, Vogues-Proskauer test and Citrate utilization test.

INDOLE TEST:

Principle:

Some bacteria can produce indole from amino acid tryptophan using the enzyme typtophanase. Production of indole is detected using Ehrlich's reagent or Kovac's reagent. Indole reacts with the aldehyde in the reagent to give a red color. An alcoholic layer concentrates the red color as a ring at the top.

Procedure:

Bacterium to be tested is inoculated in peptone water, which contains amino acid tryptophan and incubated overnight at 37oC. Following incubation few drops of Kovac's reagent are added. Kovac's reagent consists of para-dimethyl aminobenzaldehyde, isoamyl alcohol and con. HCl. Ehrlich's reagent is more sensitive in detecting indole production in anaerobes and non-fermenters. Formation of a red or pink colored ring at the top is taken as positive.

Example: *Escherichia coli*: Positive; *Klebsiella pneumoniae*: Negative

METHYL RED (MR) TEST:

Principle:

This is to detect the ability of an organism to produce and maintain stable acid end products from glucose fermentation. Some bacteria produce large amounts of acids from glucose fermentation that they overcome the buffering action of the system. Methyl Red is a pH indicator, which remains red in color at a pH of 4.4 or less.

Procedure:

The bacterium to be tested is inoculated into glucose phosphate broth, which contains glucose and phosphate buffer and incubated at 37˚C for 48 hours. Over the 48 hours the mixed-acid producing organism must produce sufficient acid to overcome the phosphate buffer and remain acidic. The pH of the medium is tested by the addition of 5 drops of MR reagent. Development of red color is taken as positive. MR negative organism produces yellow color.

Example: *Escherichia coli*: Positive; *Klebsiella pneumoniae*: Negative

VOGUES-PROSKAUER (VP) TEST:

Principle:

While MR test is useful in detecting mixed acid producers, VP test detects butylene glycol producers.

Acetyl-methyl carbinol (acetoin) is an intermediate in the production of butylene glycol. In this test two reagents, 40% KOH and alpha-naphthol are added to test broth after incubation and exposed to atmospheric oxygen. If acetoin is present, it is oxidized in the presence of air and KOH to diacetyl. Diacetyl then reacts with guanidine components of peptone, in the presence of alpha-naphthol to produce red color. Role of alpha-naphthol is that of a catalyst and a color intensifier.

Procedure:

Bacterium to be tested is inoculated into glucose phosphate broth and incubated for at least 48 hours. 0.6 ml of alpha-naphthol is added to the test broth and shaken. 0.2 ml of 40% KOH is added to the broth and shake. The tube is allowed to stand for 15 minutes. Appearance of red color is taken as a positive test. The negative tubes must be held for one hour, since maximum color development occurs within one hour after addition of reagents.

Examples: *Escherichia coli*: Negative; *Klebsiella pneumoniae*: Positive

CITRATE UTILIZATION TEST:

Principle:

This test detects the ability of an organism to utilize citrate as the sole source of carbon and energy. Bacteria are inoculated on a medium containing sodium citrate and a pH indicator bromothymol blue. The medium also contains inorganic ammonium salts, which is utilized as sole source of nitrogen.

Utilization of citrate involves the enzyme citritase, which breaks down citrate to oxaloacetate and acetate. Oxaloacetate is further broken down to pyruvate and CO_2.

Production of Na_2CO_3 as well as NH_3 from utilization of sodium citrate and ammonium salt respectively results in alkaline pH. This results in change of medium's color from green to blue.

Procedure:

Bacterial colonies are picked up from a straight wire and inoculated into slope of Simmon's citrate agar and incubated overnight at 37^{o}C. If the organism has the ability to utilize citrate, the medium changes its color from green to blue

Examples: *Escherichia coli* is citrate negative, *Klebsiella pneumoniae* Positive (47).

IMViC RESULTS FOR BACTERIA UNDER STUDY

The IMViC tests are done for identification of bacteria of Enterobacteriaceae family. The results of IMViC tests of the selected bacteria are described in the following table:

Table 3:3 IMViC test results for Enterobacteriaceae

Sl. No.	Bacteria	Indole	MR	VP	Citrate
1.	Escherichia coli	+	+	-	-
2.	Pseudomonas aeruginosa	-	-	-	+/-
3.	Klebsiella pneumoniae	-	-	+	+
4.	Proteus vulgaris	+	+	-	-
5.	Proteus mirabilis	-	+	-	-
6.	Enterobacter species	-	-	+	+

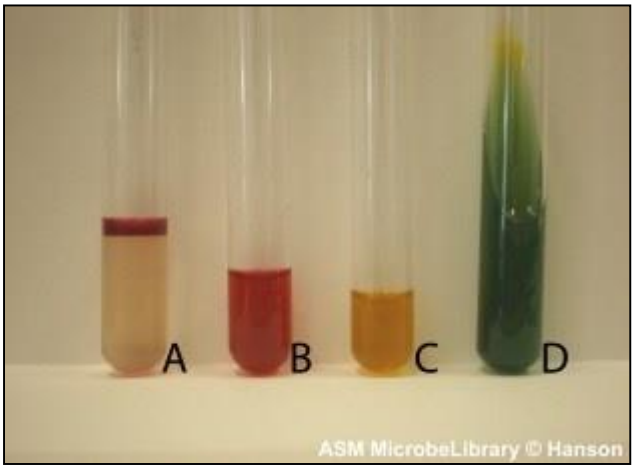


Figure 3:17 IMViC test results for Escherichia coli[2]

SPECIFIC IDENTIFICATION

[2] A- Indole(+), B- MR(+), C- VP(-) and D- Citrate(-)

The specific identification of bacteria was carried out based on following unique characteristics of different bacteria:

Escherichia coli - Lactose fermenting, Mannitol positive GNB, yellow colonies with deep yellow center on CLED agar

Staphylococcus aureus - Catalase positive, Coagulase positive, beta hemolytic on blood agar, lactose fermenting GPC

Staphylococcus epidermidis- GPC, Catalase positive, Coagulase negative, Novobiocin sensitive

Staphylococcus saprophyticus – GPC, Catalase positive, Coagulase negative, Non hemolytic, Novobiocin sensitive.

Pseudomonas aeruginosa - pigment production, Oxidase positive, Indole negative

Klebsiella pneumoniae - mucoid colonies, lactose fermenting, Indole and MR negative, VP and Citrate positive, GNB

Streptococcus viridans – GPC, Catalase negative, alpha hemolytic on blood agar, Optochin resistant.

Streptococcus agalactiae – GPC, Catalase negative, beta hemolytic on blood agar, Bacitracin resistant.

Streptococcus pyogens – GPC, Catalase negative, beta hemolytic, Bacitracin sensitive

Enterococcus species- GPC, deep pink lactose fermenting on Mac Conkey agar, Mannitol fermenting, usually non haemolytic.

Proteus vulgaris – Swarming growth on blood agar, Indole positive, MR positive, H_2S production

Proteus mirabilis- Swarming growth on blood agar, Indole negative, MR positive, H_2S production

Enterobacter species – GNB, lactose fermenting, VP and Citrate positive, mannitole motility positive

SPECIAL FEATURES OF SOME BACTERIA

Images of some bacteria taken during the study are following

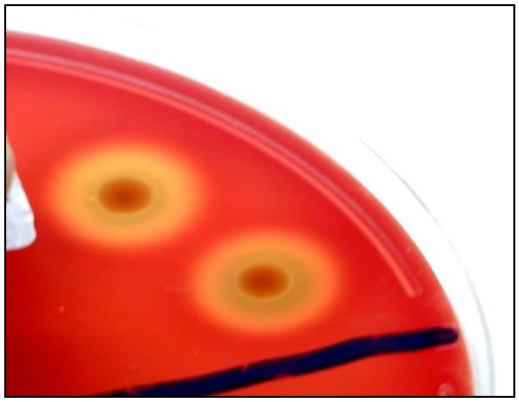


Figure 3:18 beta hemolysis shown by Staphylococcus aureus

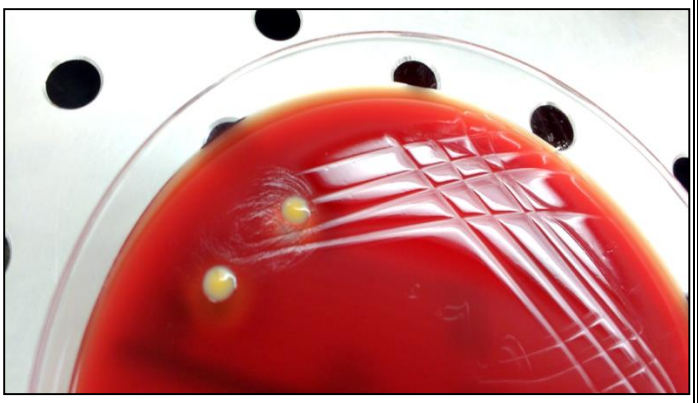


Figure3:19 golden yellow colonies of *Staphylococcus aureus*

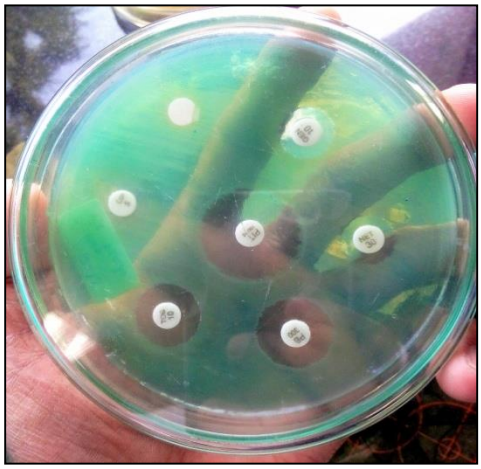


Figure 3:20 Pseudomonas growth on MH Agar.

Figure 3: 21 antibiotic sensitivity of *Streptococcus viridans with resistance only to Optochin*

Figure 3:22 swarming growth of Proteus species on blood agar along with *Staphylococcus* species

ANTIBIOTIC SUSCEPTIBILITY TESTING

The antibiotic susceptibility was performed as per CLSI Performance Standards for Antimicrobial Susceptibility Testing 2014 guidelines, using Mueller-Hinton agar (MH Agar). The method used was Kirby Bauer's disc diffusion method. For this plates were prepared using HiMedia MH agar and spread plating was done with isolated bacterial strain using swab. The composition of MH agar used in the study is given below:

Composition of MH agar

Ingredients	**Gms / Litre**
Beef, infusion from	300.000
Casein acid hydrolysate	17.500
Starch	1.500
Agar	17.000
Final pH (at 25°C)	7.3±0.1

This medium was used for all bacteria, for determining antimicrobial susceptibility. For Streptococcus species, Blood MHA was used which has, 5% defibrinized sheep blood added to it. This supported the streptococci including Enterococci, and showed haemolysis for other species of Streptococci.

The antibiotics used for susceptibility checking in the current study were as per present protocol used by the laboratory and are under four categories; antibiotics for GPC other than Streptococci, GNBs other than Pseudomonas species, antibiotics for Pseudomonas species, and antibiotics for Streptococcus species. The detailed list of antibiotics and their minimum zone of inhibition are detailed in the following tables.[3]

Table 3:4 antibiotics for GPCs other than Streptococci:

Antibiotic	Minimum zone size (mm)		Antibiotic	Minimum zone size (mm)
Amikacin	17		Gentamicin	15
Cefoxitin(II)[4]	22		Linezolid	21
Ciprofloxacin	21		Netillin	15
Chloramphenicol	18		Ofloxacin	18
Clindamycin	21		Penicillin	29
Co-trimoxazole	16		Tetracycline	19
Doxycycline	16		Vancomycin	15
Erythromycin	23			

[3] List of antibiotics used for DFI samples in accordance with CLSI guidelines

[4] Generation of antibiotic; 2nd generation Cephalosporin

Table 3:5 antibiotics for GNBs other than Pseudomonas species

Antibiotic	Minimum zone size (mm)		Antibiotic	Minimum zone size (mm)
Amikacin	17		Ciprofloxacin	21
Amoxyclav	18		Cotrimoxazole	16
Ampicillin	17		Gentamicin	15
Cefazolin(I)	23		Imipenem	23
Cefepime (IV)	18		Levofloxacin	17
Cefotaxime (III)	26		Meropenem	23
Ceftazidime (III)	21		Netillin	15
Cefuroxime (II)	18		Pip/Tazobactam	21
Chloramphenicol	18		Tetracycline	15

Table 3:6 antibiotics for Pseudomonas species

Antibiotic	Minimum zone size (mm)		Antibiotic	Minimum zone size (mm)
Amikacin	17		Ciprofloxacin	21
Amoxyclav	18		Cotrimoxazole	16
Ampicillin	17		Gentamicin	15
Cefazolin(I)	23		Imipenem	23
Cefepime (IV)	18		Levofloxacin	17
Cefotaxime (III)	26		Meropenem	23
Ceftazidime (III)	21		Netillin	15
Cefuroxime (II)	18		Pip/Tazobactam	21
Chloramphenicol	18		Tetracycline	15

Table 3:7 antibiotics for Streptococcus species

Antibiotic	Minimum zone size (mm)
Ampicillin	17
Chloramphenicol	18
Ciprofloxacin	21
Doxycycline	16
Erythromycin	23
Gentamicin	10
Linezolid	23
Penicillin	15
Tetracycline	19
Vancomycin	17

The antimicrobial susceptibility results were reported as per lab protocols; sensitive (S), intermediate (IM) and resistant (R) for the patients. These reports were analyzed to find the degree of antimicrobial susceptibility of each isolate of bacteria and were categorized under four headings; sensitive (S), multidrug resistant only (MDR), extensively drug resistant (XDR), and pan drug resistant (PDR)

These categories were selected and defined as per guidelines of the study on MDR, XDR AND PDR, by European Centre for Disease Prevention and Control, Centers for Disease Control and prevention and other organizations (45). According to this international expert proposal, MDR is defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories (antimicrobials as per CLSI guidelines), XDR is defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (i.e. bacterial isolates remain susceptible to only one or two categories) and PDR is defined as non-susceptibility to all agents in all antimicrobial categories. To ensure correct application of these definitions, bacterial isolates should be tested against all or nearly all of the antimicrobial agents within the antimicrobial categories and selective reporting and suppression of results should be avoided. The current study has followed these definitions to describe occurrence of MDR, XDR and PDR strains of bacteria in DFIs.

The following diagrams show examples of different degrees of antimicrobial susceptibility identified during the period of study and the measurement of zones.

Figure 3: 23 MDR Staphylococcus aureus, resistant to Gentamicin and Clindamycin

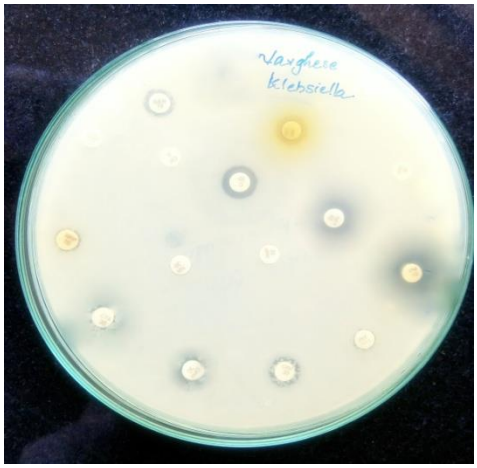


Figure 3: 14 XDR *Klebsiella pneumoniae* resistant to most antibiotics

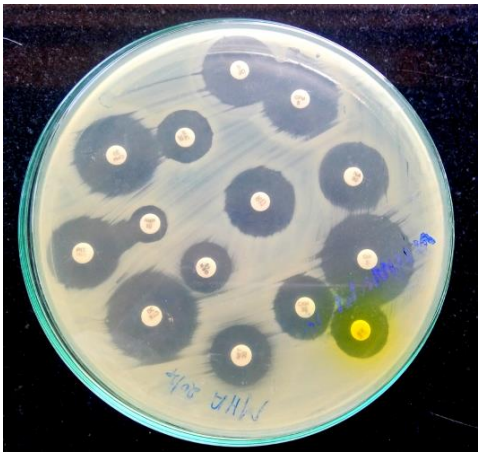


Figure 3:25 Staphylococcus aureus strain sensitive to almost all antibiotics.

Figure 3:26 measurement of antimicrobial spectrum

Relationship between MDR, XDR and PDR

The categories of antibiotic resistance are related to each other. The antibiotic sensitivity and antibiotic resistance are two broad categories. PDR is a subset of XDR and these both are subset of MDR. The diagram below illustrates this:

[5]

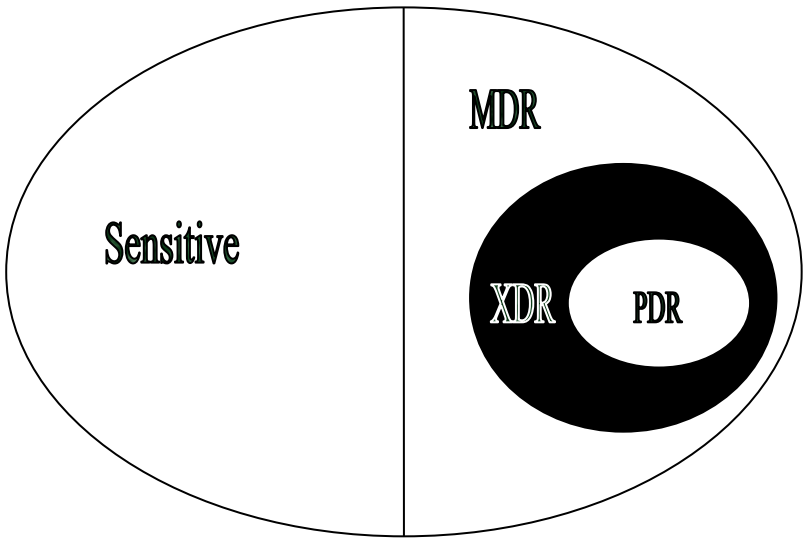


Figure 3: 27 Diagram showing relationship between MDR, XDR and PDR[2]

Confirmation of MDR, XDR and PDR strains was done as per guidelines provided by the ECDC- CDC study. The number of MDR, XDR and PDR was taken together to determine frequency of antibiotic resistant strains (48).

Statistical analysis

The association between number of bacterial isolates, and the number of antibiotic resistant strains was analyzed using Chi square analysis. For this the table prepared was as follows:

Table 7:8 statistical data table for chi square analysis

Sl.No.	Category	No. of bacteria isolated	Antibiotic resistant strains
1.	GPCs	50	37
2.	GNBs	58	53
	Total	108	90

[5] Adapted from Magiorakos et al. International standard definitions for acquired resistance Clinical Microbiology and Infection, Volume 18 Number 3, March 2012

QUALITY CONTROL

All microbiological procedures were performed aseptically following universal precautions. The quality control measures were taken to reduce the chance of contamination, for accurate results and for effective isolation of bacteria. Different types of agar plates were used to study colony morphology, and variants among bacteria. Control plates were placed in all incubators used. The antimicrobial susceptibility testing was performed as per CLSI guidelines, and the results were compared with ATCC strain antimicrobial susceptibility zones. Sterility checking of experimental setting was done periodically. The reports were counterchecked by senior microbiologists and accuracy was assured. All microbiological procedures were performed in bio safety cabinet, to avoid contamination.

Figure 3: 28 antimicrobial spectrum of ATCC Staphylococcus aureus (ATCC no. 12600)

Figure 3: 29 control plates used for quality control

CHAPTER – IV

RESULTS AND DISCUSSION

CHAPTER – IV

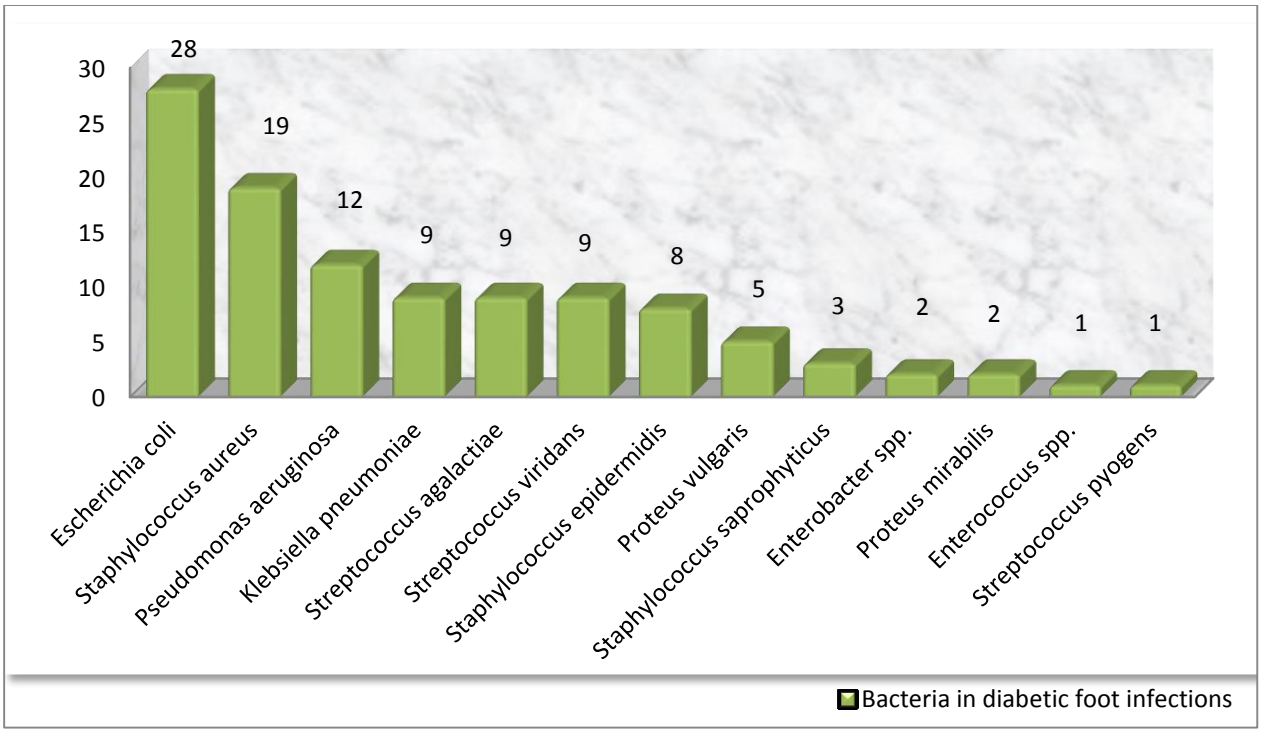
RESULTS AND DISCUSSION

The results of the study are as follows:

- There is predominance of Gram negative bacteria (54%) in DFIs. This is closely followed by Gram positive bacteria too (46%)
- The most prevalent bacteria in DFIs was *Escherichia coli* (26%) followed by *Staphylococcus aureus* (18%).
- Enterobacteriaceae comprised 42.6% of all bacteria. Coagulase negative Staphylococci were 10.19% where Streptococci comprised 18.5% of all bacteria. *Pseudomonas aeruginosa* was next predominant bacteria after *Staphylococcus aureus* (11.1%), followed by *Klebsiella pneumoniae (8.33%), Streptococcus agalactiae (8.33%), and Streptococcus viridians (8.33%).*
- *Staphylococcus epidermidis*, which was considered as common flora or contaminant, was single aetiology of 5.5% of all DFIs whereas *Staphylococcus saprophyticus*, another normal flora organism caused 1.9% monobacterial infections among all DFIs.
- Among all bacteria isolated 83% were drug resistant strains, as opposed to 17% antibiotic sensitive strains.
- Among all the bacteria isolated, 4% were resistant to all categories of antibiotics available (PDR), and 17% were resistant to most of the antibiotics (XDR)
- 67% bacteria among all isolates, showed only Multidrug resistance that is, without being XDR or PDR.
- Only 17% of all bacteria showed antibiotic sensitivity to all drugs.
- Among all patients 15% had diabetic foot Osteomyelitis.
- Study showed increased incidence of DFI in men compared to women. Among the patients 68% were males and 31% females.
- Majority of the patients (62%) were above 60 years, only 32% of patients were below 60 years.

The table following shows the prevalence of particular bacteria in this study.

Figure 4:1: diagram showing bacteria in DFIs

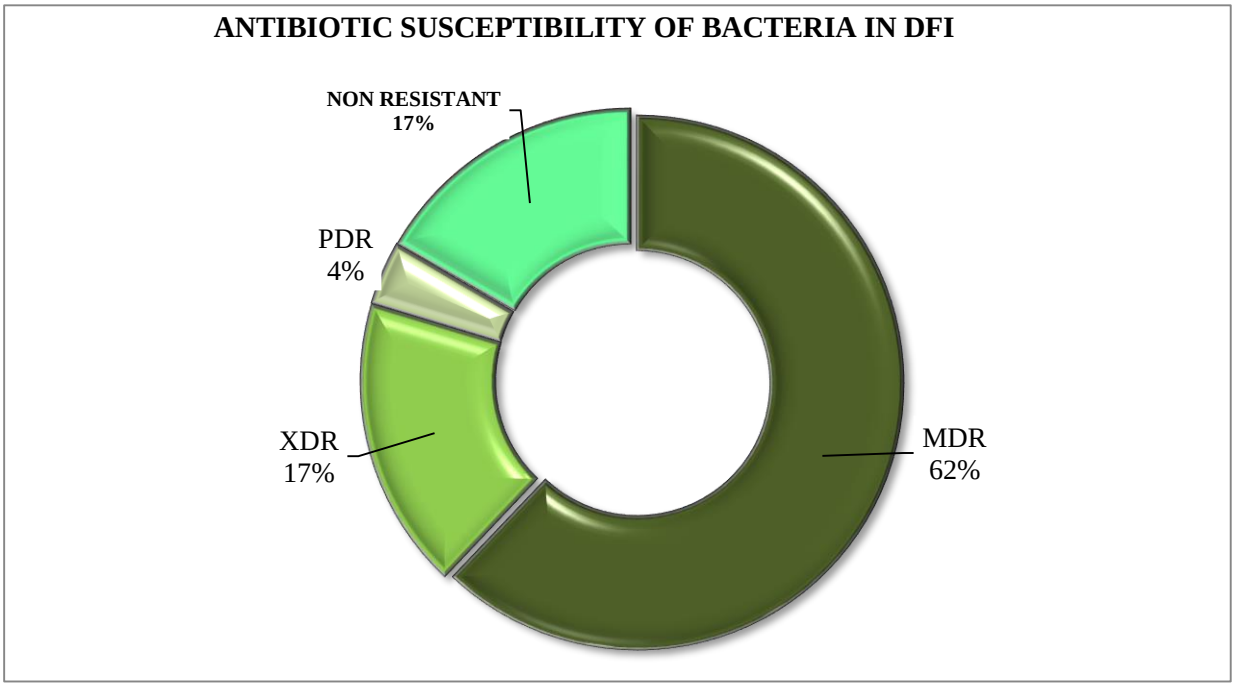


Escherichia coli were found to be most prevalent in DFIs followed by *Staphylococcus aureus* and Pseudomonas *aeruginosa. Klebsiella pneumoniae, streptococcus agalactiae and Streptococcus viridans* were isolated in 9% respectively. Only one isolate of *Enterococcus and Streptococcus pyogens* was found. *Staphylococcus epidermidis* was found in 8 samples. *Proteus vulgaris, Staphylococcus saprophyticus, Enterobacter species,* and *Proteus mirabilis* were found in less numbers.

ANTIMICROBIAL SUSCEPTIBILITY

In the perspective of antimicrobial susceptibility, 16.7% of all isolates showed susceptibility tomall given antibiotics. The rest 83.3% bacteria had drug resistance of varying degrees.For better understanding of the degree of antimicrobial resistance; bacteria were classified under three categories; Multi Drug Resistant (MDR), Extensive Drug Resistant (XDR) and Pan Drug Resistanct (PDR). MDR is defined by resistance to one or more categories of antibiotics on the list based on CLSI guideline. XDR is resistance to all but two categories of antibiotics, and PDR is resistance to all of the antibiotics in the group. The diagram below shows various degrees of antibiotic resistance shown by bacteria in DFI.

Figure 4:2: doughnut showing degrees of antibiotic susceptibility



In the case of antibiotic resistance, *Klebsiella pneumoniae, Proteus vulgaris, Proteus mirabilis and Enterobacter* species showed more drug resistance than the other species. For these organisms, all the isolates showed multi drug resistance, *Klebsiella pneumoniae* being more common. In this study 67 isolates among a total of 108 were MDR (62%), 19 isolates were XDR (17.6%) and 4 isolates were PDR (3.7%). The following table shows the statistics of each bacteria and their degree of antibiotic susceptibility.

Table 4:1Antimicrobial susceptibility of bacteria in DFI

Sl. No.	Bacteria	Number of	Non Resistant	MDR	XDR	PDR
1.	*Escherichia coli*	28	02	17	07	02
2.	*Staphylococcus aureus*	19	07	12	0	0
3.	*Pseudomonas aeruginosa*	12	03	08	01	0
4.	*Streptococcus agalactiae*	09	03	04	02	0
5.	*Klebsiella pneumonia*	09	0	06	03	0
6.	*Streptococcus viridians*	09	01	06	02	0
7.	*Staphylococcus epidermidis*	08	01	07	0	0
8.	*Proteus vulgaris*	05	0	01	02	02
9.	*Staphylococcus saprophyticus*	03	0	02	01	0
10.	*Enterobacter spp.*	02	0	01	01	0
11.	*Proteus mirabilis*	02	0	02	0	0
12.	*Streptococcus pyogens*	01	0	01	0	0
13.	*Enterococcus*	01	01	0	0	0

	TOTAL	**108**	**18**	**67**	**19**	**04**

ANTIBIOTIC RESISTANCE AND PREVALENCE OF BACTERIA

Hypothesis of the study stated that there will be significant association between number of GPCs and GNBs isolated, and their antimicrobial resistance. This can be simply stated as; most prevalent bacteria will show increased antibiotic resistance. The hypothesis was tested and proved significant at Chi square value of 0.537 at 0.05 level of significance, the critical value of chi square being 3.841. So the null hypothesis H^0 is accepted. The table below shows the statistics of the above mentioned data.

Table 4:2 antibiotic resistance in GPCs and GNBs

Sl.No.	Category	No. of bacteria isolated	Antibiotic resistant strains
1.	GPCs	50	37
2.	GNBs	58	53
	Total	108	90

The following bar diagram shows the tabulated data in a diagrammatic representation.

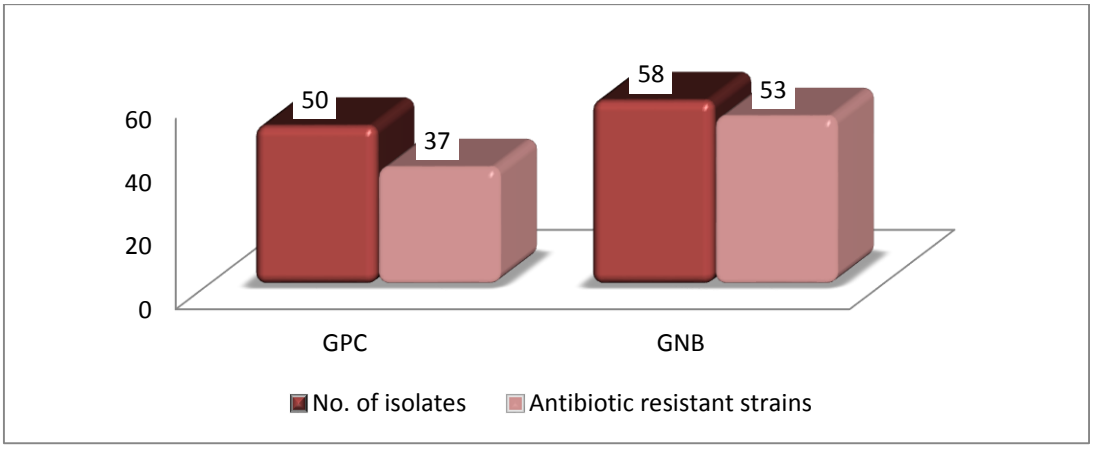
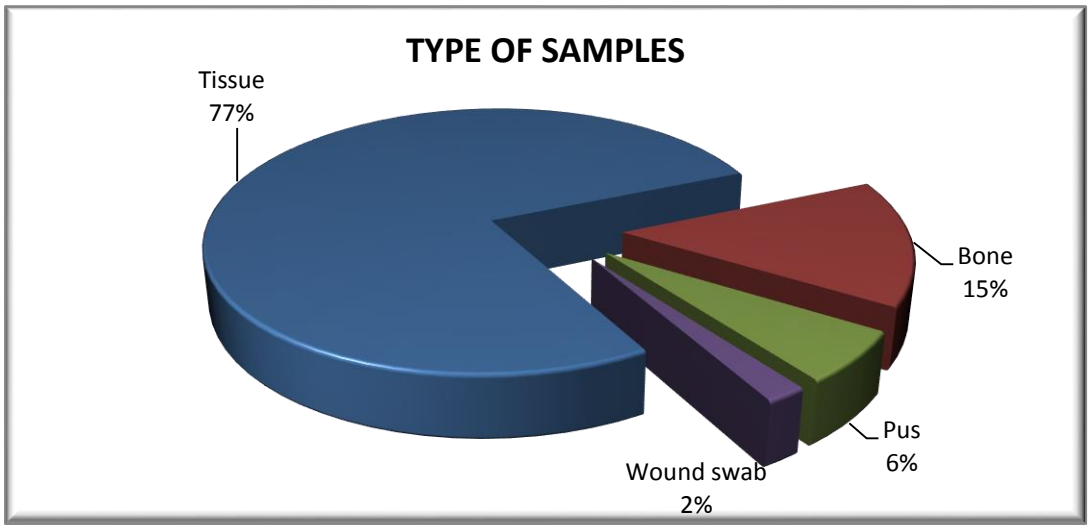


Figure 4:3: bar diagram showing frequency of GPCs and GNBs, and antibiotic resistance

SAMPLES

Among a total of hundred samples, 77 samples were tissue or deep tissue (77%), 23 samples were of bone samples (23%), 6 samples were pus (6%) and 2 were wound swab (2%). Among the bone pathogens, *Escherichia coli, Staphylococcus aureus* and streptococcus species were prevalent. Among tissue samples *Escherichia coli, Staphylococcus aureus and Pseudomonas aeruginosa* were predominant. Among pus samples, staphylococci were common and wound swabs revealed poly microbial infection with two agents. Out of hundred samples only eight were poly microbial (8%).

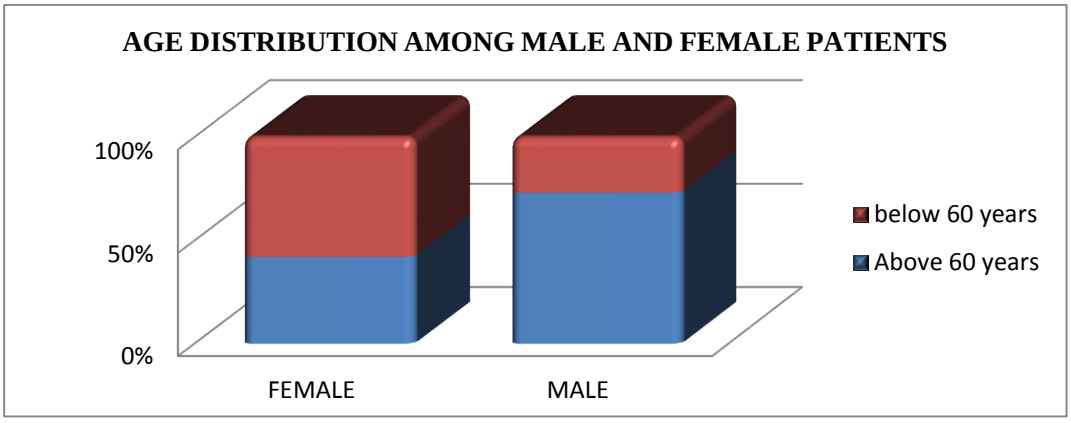
Figure 4:4: pie diagram showing type of samples and percentage



DEMOGRAPHIC DATA OF SUBJECTS

The patients from whom the sample was collected were included both genders; males were 68.3% and females were 31.67%. Majority of patients were above 60years of age, 37 out of 60 patients (61.7%) and 23 were below 60 years (38.3%). Among sixty patients, 41 were males and 19 were females. Among females 13.3% were above 60 years and 86.7% were below 60 years. Among males 73% were over 60 years. The diagrammatic representation of this data follows.

Figure 4:5 stacked columns showing demographic data of subjects



CONCLUSION

The data of the present study shows variable results in relation to generalized concepts about DFIs. The commonly found bacteria are generally Staphylococcus aureus, whereas in the present study it is *Escherichia coli*. It also shows higher number of antibiotic resistant strains (83.3%). Among the subjects, males were more affected with DFIs than females this can be contributed to poor glycemic control seen in males. 73% of male patients were above 60 years where only 42% of female patients were above 60 years. 15% subjects had diabetic foot osteomyeletis along with diabetic foot infections. About 10% of patients were having chronic DFIs and this can be a reason for increased number of Gram negative isolates in the samples.

DISCUSSIONS

The results of this study show that *Escherichia coli* are the most common bacteria found in DFIs in the selected population. This shows more number of drug resistant strains too. The next commonest bacterium is Staphylococcus aureus. The diabetic foot infections had more number of GNBs as pathogens. Among all bacteria majority were drug resistant strains. This shows that antibiotic resistance is an important point to keep in mind in the management of DFIs. Multi drug resistance, extensive drug resistance and pan drug resistance are properties developed by bacteria for their own existence. The development of these properties show that how fast bacteria are able to resist antibiotics that could extinct them, if they don't evolve with novel properties.

This is a situation which reminds us that bacteria are evolving to be smarter than our methods to kill them. The predominant pathogen in this study, *Escherichia coli* are very fast in transferring their property of antibiotic resistance by means of Plasmids. The resistance plasmids can be transferred to other bacteria by means of process called conjugation. This along with high rate of multiplication can bring about a specialized adaptive species of organisms. Evolution rate is much higher in lower level organisms than that of humans or other vertebrates. This is what makes the bacterial drug resistance a serious issue.

Findings of similar studies show that there are significant differences in prevalence of bacteria of diabetic foot infections. Though, commonly seen bacteria are *Staphylococcus aureus and Escherichia coli*. Prevalence of Gram negative bacilli of Enterobacteriaceae family is high in DFIs. In the current study also this was observed prevalence of GNBs being higher in chronic ulcers. The normal flora invades the wounds at initial stages, and later these are replaced by environmental pathogens. Most Enterobacteriaceae were drug resistant due to ESBL production, or Carbapenemase production. Most Staphylococci showed drug resistance to Penicillin and the multidrug resistance can be due to beta lactamases. This was also observed in the current study.

The occurrence of extensively drug resistant and Pan drug resistant strains may be attributed to presence of ESBL, or Metallo Beta Lactamases (MBL). The interspecies interactions can be a cause of Pan Drug resistance which was observed by different species of PDR bacteria occurring consequently in same patients who had chronic DFIs. An example of this from current study is two isolates from bone and tissue samples were Proteus vulgaris and Escherichia coli respectively. Both bacteria showed PDR. This may be due to transfer of resistance genes within these two GNBs. On the other hand cocci in the current study did not show PDR or even XDR, possibly because of absence of conjugation process in which transfer of resistance gene occur.

The study and its results are clinically relevant because it helps to understand and investigate bacteria in DFIs, and their antibiotic resistance. The investigation points which drug is effective against which bacteria and which is not. The results of this study will help to improve or modify empirical antibiotic regimen and to prevent unnecessary use of antibiotics, and thereby prevent development of antibiotic resistance.

Limitations

The study was limited to a tertiary care hospital, in central Kerala and so the results cannot be generalized. Only selected samples from four month period were included and the data may show local variations as compared to larger group studies. In the study only positive bacterial samples were selected and other pathogens like fungi, protozoa and viruses were excluded. So the study does not give a picture of whole microbiome of DFIs but only part of it. The study only assessed the number of bacteria isolated and this may or may not include chronic DFIs and frequently occurring pathogens in them. The study has examined the range or degree of antibiotic resistance and not the reasons for it.

Suggestions for further studies

Further studies can be carried out on larger groups, from different localities, including more parameters. The bacterial population and immune status of the patient can be compared. Other variables like course of infection, antibiotic regimen used previously, previous medical history, severity and stages of diabetic foot ulcers can be compared with bacterial load and antimicrobial susceptibility. Studies on different types of resistance ranging from beta lactamases to Carbapenemase and MBL will also be of value. Studies can be conducted to evaluate drug resistance to particular drug categories.

CHAPTER – V

SUMMARY AND CONCLUSIONS

CHAPTER - V

SUMMARY

The current study identified *Escherichia coli* as most prevalent (26%) bacteria in DFIs, in Ahalia diabetes hospital, Palakkad, Kerala. This bacterium was followed by *Staphylococcus aureus (18%) and Pseudomonas aeruginosa (11%).* Other bacteria seen in DFIs were *Klebsiella pneumoniae (8%), Streptococcus agalactiae (8%), Streptococcus viridians (8%), Staphylococcus epidermidis (7%), Proteus vulgaris (5%), Staphylociccus saprophyticus (3%), Enterobacter species (2%), Proteus mirabilis (2%), Streptococcus pyogens (1%), Enterococcus (1%).* The antimicrobial susceptibility shown by bacteria included higher degrees of antibiotic resistance including pan drug resistance. Only 16.7% isolates were susceptible to all antibiotics. 108 samples were obtained from 60 subjects. Among this, 68.3% were males and 31.7% were females, showing more number of men affected with DFIs. More than half of the patients were above 60 years of age (63.3%).

Drug resistance was observed increasingly evidenced by 67% Multi Drug Resistant strains, 17% Extensively Drug Resistant strains and 4% Pan Drug resistant. 17 % of bacteria showed no antibiotic resistance. Analysis of data showed 53.7% GNBs, and 46.2% GPCs in the DFIs. 91% GNBs showed MDR compared to 74% of GPCs. On statistical analysis using Chi square analysis, the null hypothesis stating relationship between frequencies of isolates and drug resistance, was accepted at 0.05level of significance. Although, the prevalence of XDR and PDR strains were independent on frequencies of isolates, *Proteus vulgaris and Escherichia coli* showing pan drug resistance. Most of the samples were tissue, followed by bone, pus and wound swabs. 15% patients had diabetic foot osteomyeletis. The current study represents the bacteria of DFIs in the selected area, and doesn't represent generally.

CONCLUSION

Infections are never new and so are the treatment methods. All the measures that we take are based on previous studies or personal experiences. The current research titled "Bacteriology of diabetic foot infections and antimicrobial susceptibility" focuses on the bacteria that cause infection in DFIs and how to manage them, by understanding their properties. The antibiotic resistance which is an important issue in current health care is analysed here, with a special reference to Multi Drug Resistant, Extensively Drug Resistant and Pan Drug resistant strains. This was planned to assess the seriousness of drug resistance and the threat these bacteria pose. The results show 63% multidrug resistant, 17% extensively drug resistant and4% pan drug resistant bacterial strains in DFIs. This shows that there is increasing rate of drug resistance revealing that newer mechanisms by bacteria are becoming prevalent in spreading antibiotic resistance. In the current study, Pan Drug Resistance was shown by two bacterial species; Escherichia coli and Proteus vulgaris. This indicates that Gram negative bacilli can be very difficult to manage when it comes to chronic infections. This ability can be attributed to presence of one or more antibiotic resistance mechanisms in bacteria which can be simple beta lactamases, Extended Spectrum Beta Lactamases (ESBL), Metallo Beta Lactamases (MBL) and other unknown factors (1, 48).

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APPENDICES

APPENDIX – I: DATA ANALYSIS TABLES

Table I A: Data collected in the month of January 2016

Sl. No	Name	MR No.	Sample	Bacteria isolated	Multi Drug Resistance (MDR)	Extensive Drug Resistance (XDR)	Pan Drug Resistance (PDR)
1	Nipin Menon		Tissue	*Staphylococcus aureus*	✓		
2	Y. Madhavan		Tissue	*Escherichia coli*	NIL		
3	Sreenivasan TK		Tissue	*1)Staphylococcus epidermidis 2)Streptococcus viridans*	NIL		
4	Bhagyalakshmi		Tissue – foot	*Staphylococcus saprophyticus*		✓	
5	Bhagyalakshmi		Tissue – leg	Pseudomonas aeruginosa	✓		
6	Bhagyalakshmi		Bone	Staphylococcus aureus	NIL		
7	Subha		Tissue - toe IV	Streptococcus viridans	✓		
8	Subha		Tissue – heel	Streptococcus viridans	✓		
9	Muraleedharan		Tissue	Staphylococcus epidermidis	✓		
10	Komalam Sankarnarayan		Tissue - toe I	Staphylococcus aureus	NIL		
11	Lakshmi		Tissue	Staphylococcus epidermidis	✓		
12	Radhakrishnan		Deep tissue	Escherichia coli	✓		
13	Subramanian		Tissue	Escherichia coli	✓		
14	Rajamma		Deep tissue	Staphylococcus aureus	✓		
15	Kunchu		Tissue	Escherichia coli	✓		

16	Lourdmary		Tissue	Escherichia coli	✓		
17	Raman kutty	104587	Tissue	Streptococcus viridans			
18	Shakunthala	116465	Bone	Staphylococcus aureus	✓		
19	Subramanian	106527	Tissue	Escherichia coli		✓	
20	Subramanian	106527	Bone	Escherichia coli	✓		
21	Sundar raj	109169	Tissue	Staphylococcus aureus			
22	Swaminathan	119017	Tissue	Enterobacter Spp.	✓		
23	Sreenivasan T K	119686	Bone	Streptococcus agalactiae	✓		
24	Youness	117808	Tissue	Staphylococcus aureus	✓		

APPENDIX 1B: Table IB: Data of the month February 2016

25	Dasan T K	118922	Tissue	1)Pseudomonas aeruginosa	✓ ✓		
				2)Enterobacter Spp.	✓		
26	Gangadharan	119603	Tissue	Klebsiella Spp.	✓		
27	Khadeeja	119439	Pus	Staphylococcus saprophyticus	✓		
28	Shanta Krishnan	119209	Tissue	1)Streptococcus viridians 2)Escherichia coli		✓ ✓	
29	Shanta Krishnan	119209	Tissue-Anaerobic Culture	Staphylococcus epidermidis	✓		
30	Aminumma	119533	Tissue	Pseudomonas aeruginosa	✓		
31	Aminumma	119533	Bone	Escherichia coli			
32	Purushothaman KG	119497	Tissue-Anaerobic culture	Enterococcus Spp.			
33	Varghese	118060	Tissue	Klebsiella Spp.		✓	
34	Muraleedharan	118630	Tissue	Staphylococcus epidermidis	✓		
35	Komalam shankarnarayan	119710	Tissue - Toe I	Staphylococcus aureus			
36	Komalam shankarnarayan	119710	Tissue - Toe III	Staphylococcus aureus	✓		
37	Bhagyalakshmi	118637	Wound swab	Pseudomonas aeruginosa	✓		
38	Bhagyalakshmi	118637	Wound swab (Anaerobic)	1)Streptococcus viridians	✓		
				2)Pseudomonas aeruginosa	✓		
39	Kannan	119747	Tissue (Toe)	Streptococcus viridians		✓	

40	Kannan	119747	Bone	Streptococcus viridians		✓	
41	Kannan	119747	Tissue (heel)	Klebsiella Spp.		✓	
42	Varghese	118060	Tissue	Streptococcus viridans	✓		
43	Varghese	118060	Bone	Klebsiella Spp.		✓	
44	Rajan K Louis	119719	Tissue	Streptococcus viridans			
45	Sasidharan N	119788	Tissue	Pseudomonas aeruginosa			
46	Sasidharan N	119788	Bone	Pseudomonas aeruginosa			
47	Murughan	111397	Tissue	1) Klebsiella Spp. 2) Staphylococcus aureus	✓ ✓		
48	Murughan	111397	Bone	1) Klebsiella Spp. 2) Staphylococcus aureus	✓ ✓		
49	Narayanan kutyy	118204	Pus	Staphylococcus epidermidis	✓		
50	Khadeeja	119439	Tissue	Staphylococcus saprophyticus	✓		
51	Khadeeja	119439	Tissue-anaerobic	Staphylococcus aureus	✓		

APPENDIX 1 C; Table I C: data of the month March 2016

52	Khadeeja	119925	Tissue	Pseudomonas aeruginosa		✓	
53	Muhammed Kutty	115402	Tissue	Staphylococcus aureus			
54	Krishnan	120041	Tissue	Escherichia coli	✓		
55	Rajamma	118384	Tissue	1)Streptococcus pyogens	✓		
				2)staphylococcus epidermidis	✓		
56	Suguna	118578	Tissue	Pseudomonas aeruginosa	✓		
57	Nipin menon	118594	Tissue	Streptococcus viridans	✓		
58	Radhakrishnan Nair	118524	Tissue	Escherichia coli		✓	
59	Radhakrishnan Nair	118524	Bone	Escherichia coli		✓	
60	Marakkar	118263	Tissue	Streptococcus agalactiae	✓		
61	Shakunthala G	116465	Deep tissue	Pseudomonas aeruginosa	✓		
62	Ramakrishnan	118389	Tissue	Escherichia coli	✓		
63	Renu sabu	118317	Pus	Staphylococcus aureus	✓		
64	Devasia	117667	Pus	Staphylococcus epidermidis	✓		
65	Narayanan kutty	118204	Tissue	Staphylococcus aureus	✓		
66.	Vijayaraghavan	118243	Tissue	Proteus mirabilis	✓		
67	Sulaiman K	118368	Tissue	Escherichia coli	✓		
68	Vijayaprasad	118271	Tissue	Klebsiella Spp.	✓		
69	Muhammed kutty	117354	Tissue – left foot	Proteus vulgaris		✓	

70	Muhammed kutty	117354	Tissue – right foot	Proteus vulgaris		✓	
71	Ramaswamy	118253	Tissue	1)Proteus vulgaris 2)Pseudomonas aeruginosa	✓ ✓		
72	Subramanian	106527	Tissue	Staphylococcus aureus			
73	Kamalam	118236	Tissue	Klebsiella Spp.	✓		
74	Muhammed ali	118171	Tissue	Escherichia coli	✓		
75	Muhammed ali	118171	Bone	Escherichia coli	✓		
76	Muhammed basher	112462	Pus	Escherichia coli			✓
77	Souda		Tissue	Proteus vulgaris	✓		
78	Balakumar	117335	Tissue	Escherichia coli	✓		
79	Muhammed basheer	112462	Tissue	Proteus vulgaris		✓	
80	Varghese	118060	Tissue	Klebsiella Spp.			✓

APPENDIX I D: Table I D: data of the month March 2016

81	Lourdmary	101096	Tissue	Escherichia coli	✓		
82	Omana	194642	Tissue	Klebsiella spp.	✓		
83	Rajamma	116642	Pus	Pseudomonas aeruginosa	✓		
84	Lakshmi	108646	Tissue	Streptococcus viridians	✓		
85	Ismail KS	110039	Deep Tissue	Staphylococcus aureus	✓		
86	Vinodhini	116923	Tissue	Escherichia coli	✓		
87	Vinodhini	116923	Bone	Escherichia coli	✓		
88	Muhammed basher	112462	Tissue	Escherichia coli			✓
89	Muhammed basher	112462	Bone	Proteus vulgaris			✓
90	Mariyamma	116977	Tissue	Escherichia coli	✓		
91	Maria Thomas	117305	Tissue	Streptococcus agalatiae	✓		
92	Lourd mary	101096	Tissue	Escherichia coli	✓		
93	Chandran	117211	Tissue	Streptococcus agalactiae	NIL		
94	Chandran	117211	Bone	Streptococcus agalactiae	NIL		
95	C D jaya	100161	Deep Tissue	Staphylococcus aureus	NIL		
96	Balakumar	117335	Tissue	Escherichia coli		✓	
97	Appukkuttan	117497	Tissue	Proteus mirabilis	✓		

98	Tk muhammed	117113	Tissue	Escherichia coli		✓	
99	Pazhani chamy	117893	Tissue	Staphylococcus aureus	✓		
100.	Aboobakkar	100188	Tissue	Staphylococcus aureus	✓		

DEMOGRAPHIC DATA OF PATIENTS

APPENDIX I E: Table I: E: demographic data of patients

Sl. No	Name	MR No.	Age in years	Gender
1.	Nipin Menon	118544	35	Male
2.	Y. Madhavan	103918	68	Male
3.	Sreenivasan TK	119686	58	Male
4.	Bhagyalakshmi	118637	58	Female
5.	Subha	119703	45	Female
6.	Muraleedharan	118630	61	Male
7.	Komalam Sankarnarayan	119710	62	Female
8.	Lakshmi	108646	65	Female
9.	Radhakrishnan	105427	63	Female
10.	Subramanian	106527	64	Male
11.	Rajamma	118384	56	Female
12.	Kunchu	108946	78	Male
13.	Lourdmary	101096	76	Female
14.	Raman kutty	104587	61	Male
15.	Shakunthala	116465	63	Female
16.	Sundar raj	109169	60	Male

17.	Swaminathan	119017	75	Male
18.	Youness	117808	46	Male
19.	Dasan T K	118922	53	Male
20.	Gangadharan	119603	58	Male
21.	Aysha	119439	48	Female
22.	Shanta Krishnan	119209	57	Female
23.	Aminumma	119533	84	Female
24.	Purushothaman KG	119497	80	Male
25.	Varghese	118060	69	Male
26.	Bhagyalakshmi	118637	58	Female
27.	Kannan	119747	64	Male
28.	Rajan K Louis	119719	50	Male
29.	Sasidharan N	119788	60	Male
30.	Murughan	111397	56	Male
31.	Khadeeja	119925	85	Female
32.	Muhammed Kutty	115402	60	Male
33.	Krishnan	120041	69	Male
34.	Suguna	118578	54	Female
35.	Radhakrishnan Nair	118524	63	Male

36.	Marakkar	118263	62	Male
37.	Ramakrishnan	118389	56	Male
38.	Renu sabu	118317	39	Female
39.	Devasia	117667	60	Male
40.	Narayanan kutty	118204	70	Male
41.	Vijayaraghavan	118243	75	Male
42.	Sulaiman K	118368	65	Male
43.	Vijayaprasad	118271	62	Male
44.	Muhammed kutty	117354	64	Male
45.	Ramaswamy	118253	70	Male
46.	Kamalam	118236	64	Male
47.	Muhammed ali	118171	54	Male
48.	Muhammed basher	112462	56	Male
49.	Souda	117439	45	Female
50.	Balakumar	117335	54	Male
51.	Omana	194642	60	Female
52.	Ismail KS	110039	73	Male
53.	Vinodhini	116923	27	Female
54.	Mariyamma	116977	72	Female

55.	Maria Thomas	117305	49	Female
56.	Chandran	117211	66	Male
57.	Appukkuttan	117497	73	Male
58.	Tk muhammed kutty	117113	65	Male
59.	Pazhani chamy	117893	78	Male
60.	Aboobakkar	100188	44	Male

APPENDIX –II: BACTERIA AND ANTIMICROBIAL SUSCEPTIBILITY

APPENDIX II A: Table II A: number and percentage of bacteria in DFI

Sl. No.	Bacteria	Number	Percentage
1.	Escherichia coli	28	26%
2.	Staphylococcus aureus	19	18%
3.	Pseudomonas aeruginosa	12	11%
4.	Streptococcus agalactiae	9	8%
5.	Klebsiella pneumonia	9	8%
6.	Streptococcus viridians	9	8%
7.	Staphylococcus epidermidis	8	7%
8.	Proteus vulgaris	5	5%
9.	Staphylococcus saprophyticus	3	3%
10.	Enterobacter spp.	2	2%
11.	Proteus mirabilis	2	2%
12.	Streptococcus pyogens	1	1%
13.	Enterococcus	1	1%
Total 13		108	100%

APPENDIX II B: Table II B Antimicrobial susceptibility of bacteria in DFI

Sl. No.	Bacteria	Number of isolates	Non Resistant	MDR	XDR	PDR
1.	Escherichia coli	28	02	17	07	02
2.	Staphylococcus aureus	19	07	12	0	0
3.	Pseudomonas aeruginosa	12	03	08	01	0
4.	Streptococcus agalactiae	09	03	04	02	0
5.	Klebsiella pneumonia	09	0	06	03	0
6.	Streptococcus viridians	09	01	06	02	0
7.	Staphylococcus epidermidis	08	01	07	0	0
8.	Proteus vulgaris	05	0	01	02	02
9.	Staphylococcus saprophyticus	03	0	02	01	0
10.	Enterobacter spp.	02	0	01	01	0
11.	Proteus mirabilis	02	0	02	0	0
12.	Streptococcus pyogens	01	0	01	0	0
13.	Enterococcus	01	01	0	0	0
	TOTAL	**108**	**18**	**67**	**19**	**04**

APPENDIX- III: CLASSES OF ANTIBIOTICS

APPENDIX III A: Table III A: CLASSES OF ANTIBIOTICS (49)

PENICILLINS	CEPHALOSPORINS	FLUOROQUINOLONES	AMINOGLYCOSIDES	MONOBACTAMS	CARBAPENEMS	MACROLIDES	OTHER
Natrual	***First generation***	Ciprofloxacin (Cipro) Levofloxacin (Levaguin) Moxifloxacin (Avelox) Norfloxacin	Amikacin Gentamicin Kanamycin Neomycin Tobramycin	Aztreonam	Ertapenem Imienem Meropenem	Azithromycin Clarithromycin Dirithromycin Erythromycin Clindamycin	Vancomycin Rifampin Doxycycline Linezolid Tetracycline Trimethoprim/ sulfamethoxacole
Penicillin G Penicillin-VK	Cephalothin Cefazolin (Ancef, Kefzol) Cephapririn Cephalexin (Keflex) other						
Penicillinase Resistant	*Second Generation*						
Methicillin Nafcillin Oxacillin other	Cefacor Cefotetan (Cefotan) other						
Aminopenicillins	*Third Generation*						
Ampicillin	Ceftriaxone (Rocephin) other						
	Fourth Generation						
	Cefpirome Cefepime						