"A CROSS SECTIONAL STUDY TO KNOW THE BACTERIAL AND FUNGAL PATHOGENS CAUSING DIABETIC FOOT ULCER"

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LIST OF ABBREVIATION

ATCC	American Type Culture Collections
H_2S	Hydrogen sulphide
CO_2	Carbon dioxide
MR	Methyl red
VP	Voges Proskauer
MRSA	Methicillin resistant Staphylococcus aureus
ESBL	Extended Spectrum Beta Lactamase
PPDT	Potentiated disk diffusion test
GNB	Gram negative bacilli
GPC	Gram positive cocci
Spp	Species

ABSTRACT

Background:

Diabetes mellitus is a chronic disorder affecting a large segment of population. Diabetic foot ulcer is one of the commonest complication of longstanding diabetes. The triad of problems leading onto diabetic foot is neuropathy, vascular changes and infections, which constitute the diabetic foot syndrome.

Objectives:

The present study was undertaken to-

1. To isolate the aerobic, anaerobic and fungal pathogens of diabetic foot ulcer
2. To study the antimicrobial susceptibility of the aerobes isolated.

Material and methods:

The present study was conducted in the Department of Microbiology, JNMC, Belgaum. Material was collected from the deep tissue biopsy in patients with grade 1-3 (Megitt Wagner classification) diabetic foot ulcer attending the surgery OPD & those who were admitted in surgery ward at KLEs Dr.Prabhakar Kore hospital & medical research centre, Belgaum over a period of one year from Jan 2011 –Dec 2011. The study comprised of 75 cases of clinically diagnosed cases of diabetic foot ulcer. Clinical samples were transported to the laboratory in fluid thioglycollate medium. Initially gram's stain and KOH wet mount was done. Aerobic, anaerobic and fungal cultures were put up. For aerobic, 5% sheep blood agar, Macconkey agar and for anaerobic, blood agar with haemin and vitamin K, Kanamycin, Vancomycin laked blood agar (KVLB) and Bacteroides Bile Esculin agar (BBE) were used. The media were placed in McIntosh Fildes jar with Internal Gas Generating System and incubated at 37ºC for minimum of 3-5days. Anaerobic growth was identified using standard techniques. Antibiotic sensitivity testing of aerobic organisms were carried

out by the Kirby Bauer's disk diffusion technique. MRSA and ESBL producers were directed using standard methods. For fungal culture, samples were inoculated onto SDA with and without antibiotics and incubated at 25º C and 37º C for four weeks. The isolates were identified using standard methods.

Results:

Seventy five samples yielded 139 isolates of which 57.34% were polymicrobial, 46.27% were monomicrobial . Out of 139 isolates, aerobes constituted for 76.98% of isolates , anerobes for 22.30% of isolates and fungus for 0.71% of isolates in this study. *Staphylococcus aureus* was the most common aerobe isolated. *Bacteroides fragilis* was the most common isolate among anaerobes. *Aspergillus flavus* was the only fungal isolate. MRSA accounted for 29.41%. ESBL accounted for 32.35%.

Conclusion:

This study has shown that diabetic foot ulcers harbour polymicrobial infections. Isolation of these organisms and their antibiotic susceptibility should be determined to start appropriate antibiotic therapy as early as possible to overcome the morbidity and mortality associated with this disease and to prevent the development of resistant strains.

Key words: Diabetic foot ulcer, Polymicrobial, *Staphylococcus aureus*, *Bacteroides fragilis*.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PHOTOGRAPHS

INTRODUCTION

Diabetes mellitus is a disease as old as mankind itself and is a major health care challenge. The chronic nature of diabetes and its tendency to affect major organs has led some people to call it "disease of complications."[1] Diabetes mellitus is recognized as an epidemic in the Asian sub-continent affecting nearly 42 millions in India alone.[2] The Indian diabetic population is expected to increase to 57 million by 2025.[3]

Foot infections are common and represent a serious problem in diabetic patients.[4] Unfortunately these infections are a common cause of morbidity in diabetic patients leading to dreaded complications like gangrene leading to amputation.[5]

The triad of problem leading to diabetic foot is peripheral neuropathy, micro and macro angiopathy and infections, which constitute the diabetic foot syndrome.[6]

India has about 42 million people with diabetes putting it first on the list of 10 nations most affected by the disease.[6] Foot ulcer is a major complication of diabetes mellitus which precedes 85% of all lower limb amputations. Diabetic foot infections are polymicrobial in nature with aerobic, anaerobic and fungal infections playing a role in it.[7]

Proper management of these infections requires isolation and identification of the microbial flora, appropriate antibiotic treatment according to sensitivity patterns, identification of chronic complications and proper surgical intervention for these complications.

However the spectrum of micro organisms depends mainly on the microbial flora of the lower limb, metabolic factors, foot hygiene and the use of antibiotics.

Inspite of a multidiscipilinary foot-care team to optimize foot care , deleterious effects of infection on soft- tissue and bone continues to be a major problem.[8] Progress of infection is associated with delayed diagnosis and under estimation of the extent of infection or suboptimal wound or antimicrobial therapy.

The present study was therefore taken to assess the role of aerobic, anerobic bacteria and fungi as pathogens in diabetic foot ulcer and to determine the antibiotic susceptibility pattern of the aerobic pathogens isolated.

OBJECTIVES

1. To isolate the aerobic, anaerobic and fungal pathogens of diabetic foot ulcer.

2. To study the antimicrobial susceptibility of the aerobes isolated.

3. To determine the presence of diabetic foot ulcers in various age groups and gender.

4. To study the frequency of diabetic foot infection with duration of diabetes mellitus.

5. To study the nature of isolates in different Wagner's grades of diabetic foot ulcer.

REVIEW OF LITERATURE

Diabetes mellitus is a disease which was recognized in antiquity. The history of diabetes mellitus up to the time of discovery of insulin is divided into four major periods: the '**ancient period'** with descriptions of diabetes and its complications, the 16^{th} to 18^{th} centuries termed as the **'diagnostic period'** in which diabetes mellitus was distinguished as a disease entity; **'experimental period'** during which the gluco regulatory role of pancreas became clear and the biochemical disturbances of diabetes were described. Finally, the 20^{th} century had seen a dramatic increase in knowledge about diabetes and metabolism in general.[9]

The Ebers papyrus, dating from about 1550 BC, testifies to the long history of diabetes. This papyrus, found in a grave in Thebes in 1862 and named after the Egyptologist, Georg Ebers contains descriptions of various diseases, including a polyuric state resembling diabetes mellitus.[9]

The term '**diabetes'** which is from the Ionian Greek meaning 'to pass through'or 'a siphon' was first used by Arataeus of Cappodocia in the second century A.D. He described excessive thirst , constant need to urinate, dry mouth , parched skin and loss of weight, and interpreted the disease as " a melting down of the flesh into the urine".[9]

The Roman Physician, Galen (AD 131-20), like Aretaeus thought diabetes to be a rare disease and apparently encountered only two cases. Galen employed alternative terms for diabetes, including '**diarrhoea urinosa'** and '**dipsakos**', emphasizing the cardinal symptoms of excessive thirst and drinking.[9]

The association of polyuria with a sweet – tasting substance in the urine was reported in Sanskrit literature dating from the 5th to 6th centuries AD at the time of two notable Indian physicians, Susruta and Charaka. The urine of polyuric patients was described as tasting like honey ("madhumeha") , being sticky to touch and strongly attracting ants. The fact that diabetic urine tasted sweet was subsequently emphasized by Arabian medical tests during the 9th to 11th centuries, when Arabic medicine was at the peak of achievement. Avicenna (AD 960-1037) described accurately the clinical features of diabetes and mentioned two specific complications of the disease, namely gangrene and disturbances in sexual function.[9]

In 17th century, Thomas Willis made reference to the sweet taste of the diabetic urine. Another celebrated physician of this period was Thomas Syndenham who speculated that diabetes was a systemic disease arising in the blood where 'chyle' was incompletely digested and its non absorbed residue had to be excreted.[9]

In 18th century John Rollo, an English physician was one of the first to use adjective 'mellitus' (from the Latin and Greek roots for 'honey'). He made other contributions to the study of diabetes, including descriptions of diabetic cataracts and the odour of acetone in the breath of some diabetic patients.

In the 19th century Claude Bernard named '**piqure diabetes**' (from the French piquer, to prick) implicating pancreas as the seat of diabetes. In 1869 Paul Langerhans had noticed small clusters of cells in the teased preparation of pancreas.[9]

In 1887, Pryce described the association between foot ulceration, neuropathy and vascular disease.

In 1889, Oskwar Minkowski and Joseph Von Mering established the role of pancreatic disorders in causing diabetes.[9]

In 1893 Pryce described about skin atrophy and tropic ulcers in diabetic patients.[11] In the same year Edwouard Lagousse suggested the name for the cells in pancreas as '**islets of langerhans**', which might constitute the endocrine tissue of the pancreas.

In 1909, Belgian physician, Jean de Meyer gave the name 'insulin' (Latin, insula - island) to the glucose lowering hormone, which he postulated was produced by the islet tissue.[9]

The discovery of insulin at the University of Toronto in 1921-22 resulted from a collaborative effort involving Fredrick G.Banting, Charles H. Best and James B.Collip.[9]

In 1943, Weiss reported an infected stump in a diabetic from which *Proteus* and haemolytic *Streptococci* was isolated.[12]

In 1958, Goldenberg and associates described endothelial proliferation and intramural deposition of a Periodic- acid Schiff positive material in small arterioles.[13]

In 1962, Peterson described the pathogenesis of foot infections. In the same year Perillie and Nolan demonstrated that there was an impaired cellular defense in ketoacidosis and there was normal cellular response once the acidosis is controlled.[13]

In 1970, Bagdade and Ivan demonstrated defective phagocytosis and defective intracellular killing of Staphylococcus by polymorphonuclear leukocytes in diabetic patients.[13]

In 1973, Cruse and Foord found that diabetics had five times the risk of infection in clean incisions.

The most commonly employed system of classification for diabetic ulceration is that described by Wagner in 1981 who described six grades of ulcer based on depth and appearance.[11]

In 1988 Calhoun concluded that intensive early intervention, antibiotics and surgical treatment of more advanced infections decreased morbidity and mortality.[11]

In 1991, Lisa G.Newman reported that by clinical judgement alone, more than two- thirds of the cases of osteomyelitis would have remained undiagnosed and not been treated.[14]

ANATOMY OF FOOT:[15,16]

The muscles of foot are arranged in four layers with the neurovascular bundles between the first and second layers and then between the third and the fourth layers. The sole of foot consists of skin, superficial fascia, deep fascia, muscles, vessels and nerves.

The skin of the sole is i) thick for protection, ii) firmly adherent to the underlying plantar aponeurosis. These features increase the efficiency of the grip of the sole on the ground.

The superficial fascia of the sole is fibrous and dense. Fibrous band bind the skin to the deep fascia or the plantar aponeurosis and divide the subcutaneous fat into small tight compartments which serve as water cushions and reinforce the spring – effect of the arches of foot during walking, jumping and running.

The deep fascia is specialised to form i) the plantar aponeurosis in the sole ii) the deep transverse metatarsal ligament iii) the fibrous flexor sheath.

Plantar Aponeurosis:

The deep fascia covering the sole is thick at the center and thin at the periphery. The thickened central part is known as the plantar aponeurosis. It protects the deeper structures and it maintains the longitudinal arches of the foot.

Deep transverse metatarsal ligaments:

These are four short flat bands which connect the plantar ligaments of the adjoining metatarsophalangeal joints.

Fibrous flexor sheath:

These are made up of the deep fascia of the toes. They retain the flexor tendons during flexion of the toes.

Muscles of the sole of foot:

The muscles are arranged in four layers.

Table 1: Muscles of the sole of foot

First layer	Second layer	Third layer	Fourth layer
i)Flexor digitorum brevis ii)Abductor hallucis iii)Abductor digiti minimi	i)Flexor digitorum longus ii)Flexor digitorum accessorius iii)Lumbricals iv)Flexor hallucis longus	i)Flexor hallucis brevis ii)Flexor digiti minimi brevis iii)Adductor hallucis.	i)Interosseous muscles ii)Tibialis posterior iii)Peroneus longus

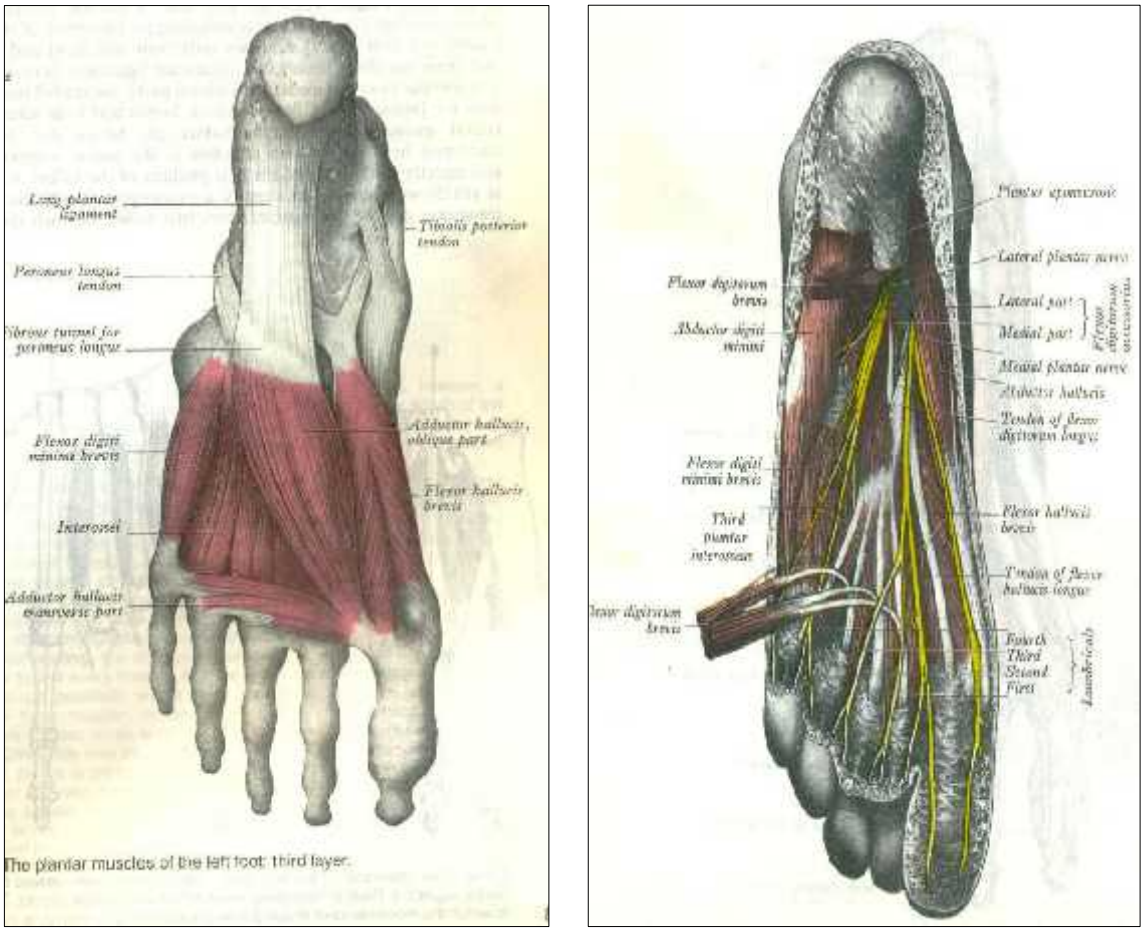


FIGURE 1: Anatomy of foot[16]

Blood supply of foot:

The chief arteries of the sole are the medial and lateral plantar arteries, the terminal branches of the posterior tibial artery.

Nerve supply of foot:

The chief nerves of the sole are the medial and lateral plantar nerves, the branches of the tibial nerve.

Definition of Diabetes mellitus:

Diabetes mellitus is a generalized chronic metabolic disorder manifesting itself in its fully developed form, by hyperglycemia, glycosuria, increased protein breakdown, ketosis and acidosis.[17]

On the basis of the pathogenic process that leads to hyperglycemia, diabetes mellitus is classified as follows

Type I diabetes due to destruction of B cell of pancreas usually leading to absolute insulin deficiency which may be idiopathic or immune mediated.

Type II diabetes may be predominantly insulin resistant with relative insulin deficiency or predominantly insulin secretory defect with insulin resistance.

Type III diabetes includes genetic defects of B cell function, genetic defects in insulin action , disease of exocrine pancreas, endocrinopathies , drug or chemical induced and infections.

Type IV diabetes: Gestational diabetes.[18]

Diagnosis:

WHO criteria for diagnosis of diabetes:

1. Symptoms of diabetes (polyuria, polydipsia and unexplained weight loss) plus random blood sugar > or = 11.1mmol/lt (200mg/dl)

Or

2. Fasting plasma glucose ≥ 7mmol/lt (126mg/dl)

Or

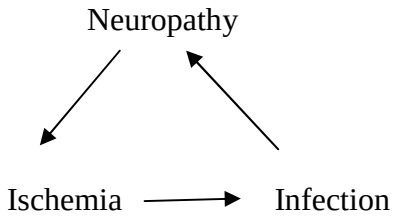
3.2 hour plasma glucose ≥11.1 mmol/lt (200mg/dl) during an oral glucose tolerance test.[19]

Prevalence of Diabetes and Diabetes foot infections:

Diabetes is one of the leading causes of death in the developing world. Diabetes is an "iceberg " disease, affecting at least 285 million people throughout the world.[20] Foot ulcers precede 85% of all nontraumatic lower limb amputations. Among the persons with diabetes, 2%-3% will develop foot ulcer each year and approximately 25% will develop a foot ulcer during their life time. Of these more than 70% require surgical intervention and result in amputation of toe or foot.[21]

Pathogenesis of diabetic foot infections:

The triad of signs leading to diabetic foot ulcer are ischemia, neuropathy and infection.[22]



1. **Neuropathy :**

Almost all patients with diabetes who develop foot ulcers have clinically significant peripheral neuropathy. Nerve damage in diabetes affects sensory, motor and autonomic fibres.[23]

i) **Sensory neuropathy:**

Painless neuropathy is the most important permissive factor in ulceration, as the loss of ability to feel a painful stimulus allows the patient to injure the foot. Small

fibres neuropathy mainly affects pain and temperature sensation.[23] Decreased sensation can lead to mechanical or thermal injuries leading to skin ulcerations.[24]

ii) **Motor neuropathy:**

Motor involvement leads to weakness and wasting of intrinsic muscles of foot which predisposes to gait disturbances and foot deformities like hammer toe and claw toes. With severe clawing, the toes become non- weight bearing and this increases the load on metatarsal heads, leading to consequent ulcers at these sites.

iii) **Autonomic neuropathy:**

The autonomic neuropathy causing loss of sweating in the foot leads to a dry foot that may fissure and crack. These breaches in the integrity of the skin allow entry of microorganisms which leads to soft tissue infections. The usual sympathetic tone is lost which causes increased peripheral blood flow and leads to arteriovenous shunting. Arterio –venous shunting reduces nutritive blood flow, contributing to foot ulcer.[23]

2. Ischaemia (Angiopathy):

Lower extremity peripheral vascular disease (PVD) is the most important factor leading to dry gangrene or ischaemic ulcerations. The ischaemic ulcer with a necrotic base presents on the dorsal, distal foot.[11]Both microangiopathy and macroangiopathy are seen in diabetics. Atherosclerosis of the lower limbs with type 2 diabetes is 20 times more common than in normal age and sex matched controls. The vessels most commonly affected are the distal superficial femoral, tibial and peroneal arteries.[23]

Glycosylation of collagen and proteoglycans produces systemic basement membrane thickening in the microcirculation of patients with diabetes. Decreased blood flow to the foot and increased vascular resistance during reactive hyperaemia has been documented in patients with diabetes.[25]

3. **Infection:**

Patients with diabetes have been shown to be at a higher risk of acquiring infection.[23]Several factors contribute to increased incidence of infection in diabetics.

Leukocyte function:

a) Hyperglycemia is known to impair leukocyte function by impairing the transport of ascorbic acid into the cells. [21, 23.]

b) Capillary basement membrane thickening may interfere with leukocyte migration through the capillary wall.

c) Poor granulation formation, prolonged persistence of abscesses and impaired wound healing are additional factors that may predispose patients to infectious complications.

CLASSIFICATION OF DIABETIC FOOT LESIONS:

There are various classifications given but Wagner's classification is the one most widely used.

1. **Wagner's classification[26]** (based on depth and appearance).

It is divided into six grades.

Grade 0- No open ulcerations present.

Grade 1- Full thickness ulceration, but depth doesn 't go beyond the loss of skin.

Grade2- Deep ulcer often with cellulitis, no abscess or bone infection

Grade3-Deep ulcer with bone involvement or abscess formation

Grade 4- Localized gangrene (toe, forefoot or heel)

Grade5- Gangrene of the whole foot.

2. **The University of Texas classification** represents an advance in the treatment of the diabetic foot. This system uses four grades, each of which is modified by the presence of infection *(Stage B)*, ischaemia *(Stage C)*, or both *(Stage D*).[27]

Table 2: UNIVERSITY OF TEXAS CLASSIFICATION OF DIABETIC FOOT

	Grade 0	Grade 1	Grade 2	Grade 3
STAGE-A	Pre-ulcerative or post-ulcerative lesion completely epithelialized	Superficial wound, not involving tendon, capsule or bone	Wound penetrating to tendon or capsule	Wound penetrating to bone or joint
STAGE-B	Infection	Infection	Infection	Infection
STAGE-C	Ischemia	Ischemia	Ischemia	Ischemia
STAGE-D	Infection and Ischemia	Infection and Ischemia	Infection and Ischemia	Infection and Ischemia

3**. Oakley W** et al [28]classified diabetic foot lesions based on aetiology into the following types,

a. Septic
b. Neuropathic
c. Ischaemic
d. Combined cause

4. **Wheat** et al[29] classified diabetic foot lesions based on clinical, roentgenographic, surgical and histopathological findings into,

a. Cellulitis
b. Osteomyelitis
c. Necrotising cellulitis or fasciitis.

5. Lipsky BA et al [30] have attempted a clinic microbiological classification based on clinical signs and bacteriological studies of the ulcer.

Lesions were classified as

Cured- if all the signs and symptoms of infection had resolved.

Improved – if most signs and symptoms had resolved.

Failed- no substantial improvement in infection and a change in antibiotic treatment or surgical intervention was believed necessary in such cases.

6. **Warren Joseph** classified diabetic foot infection based on clinical criteria as, [31]

Mild infection- superficial ulceration, localised cellulitis, minimal purulence with no systemic signs or symptoms.

Moderate infection- deep ulceration, plantar abscess, cellulitis of foot to ankle, low grade fever.

Severe infection- proximal spread to leg, marked signs of septicemia, high fever.

7. Jain AKC et al classified, diabetic foot lesions into 3 types: [32]

Type 1 - Diabetic foot complications that are infective: this includes cellulitis, abscess, necrotizing fasciitis, etc.

Type 2 - Diabetic foot complications that are non infective. Based on the structure affected they have been categorized into 4 subtypes. The diabetic Charcot foot,

peripheral arterial disease, neuropathy, etc. belong to this group.

Type 3 - Diabetic foot complications that are mixed, where both type 1 and type 2 Complications can occur in combination. A common example might be a callus ulcer with underlying osteomyelitis.

Microbiology:

Bessman AN et al reviewed 278 diabetic patients with orthopaedic vascular problems and 17% were showing non- clostridial gas infections. About 83 organisms were isolated from 48 patients in which *Proteus spp* was predominant followed by *Enterococcus spp, E.coli* and *S.aureus*. Among anaerobes *Bacteroides spp* and *anerobic Streptococci* was isolated.[33]

Louie TJ et al (1976) studied 20 diabetic foot ulcers. There were a total of 116 isolates of which 64 were aerobes and 52 isolates were anaerobes. *S. faecalis* and *Proteus spp* were predominant aerobes. Major anaerobes were *Bacteroides* and *Peptococcus* spp.[34]

Bamburger DM et al (1987) studied 51 diabetic foot ulcers from which 153 organisms was isolated. *S.aureus* was the predominant aerobe and *Peptococcus spp* was the predominant anaerobe.[35]

Ramani A et al (1991) studied 75 diabetic foot ulcers of Wagner grade 2 or more. A total of 223 isolates with an average 2.97 bacteria/ specimen were isolated. *S.aureus, P.mirabilis* and *K.pneumoniae* were predominant aerobic organisms. Anaerobes isolated were *B.fragilis* and *Peptococcus spp*.[36]

Pathare NA et al (1998) studied 252 diabetic foot infections of Wagner grade 1-5. Among them 71.09% were aerobes and 28.91% were anaerobes. *S.aureus* was the predominant aerobe followed by *Streptococci sps , Klebsiella sps*.[37]

Vijaya D et al (2000) studied 80 diabetic cases with foot infections. Of the 100 isolates, 85 were aerobes and 15 were anaerobes. Among aerobes, *Staphylococcus aureus* was the commonest pathogen followed by *Citrobacter species, Pseudomonas aeruginosa, Klebsiella species, Proteus species, Acinetobacter species, Escherichia Coli, Corynebacterium species, Enterococcus* sps. Out of the 15 anaerobes isolated, *Bacteroides species* predominated, followed by *Peptostreptococci, Clostridium species* and *Peptococci.*[38]

Senniville E et al (2006) compared the results of bone biopsy cultures with those of the corresponding cultures of superficial swab samples for patients with diabetic foot osteomyelitis. Pathogens from bone biopsy were identified from the corresponding swab cultures in only 30.4%.[39]

In a study conducted in Mumbai in 2008, among the 134 isolates, 54 were *E.coli* and 80 were *K.pneumoniae*. Of which 31 were ESBL producers by phenotypic confirmatory test.[40]

Zubair M et al (2010) isolated 75 isolates from 60 diabetic foot ulcers. In grade 1, prevalence of *S.aureus* was predominant whereas in grade 2 and 3, *E.coli* and S. *aureus* showed equal number of prevalence. Higher percentage of resistance was seen among gram negative bacilli (55.9%) with maximum resistance to Cephalosporins followed by quinolones, macrolides and monobactums.[41]

In a study conducted by Gopi Chellan in Kerala, the prevalence of fungal infections from deep tissue of diabetic wounds was found to be 27.2%. *Candida spp* was predominant (76.6%), followed by *Trichosporon spp* (12.8%) and *Aspergillus spp (*5%).[42]

Both clinical and experimental evidence indicate that true synergy between certain aerobes and anaerobes do exist. Most possible mechanisms of synergy are

a) Some anaerobes can inhibit phagocytic killing of aerobes in vitro. Ex: *B. fragilis* inhibits uptake of *E.coli*. Other studies have demonstrated the ability of anaerobes to inhibit phagocytosis by measuring the uptake of [3H] thymidine-labelled bacteria by polymorphonuclear leucocytes in the presence of an inhibiting anaerobe.

b) Bacteria may also interact by providing nutrients for each other. Ex: Vitamin K is an important growth factor for *B. melaninogenicus*. This vitamin is produced by various diphtheroids. *Klebsiella sps* produces succinate, which is a growth factor for *B.asaccharolyticus*, a heat labile growth factor produced by *S. aureus* will stimulate growth of microaerophilic *Streptococci spp*.

c) One species of bacteria optimizes the local environment for the other. Pasteur stated aerobic organisms can utilize the available oxygen to allow obligate anaerobes to proliferate. This also inhibits leucocytic oxidative function, thus further enhancing susceptibility to infection.

d) Protection of bacteria against antibiotics through bacterial interaction may result in enhancement of virulence, acquiring enzymes degrading the antibiotic, such as beta lactamases that are derived from one bacterium can protect other bacteria in mixed infections.[43]

BRIEF ACCOUNT OF ORGANISMS ISOLATED FROM DIABETIC FOOT ULCER

Identification of isolates[44, 45, 46]

I) Gram positive cocci:

1. *Staphylococcus aureus*:

Gram positive spherical cocci about 1 µ in diameter usually arranged in grapelike clusters. Nonmotile, nonsporing and usually non-capsulated except for young cultures. Aerobe and facultative anaerobe. Temperature range of 10-42ºC optimum being 37º C and pH 7.4-7.6. On nutrient agar, after 24 hrs incubation, colonies are 2-4mm diameter, circular, convex, smooth, shiny, opaque and easily emulsifiable. Most strains form golden yellow pigment. They are beta haemolytic on blood agar. On MacConkey agar, they form small pink lactose fermenting colonies. Selective media include media containing 8-10% NaCl (Salt milk agar, salt broth) and tellurite (Ludlam's medium) and polymyxin. For primary isolation sheep blood agar is recommended. On mannitol salt agar, most strains of *Staphylococcus aureus* ferment mannitol and so form colonies surrounded by yellow zones due to acid production.

Biochemical reactions- Coagulase positive, catalase positive, hydrolyse urea, reduce nitrates to nitrites, liquefy gelatin, Methyl Red and Voges Proskauer positive, indole negative. They produce phosphatase and thermostable nucleases. Virulence factors are cell associated polymers (cell wall polysaccharide peptidoglycan, teichoic acid, capsular polysaccharide), cell surface proteins (protein A, clumping factor or bound coagulase) , extracellular enzymes (coagulase, lipases, hyaluronidase, nuclease, protein receptors), toxins (cytolytic toxins- Alpha, Beta, Gamma, Delta haemolysins, leucocidin, enterotoxin, toxic shock syndrome toxin and exfoliative toxin).

***Klebsiella pneumonia* :** Gram negative straight bacilli about 1.2x0.8µ, nonsporing, nonmotile, capsulated and possess fimbriae. Colonies on nutrient agar and blood agar are large, raised, mucoid, greyish white. On MacConkey agar they form mucoid, large, dome shaped, pink lactose fermenting colonies. They are Indole and Methyl red negative, Voges Proskauer and citrate positive. They ferment sugars (glucose, lactose, sucrose, mannitol) with production of acid and abundant gas. *Klebsiella oxytoca* has similar reactions except that indole is positive.

Pseudomonas aeruginosa Gram negative bacilli of 1.5-3 x 0.5 µ, nonsporing, noncapsulated, actively motile with polar flagella. Some strains have mucoid slime layer. They are known to cause nosocomial infections. It is an obligate aerobe, but can grow anaerobically if nitrate is available. Growth occurs at wide range of temperatures 6-42ºC, optimum being 37ºC.

On nutrient agar, colonies are large, opaque, and irregular with a distinctive mawkish or earthy smell. Iridescent patches with a metallic sheen seen. *P. aeruginosa* produces a number of pigments, the best known being pyocyanin and fluorescein which diffuse into the surrounding media in which it grows. On MacConkey agar and DCA, nonlactose fermenting colonies are formed. Many strains are haemolytic on blood agar. They are catalase and oxidase positive. Negative for Indole, MR and VP tests and do not produce hydrogen sulphide or hydrolyse arginine. They utilize citrate, don't ferment lactose but glucose is utilized oxidatively with production of acid.

Nitrates are reduced to nitrites. They are intrinsically resistant to a number of antibiotics.

***Bacteroides fragilis* group**

Comprises of nonpigmented, strongly saccharolytic and bile tolerant species of Bacteroides and includes *Bacteroides fragilis, Bacteroides vulgatus, Bacteroides distesonis, B. thetaiotamicron, B. ovatus, B. uniformis, B. variabilis, B. eggerthi and B.splanchnicus.*

Among this group of saccharolytic Bacteroides, B. fragilis is the most common isolate from infections. They are nonmotile and nonsporing. They grow well on freshly prepared blood agar incubated anaerobically with 10% CO_2 at 37ºC. Colonies are low convex, white to grey, semi opaque and glistening and some strains may be haemolytic. They grow more rapidly than most nonspore forming anaerobes and growth is stimulated by bile.

Virulence factors are still debated. The capsular polysaccaride especially appears to confer added virulence to this species as evidenced from experimental studies in animals. Aggresins include a range of proteases, neuraminidase, DNAase, heparinase and other enzymes. They produce beta lactamases.

Aspergillus species:[47]

Members of this genus are *Aspergillus fumigatus, Aspergillus flavus, A.niger, A.terreus, A. glaucus, A.nidulans, A.oryzae and A.clavatus*. It mainly affects the imunocompromised host.

Aspergillus flavus produce yellow to green or brown colonies on SDA. On LPCB The conidiophores are of variable length and rough. Phialides are single and double cover the entire vesicle.

MANAGEMENT[48,49]

To formulate a wound care plan- The infected wound should be dressed in a manner that allows daily inspection and encourages a moist wound healing environment. Removal of pressure from a foot wound (Offloading) is crucial for healing process.

To determine the need for hospitalization- Patients with infections that are either severe or complicated by critical limb ischaemia should generally be hospitalized. Some patients with apparently mild infections and patients with moderate infections may also need hospitalization; this may be for observation, urgent diagnostic testing or because complicating factors are likely to affect their wound care or adherence to antibiotic treatment.

Antibacterial treatment:

Mild soft tissue infections can be treated with the administration of oral **antibiotics** for approximately 1-2 weeks. More severe infections often require **parentral**, broader spectrum therapy and a longer duration of treatment.

Surgical treatment:

1. Incision and drainage of soft tissue abscess
2. Surgical debridement of the ulcer
3. Amputations

   -Toe amputations

   -Transmetatarsal amputation

   -Below-knee amputation

   -Above-knee amputation.

Prevention

Foot ulceration results from a combination of factors:

1. Long duration of diabetes
2. Poorly controlled blood sugar
3. Neuropathy
4. Peripheral vascular disease
5. Trauma
6. Infection
7. Structural abnormalities of the foot
8. Abnormal gait
9. Illfitting footwear.[50]

"Prevention is better than cure"is a well known saying that applies equally well to diabetic foot infections also. Early detection of potential risk factors for ulceration can decrease the frequency of wound development.[51]

Successful healing of a foot ulcer or amputation does not mean the problem is over. Because neuropathy is not reversible, patients remain at risk. Following amputation, approximately 40% patients will lose the opposite leg in one to three years and 60% in three to five years due to increase pressure to the foot predisposing to callus formation and ulceration. Hence patient education is important. Special attention to the remaining leg and foot along with special shoes reduce the early amputation rate of the opposite limb.[52,53,54]

Some of the DO's and DON'Ts of diabetic foot ulcer are:

DO'S:

- To inspect feet daily using mirror.
- To apply lotion/ oil to feet to prevent drying.
- To inspect shoes daily
- To have feet checked at each clinical visit.
- To avoid exposure to extremes of temperature.

DON 'Ts

Smoking

Using hot water bottles or healing pads

Using chemical agents to treat calluses or corn

Walking bare foot

Wearing tight stockings.

MATERIAL AND METHODS

The present study was conducted at the Department of Microbiology, Jawaharlal Nehru Medical College, Belgaum.

Source of data: Deep tissue biopsy was taken from patients with grade 1-3 (Megitt Wagner classification) diabetic foot ulcer attending the surgery OPD & those who were admitted in surgery ward at KLEs Dr.Prabhakar Kore hospital & Medical Research Centre, Belgaum over a period of one year from Jan 2011 –Dec 2011.The study comprised of 75 cases of clinically diagnosed cases of diabetic foot ulcer.

Detailed clinical history regarding age, sex, chief complaints, past history and treatment history was obtained from each patient. Deep tissue biopsy taken from diabetic foot ulcer was studied in the Department of Microbiology, JNMC, Belgaum.

Sample size:

Allowing 10% absolute error and 25% being the prevalence of diabetic foot ulcer among diabetics, with all of them having infection. Hence P is taken as 25 and the sample size is calculated by using formula,

$n=4pq/d^2$

$=4\times25\times75/ (10)^2$

$=7500/100$

$=75$

Sample size n=75

n=sample size

p=prevalence

q=100-p

d=10% of error.

Method of collection of data:

Inclusion criteria:

All clinically diagnosed new cases of diabetic foot ulcer of grade 1-3 (Meggitt Wagner's classification).

Exclusion criteria:

Patient on antibiotic therapy 48 hrs prior to collection of sample.

Among 90 cases screened , 75 cases were included in the study. A total of 15 cases were excluded on the basis of exclusion criteria

Collection of sample:

The surface of the ulcer was cleaned with normal saline and deep tissue biopsy was collected with the help of No 11 blade and placed in normal saline. For anaerobic culture the sample was placed in sterile test tubes containing fluid thioglycollate medium.

Laboratory methods:[44,46,55]

Day 1:

Microscopic examination:

Part of the deep tissue specimen was crushed or ground with a sterile mortar and pestle. Direct smear was made from crushed specimen. The smear was heat fixed

and stained by Gram method for an immediate presumptive diagnosis of the type, number of bacteria, presence or absence of inflammatory cells.

Aerobic culture:

The crushed specimen was inoculated on Blood agar (BA), Mac Conkey agar (MA), Chocolate agar (CA). The BA and MA plates were incubated at 37º C for 24 hrs aerobically. The chocolate agar was incubated in a candle jar with 5-10% CO_2.

Day 2

The isolates were identified by colony characteristics, Gram staining and biochemical tests by standard procedures.[44,46,55.]

1. Colony characters observed
2. Smear for gram stain done
3. Hanging drop for motility
4. Tests for enzymes like Catalase, Oxidase, Coagulase
5. Biochemical tests done
6. Sugar fermentation test- glucose, lactose, sucrose, maltose, mannose, arabinose, xylose . Antibiotic susceptibility testing done by using Kirby Bauer disc diffusion method on Mueller-Hinton agar plate.

GRAM POSITIVE COCCI: For gram positive bacteria that are gram-positive cocci catalase test, was done. For cocci in clusters, catalase positive, slide and tube coagulase tests were done. The colonies which were seen on the aerobic culture plates were examined in detail by the methods specified by Mackie and McCartney.[44]

GRAM NEGATIVE BACILLI: For lactose fermenting and non-lactose fermenting colonies, further Gram staining, Hanging drop for motility and catalase and oxidase tests were done. After which the following biochemical tests were done. They include

- Indole test
- Methyl Red
- Voges Proskauer
- Citrate
- Urease
- Nitrate reduction
- Phenylalanine dehydrogenase
- Mannitol motility
- Triple sugar iron
- Oxidative-Fermentative glucose
- Sugar fermentation

1. Gram's stain:

A thin smear from a single colony was made on a glass slide and fixed by flaming over the Bunsen burner. After fixing crystal violet was poured over the smear, care was taken to completely cover the smear; it was allowed to stand for 1min, washed with tap water. Then Gram's iodine was poured over the slide and kept for one minute, washed with water and was decolorized by 95% alcohol till the blue color of crystal violet disappeared. The slide was washed with water and counter stained by safranine for 1min. Then the slide was washed with water, dried and was observed under oil immersion objective. In all those smears, gram negative bacilli were seen, further motility of these organism were noted by hanging drop method.

Quality control:

Positive control: *Staphylococcus aureus* ATCC 25923

Negative control: *Escherichia coli* ATCC 25922

2. Catalase test:

Principle: The enzyme catalase mediates the breakdown of hydrogen peroxide (H_2O_2) into oxygen and water. The presence of the enzyme in bacterial isolates is evident when a small inoculum is introduced into H_2O_2, and the rapid effervescence of oxygen bubbles occur. The lack of catalase is evident by a lack of or weak bubbles.

Procedure: Presence of catalase was demonstrated by test tube method. A small amount of the culture to be tested was picked from a nutrient agar plate with a clean sterile platinum loop or a clean, thin glass rod and was inserted in to 3% hydrogen peroxide solution held in a small, clean tube.

Interpretation: The production of gas bubbles from the surface of the solid culture material indicates a positive reaction and negative reaction when there were no gas bubbles.

Quality control:

Positive control: *Staphylococcus aureus*

Negative control: *Streptococcus pyogenes*

This test was used to differentiate *Staphylococcus spp.* from *Streptococcus spp*.

3. Coagulase test:

This was done to differentiate between the *Staphylococcus* species. Both slide coagulase test and tube coagulase test was done.

a) Slide Coagulase test:

Principle: Staphylococcal coagulase is a protein that has prothrombin like activity which can convert fibrinogen to fibrin. A visible clot will result. Slide coagulase test detects bound coagulase which is attached to the bacterial cell wall and is not present in culture filtrate. Fibrin strands are formed between the bacterial cells when suspended in plasma (fibrinogen), causing them to clump into visible aggregates.

Procedure : A smooth milky suspension of the growth was made in normal saline over a clean glass slide. Make similar suspension of control positive and negative strains to confirm the proper reactivity of the plasma. To the test suspension a loop full of undiluted human plasma was added and the suspension was observed for the appearance of coarse clumps.

Interpretation : Read as positive when a course clumping of cocci was visible to the naked eye within 10 seconds. Read as negative when there was absence of clumping of any reaction within 10 seconds or slow reaction was seen after 10 seconds. Negative and slow reacting strains were re-examined by the tube test.

b) Tube Coagulase test:

Principle: Detects free coagulase, a thrombin like substance, present in culture filtrate. Free coagulase reacts with serum substance (coagulase reacting factor) to form a complex that, in turn, reacts with fibrinogen to produce the fibrin clot.

Procedure: Prepare a 1 in 6 dilution of the plasma in saline (0.85% NaCl) place 1ml volumes of the diluted plasma in a small tube. Emulsify a colony of the *Staphylococcus* under test in a tube of the diluted plasma. With each batch of test include positive and negative control, a tube of unseeded diluted plasma to confirm than it does not clot spontaneously. Incubate the tube at 37^{o}C preferably in a water bath, for upto 4hrs. Examine at 1, 2 and 4 hrs for clot formation by tilting the tube through 90^{o}C. Leave the negative tubes at room temperature over night and re-examine.

Interpretation:

Positive: Any degree of visible clot formation or stiff gel formation or if clots was seen floating in the medium.

Negative: When plasma remains wholly liquid or showed only a flocculent or ropy precipitate.

Quality control:

Positive control: *Staphylococcus aureus*

Negative control: Coagulase negative *Staphylococcus.*

4. Oxidase test:

Principle: To determine the presence of an enzyme oxidase, which catalyse the transport of electrons between electron donors in the bacilli and redox dye. The dye is oxidized to indophenol blue producing deep purple colour.

Procedure: Wet filter paper method was used for this test. Strip of Whatman's No. 1 filter paper was soaked with a little freshly made 1% solution of tetramethyl-para-phenylene-diamine dihydrochloride and then with a help of sterile glass rod a single colony from the medium was rubbed over the strip.

Interpretation: A positive reaction was indicated by an intense deep purple blue, appearing within 5-10 seconds and a negative reaction by absence of colouration or by colouration later than 60seconds.

Quality control:

Positive control: *Pseudomonas aeruginosa ATCC 27583*

Negative control: *Escherichia coli ATCC 25922*

5. Hanging drop: For this, a small amount of paraffin wax was placed around the lip of the well on the concavity slide. A smooth saline suspension of an individual colony was prepared with the help of a clean sterile loop and loopful of the material was placed on a cover slip. The slide was inverted and pressed over the cover slip, guiding the drop of bacterial suspension in to the centre of the well. The slide was carefully examined by hanging drop method to know the motility of the different gram negative

bacilli. In doubtful cases, motility was confirmed by inoculating onto semisolid media.

One peptone water tube was inoculated with the growth. Care was taken to select individual discrete colonies. The tube was kept at 37ºĊ for 2 hours and turbidity was compared with 0.5 McFarland standards. It was used for sugar fermentation tests, biochemical tests and also for antibiotic sensitivity.

BIOCHEMICAL TESTS:

6. Indole test:

Principle: This test was done to demonstrate the ability of certain bacteria to decompose the amino acid tryptophan in to indole. Here tryptophan rich medium was used**.**

Procedure: Kovac's reagent method was employed.

Kovac's reagent- preparation,

Amyl or isoamyl alcohol	150ml,
β-dimethylaminobenzaldehyde	10gm,
Concentrated HCL	50ml.

Individual colonies were inoculated on to tryptophan broth were incubated at 37º C for 18-24hrs. To this 0.5ml of Kovac's reagent was added and gently shaken.

Interpretation: Appearance of red color was taken as indole producer.

Quality control:

Positive control: *Escherichia coli*

Negative control: *Klebsiella pneumoniae*.

7. Urease test: Principle:

This was done to determine the ability of bacteria to decompose urea in ammonia. Here Christensen's urea agar medium was used.

Procedure: Inoculate heavily over the entire surface of Christensen's urea agar medium with the peptone water culture and incubate at 37^{o}C. Examine after 4hrs and then overnight incubation.

Interpretation:

Positive: when the indicator turned to purple-pink

Negative: no change in colour.

Quality control:

Positive control: *Proteus species*,

Negative control: *Escherichia coli.*

8. Citrate utilization test:

Principle: To determine the ability of bacteria to utilize citrate as sole source of carbon for its growth. Simmon's citrate medium was used to know the utilization of citrate.

Procedure: The citrate slant was inoculated with the suspected single colony and medium was incubated at 37^{o}C for 24 to 48hrs. A positive reaction was indicated by blue colour and streak of growth. A negative reaction is indicated if original green color and no growth is observed.

Quality control:

Positive control: *Enterobacter aerogenes*,

Negative control*: Escherichia coli.*

9. Triple sugar iron agar test:

Principle: This was done to determine the ability of bacteria to ferment carbohydrates incorporated in a growth medium and production of hydrogen sulfide**.** Triple sugar iron (TSI) agar medium contains 10 parts Lactose, 10 parts sucrose, 1 part glucose and peptone. Phenol red and ferrous sulphate serve as indicators of acidification and H_2S production respectively. With a sterile straight inoculating wire, touch the top of a well-isolated colony.

Procedure: Inoculate TSI by first stabbing through the centre of the medium to the bottom of the tube and then streaking the surface of the agar slant. Incubate the tube at 37^oC for 18 to 24 hours the results were interpreted as follows.

Interpretation:

Slant/butt	**Color**	**Utilization**
Alkaline slant/No change in butt (K/No change)	Red/No Change	Glucose, lactose, sucrose non-utilizers.
Alkaline slant/Acid butt(K/A)	Red/Yellow	Glucose only fermented; peptones utilized
Acid slant/Acid butt(A/A)	Yellow/Yellow	Glucose fermented, lactose and/or sucrose fermented
Alkaline slant/Alkaline butt (K/K)	Red/Red	No fermentation of glucose, lactose or sucrose. Peptones utilized

A black precipitate in the butt indicates production of ferrous sulphide and H_2S gas (H_2S^+). Bubbles or cracks in the media indicate the production of CO_2 or H_2.

10. Methyl Red test:

Principle: To determine the ability of bacteria to produce and maintain the low pH after prolonged incubation.

Procedure: Inoculate the glucose phosphate peptone water medium with a young culture and incubate at 37^oC for 48 hrs. To this, add about five drops of the methyl red reagent. Mix and read immediately. Positive tests are bright red and negative tests are yellow.

Quality control:

Positive control: *Escherichia coli* .

Negative control: *Enterobacter aerogenes*

11. Voges Proskauer test:

Principle: This test was done to determine the ability of an organism to produce neutral end products like acetyl methyl carbinol or it reduction product 2, 3 butylene glycol from glucose fermentation.

Medium: Glucose phosphate peptone water.

Procedure: Inoculate the glucose phosphate peptone water medium with a young culture, and incubate at 37^oC for 48hrs, to this, add 1ml of 40% potassium hydroxide and 3ml of solution of α-naphthol in absolute ethanol.

Interpretation: A positive reaction is indicate by the development of a dark green color in 2-5min.

Quality control:

Positive control: *Enterobacter aerogenes,*

Negative control: *Escherichia coli.*

12. Sugar fermentation test:

Principle: This test was done to determine the ability of an organism to ferment a specific carbohydrate that is incorporated in a basal medium, thereby producing acid with or without visible gas.

Procedure: The test was performed on conventional culture media with test sugar. The common sugar fermentation media used for present study were glucose, sucrose, lactose, maltose, mannose, arabinose and xylose. From the peptone water tube (which was incubated for 2hrs. after inoculation) all the sugar fermentation media were inoculated with the help of a clean sterile loop. Care was taken to sterilize the loop every time, after dipping in different sugars. After different media were inoculated, these were incubated at 37^{o}C for 18-24hrs. After 24hrs, the sugar media were examined for the production of acid indicated by pink color and gas (presence of an air bubble inside the durham's tube).

Interpretation: Positive test in indicated by change in color to pink with or without gas formation in durham's tube. Negative test is indicated by growth, but no change in colour.

13. Oxidation/ Fermentation test (Modified Hugh and Leifson) :

Principle: This test was done to know if the organism uses carbohydrate substrate to produce acid byproducts either oxidatively or fermentatively.

Procedure: Hugh-Liefson's basal medium was prepared and carbohydrate to be added was sterilized separately and added to give a final concentration of 1%. The medium was then tubed to a depth of about 4cm.

Duplicate tubes of medium were inoculated by stabbing. One tube was promptly covered with a liquid paraffin to a depth of 1cm and were incubated at 37^{o}C for 18-24hrs.

Interpretation:

- Fermenting organisms produce an acid reaction throughout the medium in both covered (anaerobic) as well as the open (aerobic) tubes.
- Oxidative organism produce an acid reaction only in the open tube.
- Organisms that cannot breakdown the carbohydrate aerobically or anaerobically produce an alkaline reaction in the open tube and change in covered tube.

14. Nitrate Reduction test:

Principle: The test is used to determine the ability of an organism to reduce nitrate. The reduction of nitrate to nitrite is determined by adding sulfanilic acid and alpha-napthylamine. The sulfanilic acid and nitrate react to form a diazonium salt. The diazonium salt then couples with α-napthylamine to produce a red, water soluble azo dye.

Procedure: This liquid medium was inoculated with the suspected single colony and the medium was incubated for 18-24hrs. Add 0.1ml of the test reagent to

the test culture. The test reagent was prepared by mixing equal volumes of solution A (8.0gm of sulphanilic acid in 1liter of acetic acid 5mol/liter) and solution B (5.0gm of α-napthylamine in 1 liter of acetic acid 5mol/liter). A red color developing within a few minutes indicates the presence of a nitrate. No colour indicated that nitrate has not been reduced or reduced to products other than nitrites, such as ammonia, molecular nitrogen, nitric oxide or nitrous oxide and hydroxylamine. Addition of zinc dust to all negative tests was necessary. Zinc ions reduced nitrates to nitrites and development of red colour after addition of zinc dust indicated the presence of residual nitrate.

Quality control:

Positive control: *Escherichia coli*

Negative control: *Acinetobacter baumannii*

15. Phenylalnine Deaminase test (PDA) :

Principle: To determine the ability of bacteria to deaminate phenylalanine to phenyl pyruvic acid.

Procedure: This test was done to know the ability of the organism to deaminate phenylalanine with the production of phenyl pyruvic acid, which reacts with ferric salts to give green color. Inoculate the agar slope medium containing DL-phenylalanine with a fairly heavy inoculum and incubate 37^{o}C for 18-24hrs. After incubation, allow a few drops of 10% solution of ferric chloride to run down over the growth on the slope if the test is positive, a green color will develop in the fluid and in the slope.

Quality control:

Positive control: *Proteus species.*

Negative control: *Escherichia coli.*

16. Amino acid decarboxylase and Arginine dihydrolase test:

Principle: Decarboxyases are a group of substrate- specific enzymes that are capable of reacting with the carboxyl portion of amino acids, forming alkaline-reacting amines. The conversion of arginine to citrullin is a dehydrolase, in which an NH_2 group is removed from arginine.

Procedure: Here Moellar decarboxylase broth base was used. Test organism was inoculated in the medium with straight wire, then overlay tubes with sterile mineral oil to cover above 1 cm of the surface. Incubate and read daily for 4 days. The medium first becomes yellow due to acid production during glucose fermentation later if decarboxylation occurs; the medium turns to violet colour.

Quality control:

Positive control: *Pseudomonas aeruginosa.*

Negative control: *Klebsiella pneumoniae*.

Antibiogram testing:

Antibiotic sensitivity was tested by Kirby-Bauer's disk diffusion method. Mueller-Hinton agar plate was used. One-two colonies from the culture plate were inoculated into 2 ml of peptone water and incubated at 37^oC for 2hours. Turbidity was compared to that of 0.5 McFarland's standard (1.5×10^8 CFU/ml). A cotton swab was immersed, rotated in this inoculum, the swab was then pressed to the sides of the tube so on to remove excess inoculum. The swab was then used to inoculate the plate of Mueller-Hinton agar, in three different directions to ensure an even and complete

distribution of the inoculum over the entire plate. The antibiotic disks were applied within 15 minutes of inoculation of plate and the plate inverted and incubated for 18-24 hrs at 37ºĊ.

Commercially obtained Hi-media disks were used. The strength of disks used and their zone size interpretative standards were according to guidelines by CLSI guidelines standards.

If these organisms were not sensitive to any of the drugs, then a second line of antibiotics was put up using the same procedure as observed. The drugs used for gram positive organisms were-

- Amoxicillin (Amx) (10µg)
- Erythromycin(E) (15 µg)
- Ciprofloxacin (Cf)(5µg)
- Cotrimoxazole (Cot)(30µg)
- Amoxyclav (Ac)(30µg)
- Oxacillin (Ox) (1µg)

The drugs used for gram negative organisms were-

- Amikacin (Ak) (30 µg)
- Ciprofloxacin (Cf)(5 µg)
- Ceftazidime(Caz)(30 µg)
- Levofloxacin(Le)(5 µg)
- Pipercillin(Pc)(100 µg)
- Pipercillin tazobactum (Pt) (100/10µg)

Methicillin Resistant Staphylococcus aureus- Disk diffusion test:

This test was done to know the Methicillin resistant Staphylococcus strains. A direct colony suspension of each *S.aureus* isolated was prepared to a 0.5 Mc Farland standard and placed on Mueller- Hinton agar containing 2-4% NaCl. An Oxacillin (1 µg) disc was placed on the surface and incubated at 35-37º C for 24 hrs. The zone of diameter was recorded. If zone diameter lesser or equal to 10 mm is considered as resistant, greater or equal to 13 mm is considered as susceptible, whereas 11-12 mm was considered as intermediate zone.

Extended Spectrum Beta-Lactamase – Potentiated Disk Diffusion Test (PDDT):

The test was done for Gram negative bacilli to know the presence of Extended spectrum beta – lactamase resistance to one or more beta lactum antibiotics but susceptibility to other third generation cephalosporins. Lawn culture was made in Mueller-Hinton agar plates with standardized innoculum (corresponding to 0.5 McFarland). A disk of Ceftazidime (30µg) alone and ceftazidime+ clavulinic acid (30+10µg) were placed at adistance of 25 mm centre to centre and incubated at 37ºC for about 16-18 hrs. An increase in the inhibition zone diameter of > 5mm for a combination disc versus ceftazidime disc alone confirmed ESBL production.

Culture of anaerobic organisms:[56]

Each sample was inoculated onto

1) Blood agar supplemented with Haemin and Vit K

2) Kanamycin Vancomycin laked blood agar(KVLB)

3) Bacteroides bile esculin agar (BBE)

↓

Single colony of each distinct type

↓

Aerotolerance testing

↓

Purity blood agar plate

↓

Add antibiotic identification discs	**Perform**	**Record**
-Sodium polyanethol sulphonate	1. Grams stain	1. Colony morphology
(SPS) disc	2. Spot indole test	2. Pigment
-Nitrate disc	3. Catalase test	3. Haemolysis
-Susceptibility to Kanamycin(1mg),	4. Fluorescence	
Vancomycin(5µg) , Colistin(10µg)	5. Pitting	

1) Blood agar supplemented with Haemin(5µg) and Vitamin K (10mcg/ml). Blood agar plates used for anaerobic isolation were prepared with Brucella agar base.
2) Kanamycin (75mcg/ml) Vancomycin(7.5mcg/ml) laked blood agar (KVLB). KVLB agar inhibits the growth of most facultative bacteria and allows for earlier pigmentation of *Prevotella melaninogenicus*. Most *Bacteroides spp*. grow well on it, while *Fusobacterium spp*. and most gram positive anaerobes are inhibited.

Preparation: Brucella agar base was prepared to which 75µg /ml Kanamycin was added and autoclaved. Vancomycin7.5µg/ml and laked blood (5%) was aseptically added after autoclaving. Laked blood was prepared by freezing whole blood overnight and then thawing.

3)Bacteriodes bile esculin agar (BBE) containing Gentamycin 100µg/ml, 20% bile, 0.1%esculin, 0.05% ferric ammonium citrate (Hi media) was used. BBE agar is useful for rapid detection and isolation of members of the B.fragilis group. Members of the *B.fragilis* group grow well , producing dark colonies with brown to black halo.
The method used for obtaining anaerobiasis in the jar was "internal gas generating system" described by Lakshminarayana and Vaidhyalingam .[57]

Catalyst:

Cold catalyst reactivated every time before use by drying at 150-160 º C for 1-2 hrs.

Indicator:

Methylene blue prepared mixing equal volumes of

a. 6% glucose containing 1mg/ml thymol as preservative.
b. Sodium hydroxide solution prepared by diluting 6ml of 0.1 N NaOH to 100 ml of distilled water.
c. Methylene blue prepared by adding 3ml of 0.5 % w/v solution of methylene blue to 97 ml of distilled water. Mixture is placed in anerobic jar after it is made colourless by heating in a boiling water bath.

Principle: In this system the hydrogen and CO_2 gas mixture required for creating anaerobiasis is obtained from the following reactions.

Citric acid + sodium bicarbonate

Sodium citrate +carbon dioxide

Sodium borohydride+hydrogen

Sodium metaborate +hydrogen

Operation of the gas generator

a) 1g sodium borohydride was taken in 30ml test tube.
b) 1 g sodium bicarbonate and 1g citric acid were taken in the 5ml test tube , which was placed inside the 30 ml test tube.
c) The stem of 20 ml funnel was plugged tightly with cotton to control the flow of water. The funnel was placed in 30 ml test tube in such a way that the stem of the funnel dips into the 5ml test tube. Entire unit was kept inside the jar with the indicator. 20 ml of distilled water was poured in the funnel just before closing the lid of the jar.

The water poured into the funnel drips into the 5ml test tube liberating CO_2. CO_2 being heavier stays within, displacing the air. Once the 5ml test tube is filled with water, it overflows into the 30 ml test tube liberating hydrogen, which being lighter gas, rushes out with CO_2. The palladium catalytically reduces the oxygen present within the jar to form water. Catalyst is exothermic, so warming of the lid can be felt.[57]

After 72 hours of incubation at 37 º C anaerobic jar was opened. The plates were examined for the presence of colonies. When the colonies appeared on the anaerobic plates each predominant distinct colony was

subcultured to purity blood agar plate (BAP). From a pure culture on a BAP, following was recorded.

- Colony morphology including size of colony , shape, colour, appearance
- Pigment
- Haemolysis
- Fluorescence
- Pitting

  Single colony of each distinct type was plated on to blood agar plates with antibiotic identification discs.

- Sodium polyanethol sulphonate (SPS) disc for rapid presumptive identification of *Peptostreptococcus anaerobius*.
- The 3 anitbiotic discs Kanamycin 1mg, Colistin 10µg and Vancomycin 5µg were placed on the lawn culture.

Chocolate agar was inoculated for incubation in candle jar at 37 º C to test for aerotolerance.

The following tests were done from the purity plate.

Catalase test: The colony was touched in a glass rod and placed to a drop of 15% hydrogen peroxide in a test tube and observed for evolution of bubbles.

Spot indole test: The colony was touched on a glass rod and smeared on a filter paper saturated with 1% para dimethyl amino cinnamaldehyde in 10 %(V/V) concentrated hydrochloric acid. Appearance of blue colour indicates positive reaction. Negative reaction gave no colour or a pinkish colour.

Nitrate test: The test was done using nitrate discs. The disc was removed from the surface of plate and placed in a clean petri dish. One drop each of reagent A and B were added. Development of pink to red colour indicates nitrate had been reduced to nitrite.

Nitrate reagents

Solution A

Sulphanilic acid	0.5g
Glacial acetic acid	30 ml
Distilled water	120ml

Solution B

1,6 Cleve ‘s acid	0.2 g (5- amino- 2- naphthalenesulfonic acid)
Glacial acetic acid	30 ml
Distilled water	120ml

CULTURE OF FUNGAL ORGANISMS:[58,59]

1. **Direct examination:**
    - Gram staining to see for the presence of yeast and pseudohyphae of Candida species.
    - KOH examination for the presence of fungal elements.
2. **Fungal culture:**

The crushed specimen is placed were directly placed into SDA and SDA with antibiotics and incubated at 25ºC and 35º C and observed for four weeks. The Lactophenol cotton blue (LPCB) mount is prepared fom the colonies to examine for filamentous fungi. The filamentous fungus was identified by slide culture on SDA.

Slide culture:

A 100 mm diameter sterile petri plate with filter paper is taken. A bent glass rod with a clean grease free slide is placed on the petri plate. A block of 1× 1 cm of Potato dextrose agar was placed on the slide and innoculated with the fungus. A flamed coverslip is placed over the slide and a cotton moistened with distilled water was placed in the bottom of the petri plate and incubated at room temperature.

When reproductive structures are well developed, the coverslip was removed and placed on a slide with a drop of LPCB and examined under low power objective.

RESULTS

A total of 75 cases of diabetic foot ulcer was included in the present study.

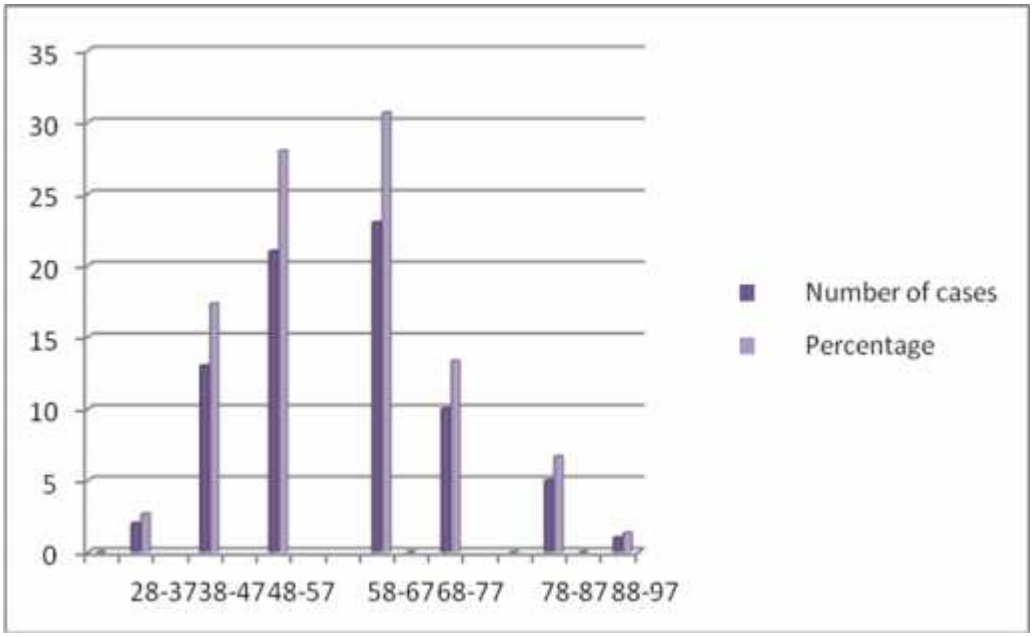
AGE DISTRIBUTION:

Our cases ranged between 30-95 years. Maximum numbers of cases were in the age group of 58- 67 years.

Table 3: Age distribution of cases

Age	Number of cases	Percentage
28-37	2	2.67
38-47	13	17.33
48-57	21	28
58-67	23	30.67
68-77	10	13.33
78-87	5	6.67
88-97	1	1.33

Figure 2: Age distribution of cases



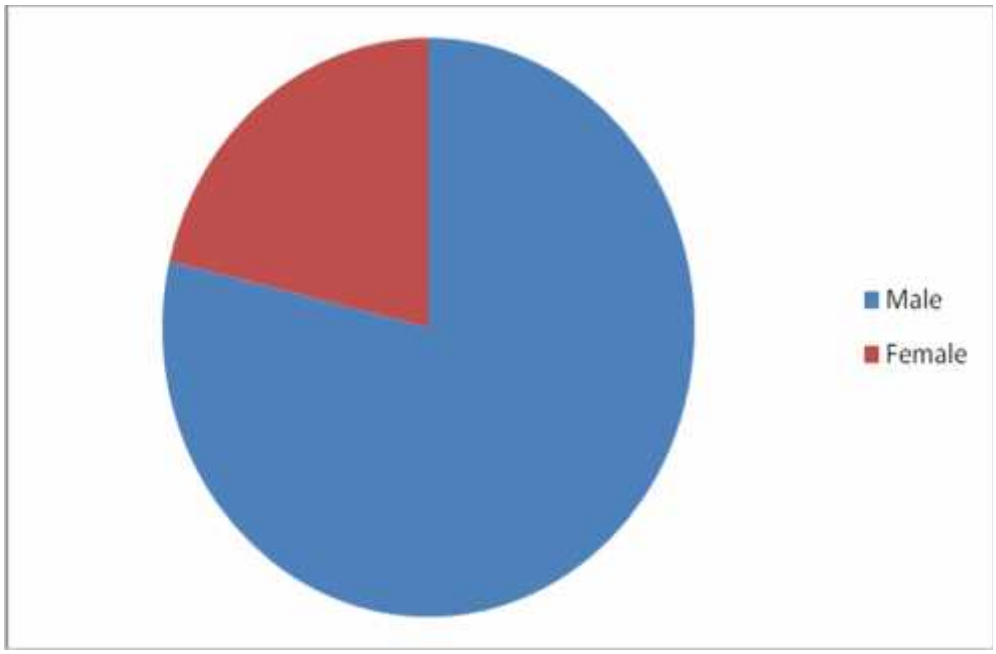
SEX DISTRIBUTION:

Out of 75 cases 59(78.7%) were male and 16 (21.33%) were female.

Table 4: sex distribution of cases

Male (%)	Female (%)
59 (78.7%)	16 (21.33)

Figure 3: sex distribution of cases

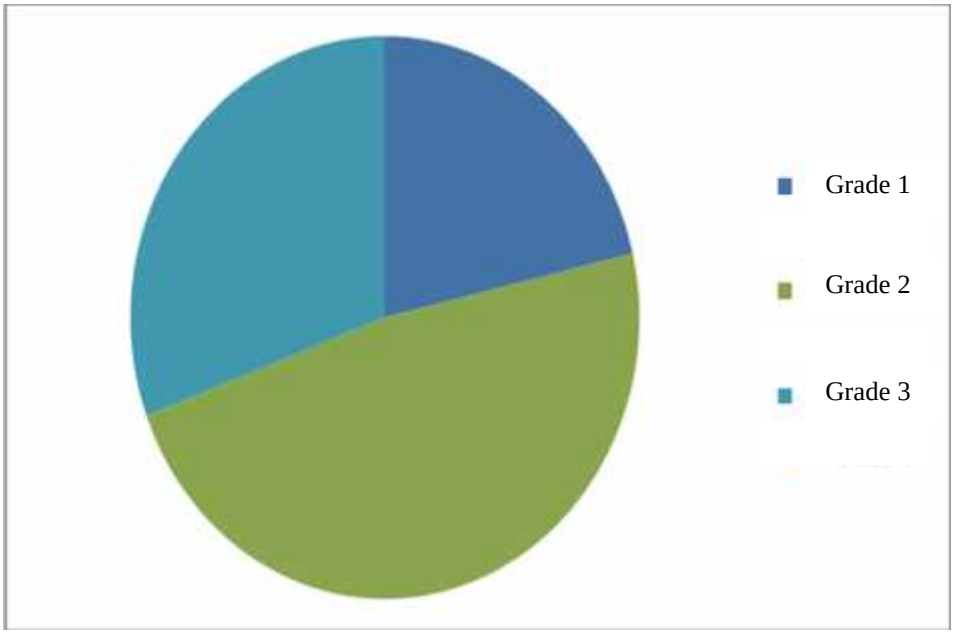


GRADE OF ULCER:

Table 5: Distribution of cases according to different grades of ulcer

Grade of ulcer	Number of cases	Percentage
Grade 1	16	21.33
Grade 2	36	48
Grade 3	23	30.67

Figure 4: Distribution of cases according to different grades of ulcer



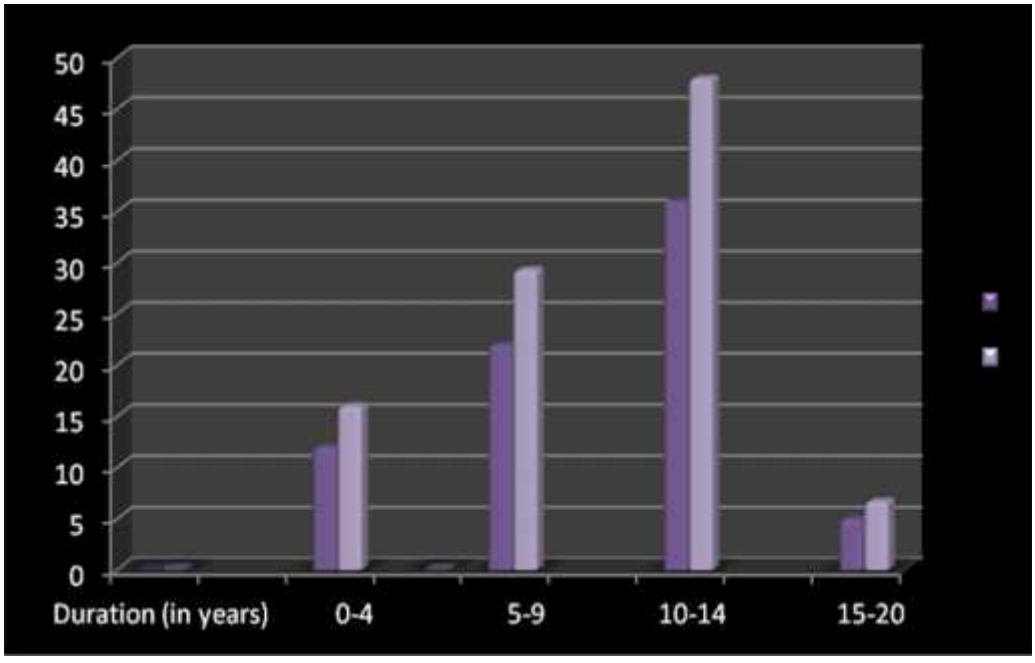
DURATION OF DIABETES:

The maximum number of cases had diabetes for 10-14 years.

Table 6: DURATION OF DIABETES

Duration (in years)	No of cases	Percentage
0-4	12	16
5-9	22	29.3
10-14	36	48
15-20	5	6.7

Figure 5: DURATION OF DIABETES



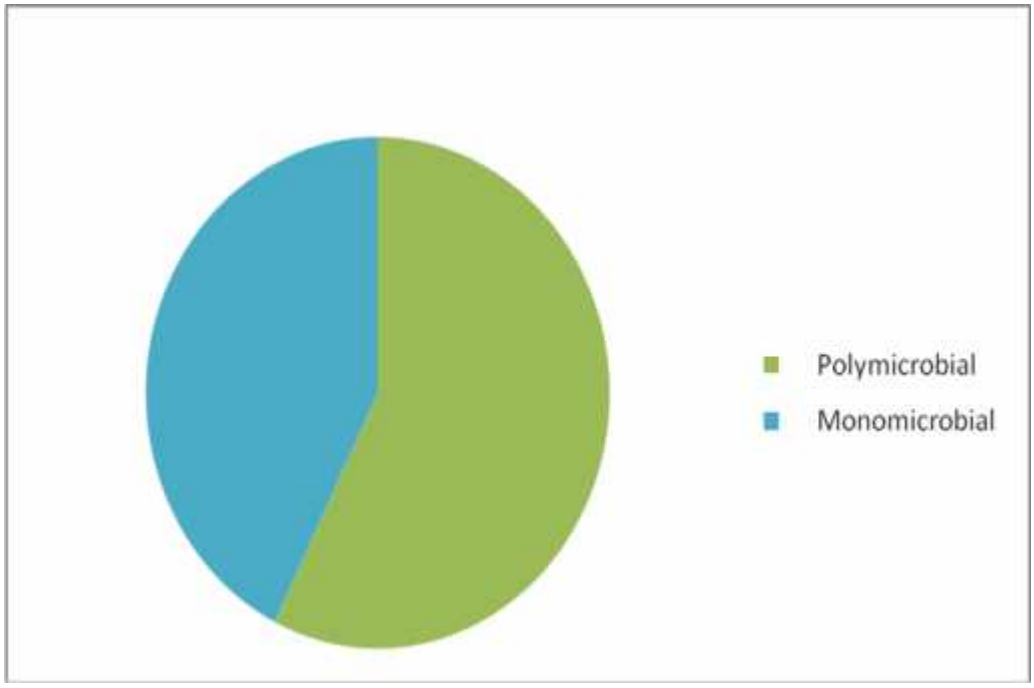
ISOLATES OBSERVED:

75 samples yielded 139 isolates of which 43 isolates (57.34%) were polymicrobial, 32 isolates (42.67%) were monomicrobial .

Table 7: Distribution of isolates

Type of isolates	Number of cases	Percentage
Polymicrobial	43	57.34
Monomicrobial	32	46.27

Figure 6: Distribution of isolates



DISTRIBUTION OF TYPE OF ISOLATES:

Table 8: DISTRIBUTION OF TYPE OF ISOLATES:

Type of isolates	Type of isolates	Percentage
Aerobic	107	76.98
Anaerobic	31	22.30
Fungal	1	0.71

Figure 7: Distribution of type of isolates

Distribution of isolates in different grades of ulcer:

Table 9: Distribution of isolates in different grades of ulcer

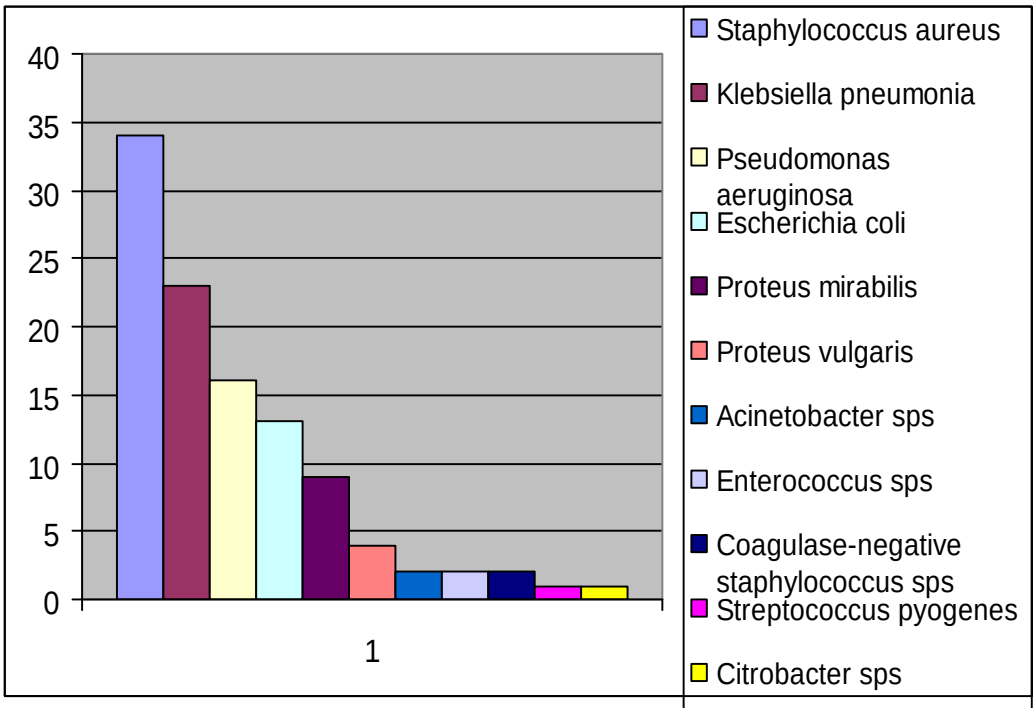
	Grade 1	Grade 2	Grade 3
Aerobes	20	53	34
Anaerobes	1	14	16
Fungus	-	1	-

Aerobic isolates:

Table 10: Aerobic isolates

Isolate	Number of cases	Percentage
Staphylococcus aureus	34	31.77
Klebsiella pneumonia	23	21.49
Pseudomonas aeruginosa	16	14.96
Escherichia coli	13	12.14
Proteus mirabilis	9	8.14
Proteus vulgaris	4	3.73
Acinetobacter baumanii	2	1.87
Enterococcus sps	2	1.87
Coagulase-negative staphylococcus sps	2	1.87
Streptococcus pyogenes	1	0.93
Citrobacter sps	1	0.93

Figure 8: Aerobic isolates:



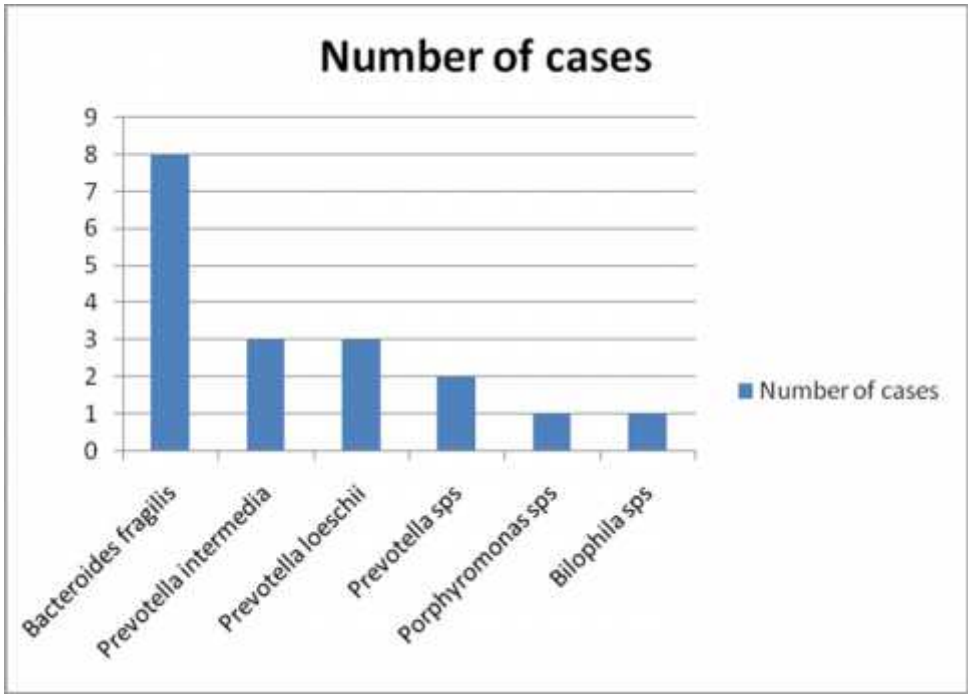
Anaerobic isolates:

Out of the 31 anaerobic isolates 18 (58.06%) were GNB and 13 (42%) were GPC. Among GNB Bacteroides fragilis was the most common isolate.

Table 11: Anerobic GNB isolates

Isolate	Number of cases	Percentage
Bacteroides fragilis	8	44.45
Prevotella intermedia	3	16.67
Prevotella loeschii	3	16.67
Prevotella sps	2	11.12
Porphyromonas sps	1	5.56
Bilophila sps	1	5.56

Figure 9: Anaerobic GNB isolate

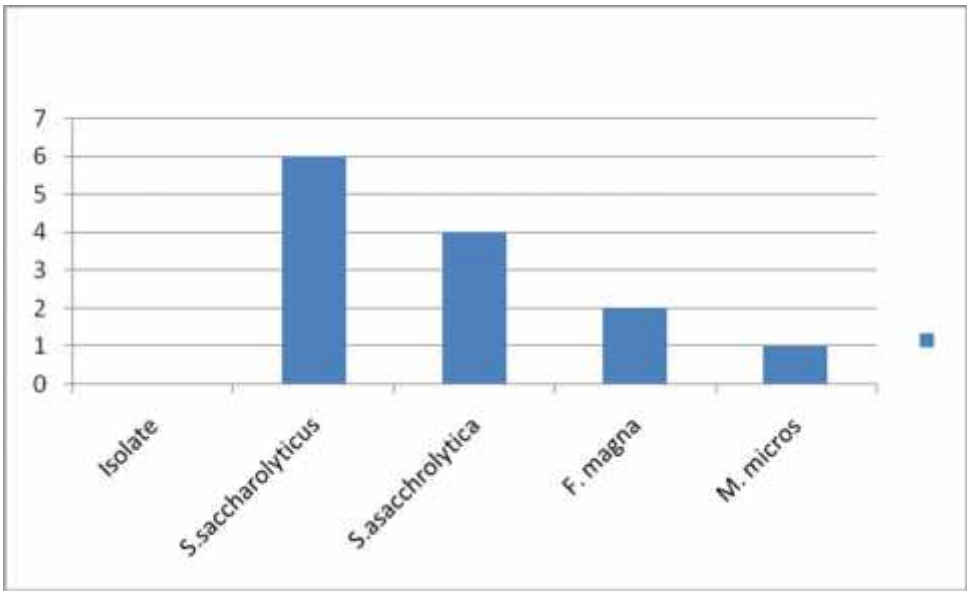


Anaerobic GPC isolates:

Table 12: Anaerobic GPC isolates

Isolate	Number of cases	Percentage
Staphylococcus saccharolyticus	6	46.15
Schleiferella asacchrolytica	4	10.80
Finegolda magna	2	15.39
Micromonas micros	1	7.70

Figure 10: Anaerobic GPC isolates



Fungal isolates:

The only fungal isolate is ***Aspergillus flavus*** (0.7%)

Table 13: Antibiotic susceptibility pattern of aerobic GPC isolates

		Antibiotic Sensitivity															
Isolate	No. of Isolate	E		Cf		Cot		Am		Ac		M		V		G	
		S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R
S.aureus	34	23	11	19	5	27	7	20	17	24	10	24	10	-	-	-	-
Enterococus sps	3	1	2	2	1	2	1	2	1	2	1	-	-	3	0	1	2
CONS	2	1	1	2	0	1	1	2	0	2	0	2	0	-	-	-	-
S pyogenes	1	1	0	1	0	1	0	1	0	1	0	-	-	-	-	-	-

(E- Erythromycin, Cf – Ciprofloxacin, Cot- Cotrimoxazole, Am- Amoxicillin, Ac-Amoxyclav, M-Methicillin, V- Vancomycin, G- Gentamycin)

Table 14: Antibiotic susceptibility pattern of aerobic GNB isolates

Isolate	No. of Isolate	Antibiotic Sensitivity													
		Ak		Cf		Caz		Pc		Le		Cot		Pt	
		S	R	S	R	S	R	S	R	S	R	S	R	S	R
K. pneumoniae	23	13	10	8	15	7	16	12	11	12	11	12	11	-	-
E coli	13	10	3	5	5	5	8	0	4	7	6	7	6		
P aeruginosa	17	9	8	7	9	9	8	14	2	10	7	-	-	14	2
P mrabilis	9	7	2	4	5	5	4	8	1	8	1	-	-	9	0
P vlgaris	4	4	0	2	2	3	1	4	0	3	1	-	-	4	0
A .baumanii	2	1	1	1	1	1	1	2	0	1	1	-	-	2	0
Citrobacter sps	1	1	0	1	0	1	0	1	0	1	0	1	0	-	-

(Ak- Amikacin ,Cf- Ciprofloxacin, Caz- Ceftazidime, Pc- Pipercillin, Le-Levofloxacin, Pt –Pipercillin/tazobactum)

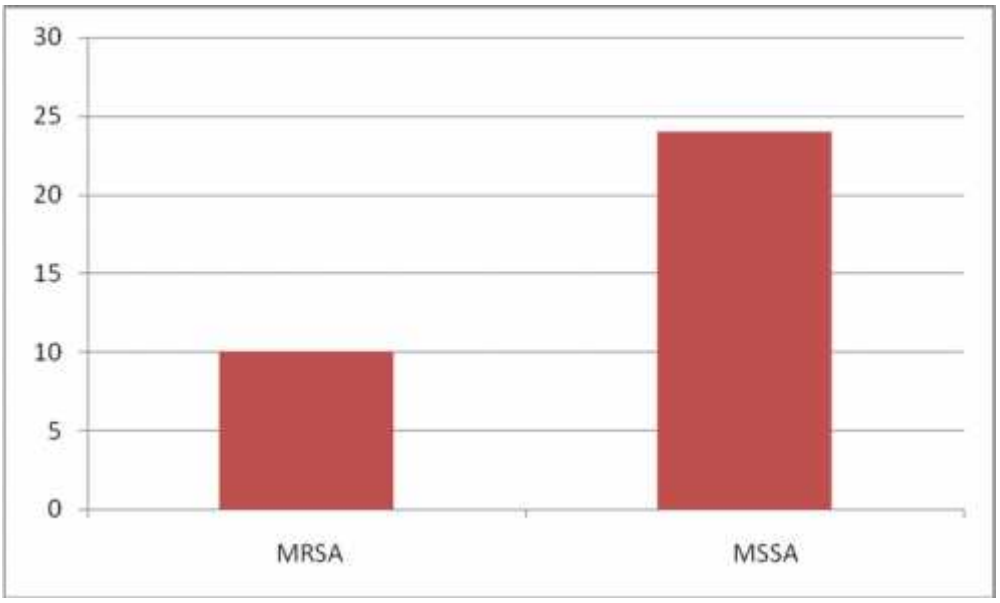
MRSA Producing Staphylococcus aureus:

Out of 34 *S.aureus* isolates, 10 were MRSA.

Table 15: MRSA Producing Staphylococcus aureus:

Total no of S. aureus isolated	MRSA	MSSA
34	10 (29.41%)	24 (70.58%)

Figure: 12 MRSA Producing Staphylococcus aureus:



ESBL Producers in GNB isolates:

Table 16: ESBL Producers in GNB isolates

Total no of GNB isolates	ESBL producers	Non-ESBL producers
60	22 (32.35%)	38 (67.655%)

PHOTOGRAPHS

Photo 1: A case of diabetic foot ulcer

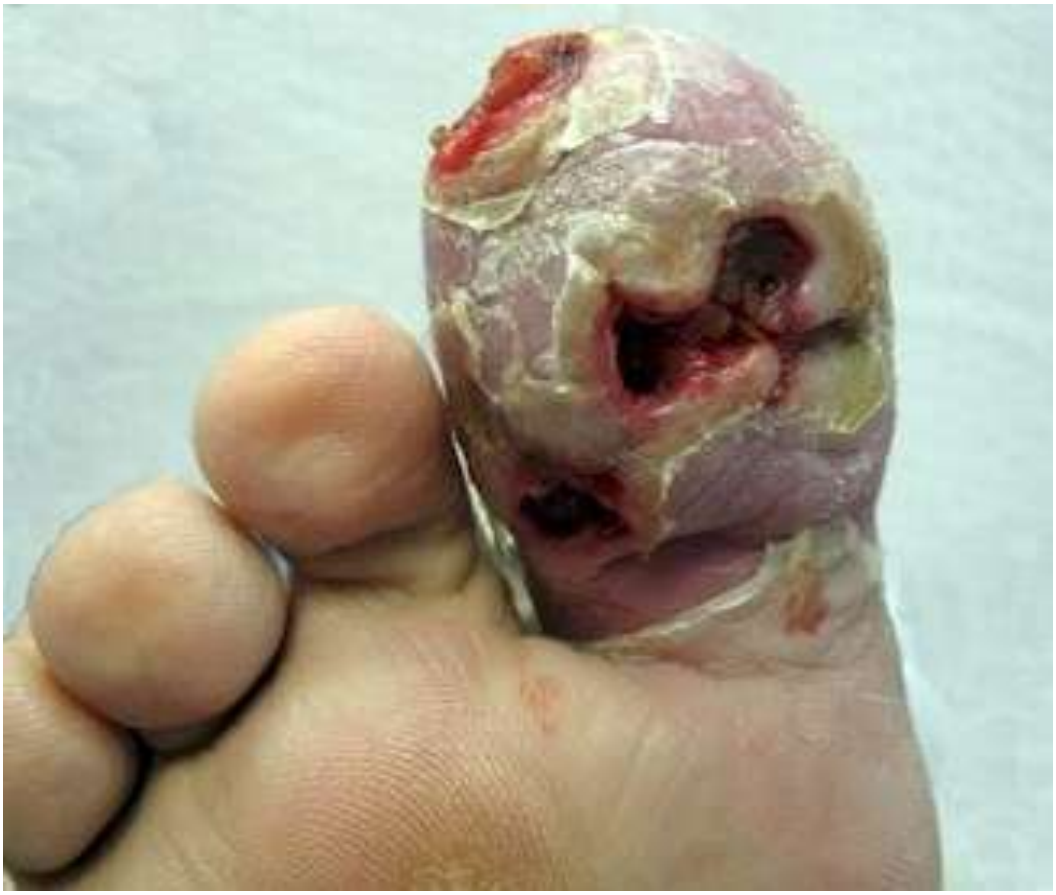


Photo 2: Growth of Staphylococcus aureus on Blood Agar

Photo 3: Biochemical reactions of S. aureus

Photo 4: Tube coagulase test

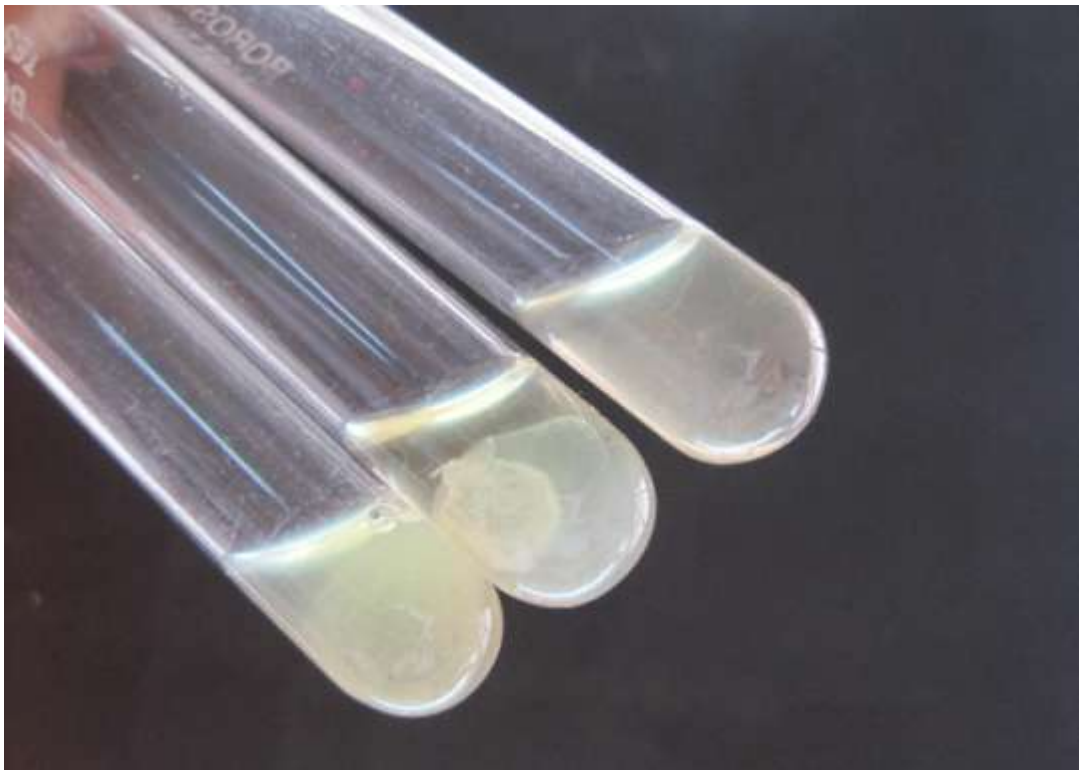


Photo 5: Methicillin resistant Staphylococcus aureus

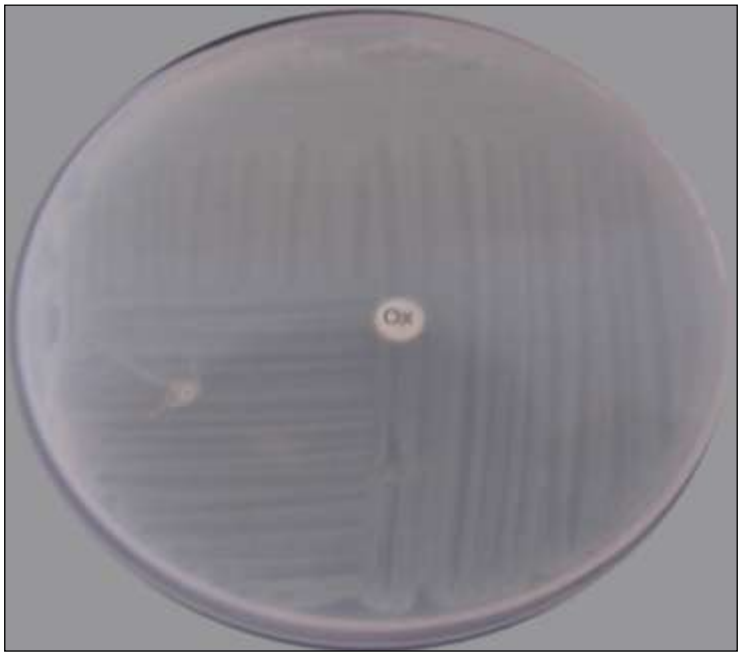


Photo 6: growth of K.pneumoniae on MacConkey agar

Photo 7: Biochemical reactions of K.pneumoniae

Photo 8: Growth of P.aeruginosa on Nutrient agar plate

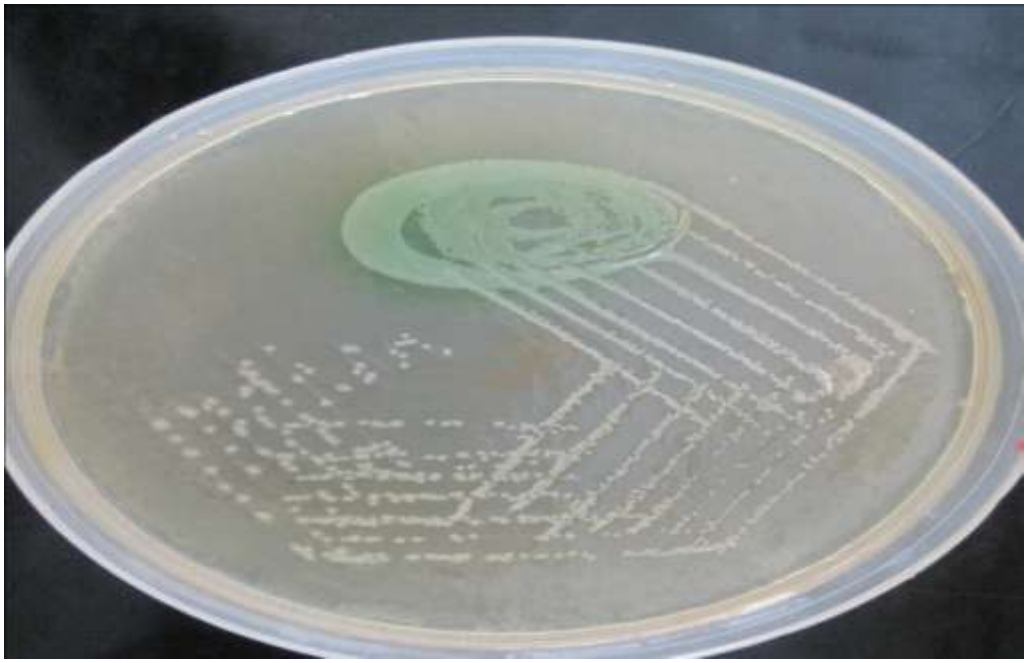


Photo 9: Biochemical reactions of P.aeruginosa

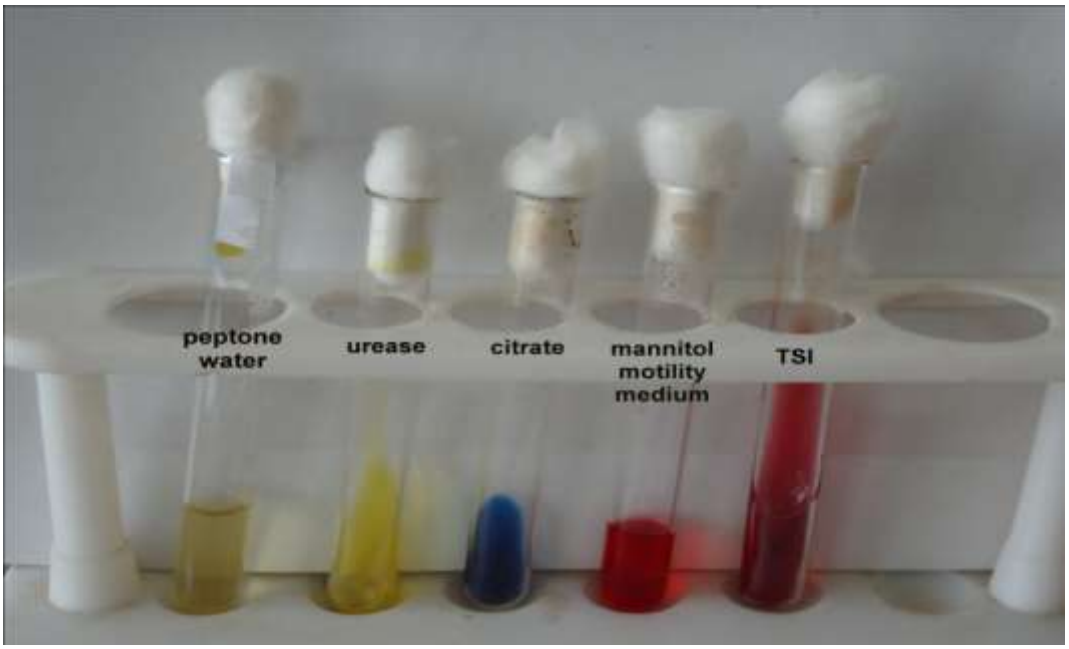


Photo 10: Thioglycollate medium

Photo 11: McIntosh Fildes jar

Photo 12: Growth of B. fragilis on Brucella Blood agar

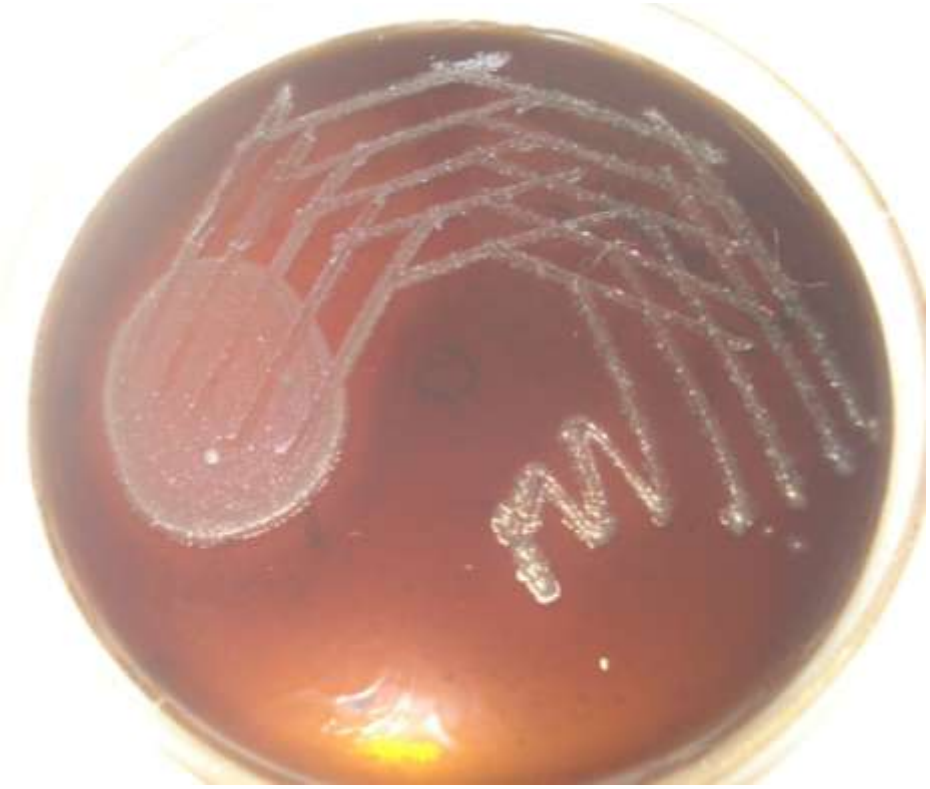


Photo 13 : Growth of B. fragilis on Bacteroides bile esculin agar

Photo 14 : Bacteroides fragilis showing resistance to Kanamycin (K), Vancomycin (V), and Colistin (C) discs

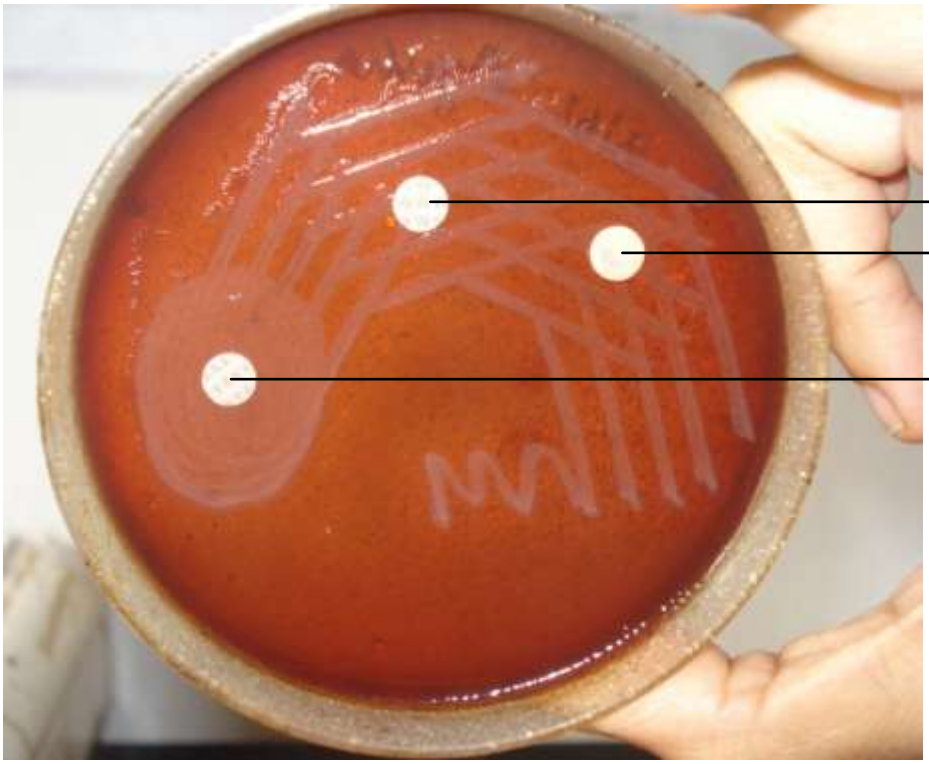


Photo 15 : Growth of Prevotella intermedia on Brucella blood agar

Photo 16 : Growth of Porphyromonas species on Brucella blood agar

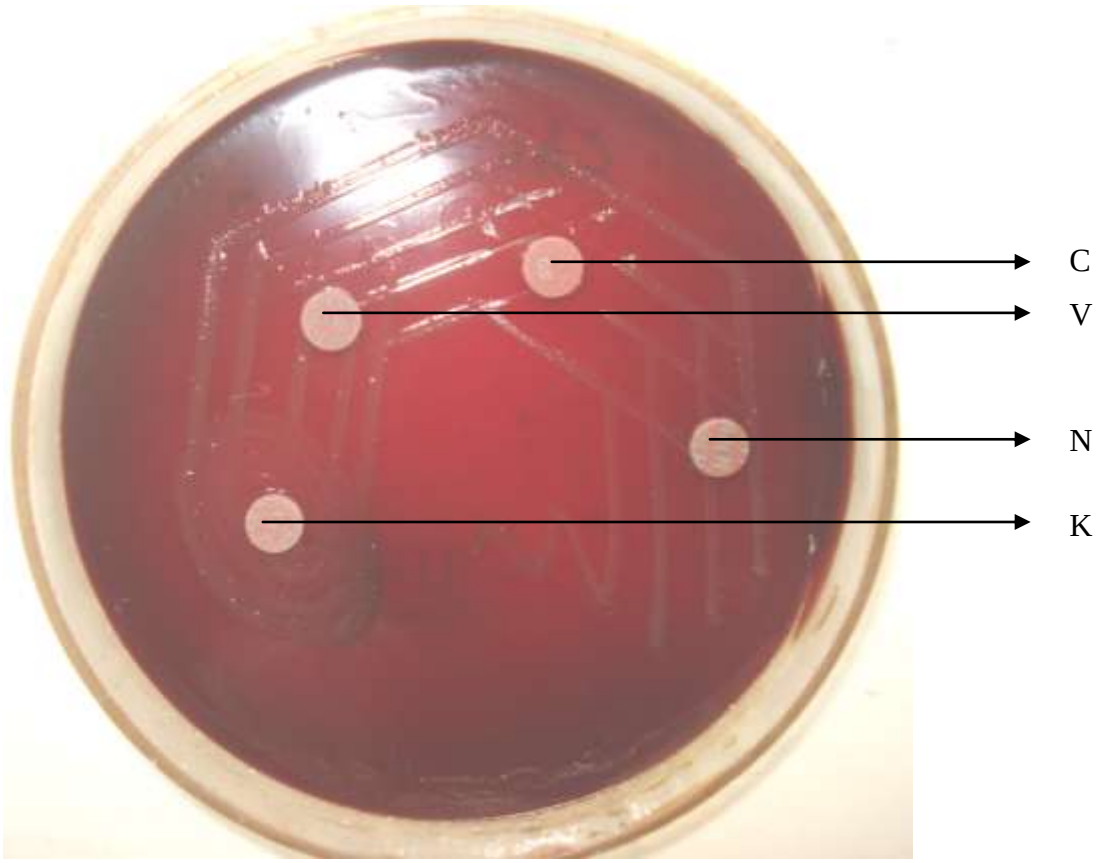


Photo 17 : LPCB mount of Aspergillus flavus

DISCUSSION

Foot ulcers are a significant complication of diabetes which are the most common cause of non traumatic lower extremity amputations. Foot infections are the most common complications of diabetic foot and plays a main role in the development of moist gangrene.[60]These infections are responsible for 20% of all hospital admissions.[61] In general , people with diabetes have infections that are more severe and take longer to cure than equivalent infections in other people.

Infections leads to early development of complication even after a trivial trauma with disease progression and becomes refractory to antibacterial therapy. It is essential to assess the magnitude of bacterial infection of the lesions to avoid further complications and save the foot.[60]

The present study consisted of 75 cases . The age group ranged from 25- 95 years with maximum number of cases in the age group of 58- 67 years. The results were similar to the study conducted in Tamilnadu where the maximum number of cases ranged from 56-65 years.[62] In another study conducted in Aligarg the maximum number of cases were in the age group of 41-60 years.[37]

The mean age of the patients was 59.5 years in Kahn et al , 80.3 years in Delbridge et al, 58 years in Ramani et al , 75.02 years in Pathare NA et al, 58 years in Dipali AC et al and 43 years in Anandi C et al.[4,6,32,63,64,65]

The male to female ratio in our study is 3.6:1.ie males are affected more than females. Our results are comparable with the observations made by Ramani et al , Pathare et al, Vijaya et al, Dipali AC et al, Faiz ur Rehman et al, Anandi et al.[4,6,32,34,63,66] The male population predominating the studies may be due to the fact

that males are more involved in outdoor activities in the Indian scenario and are thus more prone to injuries which can predispose to ulcers.

Rehman F et al found that most patients had diabetes for >11 years in his study.[66] Dipali AC et al , Echard M et al observed that 70.5% of the cases had diabetes for more than a decade.[63,67]Chander J et al found that 49% of patients were diagnosed to have diabetes for more than 10 years.[68] The mean duration of diabetes in our patients was 11 years. The maximum number of cases (48%) had diabetes for 10- 14 years .

Among the total study population, 96% had type 2 diabetes, whereas only 3 had type 1 diabetes. This is in accordance with the findings of Chander J et al, Ramani A et al, Zubair M et al, Falta S et al and Girish MB et al.[32,37,38,69,70]

In the present study, the maximum number of aerobes were isolated from Grade II ulcers and maximum number of anaerobes were isolated from Grade III. This is in accordance with Colayco CAS et al , Rouhipour N et al.[71,72]The poor defense leads to a rapid increase in the number of microbes, both aerobes and anaerobes with progressive deterioration of diabetic foot wound. Anaerobic organisms flourish in deep seated infections.[73] This indicates that with an increasing grade of ulcer, the anaerobic conditions are produced as a result of increase in the depth of the wound and decrease in peripheral blood flow, leading to higher rate of infections by anaerobes .

Diabetic foot infections are known to be polymicrobial in nature.[1,74,75,76.] In this study, 57.34% were polymicrobial in nature. The interactions of organisms within the microbial mixtures leads to production of virulence factors such as hemolysins, proteases and collagenases that cause inflammation, impede wound

healing and contribute to the chronicity of infection.[77-80]In such mixtures biofilms may form which impede the penetration of antimicrobial agents into the infected site.[81]

Aerobes constituted 77.53% of isolates and anerobes constituted 22.46% of isolates in this study. This is in accordance with the studies conducted in Mumbai and Saudi Arabia , where they reported 71.09% of aerobes, 28.91% of anaerobes, and 87.58% , 11% of anaerobes respectively.[4,82]

In the present study, *Staphylococcus aureus* was the predominant aerobic isolate (22.01%). Our study is in correlation with Wheat et al, Ramani et al Dipali AC et al and Vijaya et al, Raja NS et al who isolated *Staphylococcus aureus* as their major aerobe.[28,32,34,63,83] Other Gram positive cocci isolated were *Enterococci sps* (1.83%), Coagulase negative *Staphylococcus sps* (1.83%), *Streptococcus pyogenes* (0.93%).

Gram negative bacilli were the common organisms next to *Staphylococcus aureus*. Among the 68 of the aerobic gram negative pathogens, *Klebsiella pneumoniae* was the commonest isolate accounting for 21.49% followed by *Pseudomonas aeruginosa* (14.96%). Other isolates in the present study were *Escherichia coli* (12.14%), *Proteus mirabilis* (8.14%), *Proteus vulgaris* (3.73%), *Acinetobacter baumanii* (1.87%), *Citrobacter sps* (0.93). All these isolates has been reported in other studies.[84,85,86,87]

Among anaerobes *Bacteroides fragilis* was the common isolate accounting for 44.45%. This is in accordance to the study conducted by Ramani A et al, Tahawy AT et al, Senneville E et al .[32,35,82] Other gram negative anaerobes isolated are *Prevotella*

intermedia (16.67%), *Prevotella loeschii* (16.67%), *Prevotella sps* (11.12%), *Porphyromonas sps* (5.56%), *Bilophila sps* (5.56%).

Among the gram positive anerobes *Staphylococcus saccharolyticus* was predominant (46.15%) while the study done by Wheat J et al, Louie et al, Lily SY et al, Scher et al showed *Peptostreptococcus spp* (46%) as the predominant isolate.[26,28,88,89] Other gram positive anaerobic cocci isolates were *Schleiferella asaccharolytica* (10.80%), *Finegolda magna* (15.39%), *Micromonas micros* (7.70%).

Anandi *et al* noted 20.8% incidence of MRSA. Shanker *et al* noted 10.3% of MRSA in his study. Dang CN *et al* isolated *Staphylococcus aureus* as the commonest isolate (79%) with MRSA accounting for 30.2%. Ravisekar G et *al* isolated 25 (13.7%) *Staphylococcus aureus* with methicillin resistance in 14 (56%) of them.[6,90,91,92] In this study *Staphylococcus aureus* was the predominant isolate (31.77%) with MRSA accounting for 29.41%. Our study is in correalation with the above studies. All the isolates of MRSA showed 100% susceptibility to Vancomycin. *Staphylococcus aureus* isolates showed 76.47% susceptibility to Methicillin, 55.85% susceptibility to ciprofloxacin and 79.41% to cotrimoxazole.

Enterococcus sps showed 66.67% sensitivity to ampicillin, ciprofloxacin, 33.33% to gentamycin and 100% sensitivity to vancomycin. Coagulase negative *Staphylococcus sps* showed 100% sensitive to methicillin, ciprofloxacin and 50% sensitivity to cotrimoxazole and erythromycin.

Chopdekar found that majority of the isolates of *Pseudomonas aeruginosa* (79%) were resistant to ciprofloxacin, followed by gentamicin (70%), ceftazidime (32%) and cefoperazone (21%).[93] Pappu AK eta al, Nema et al 67 found that the gram

negative bacilli were most sensitive to aminoglycosides and sensitivity to cefotaxime was 63.12%.[94,95]

In our study, *Klebsiella pneumoniae* (19.7%) was the major gram negative aerobe. More than 50% of isolates of *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* were resistant to ciprofloxacin, ceftazidime and amikacin .

Motta et al found the prevalence of ESBL in only 6% amongst *E.coli* isolates.[96]Uma shankari et al described 53% of ESBL producers among *E.coli* and *Klebsiella pneumoniae*.[97]Varaiya et al found that among 43 *E.coli* isolated, ESBL production was observed in 46.51% whereas from 27 isolates of *K.pneumoniae* , ESBL production was found in 44.44%.[98] In this study ESBL producers were found to be 32.35% . Among the isolates 45.45% was Klebsiella *pneumoniae, 27.27% was Pseudomonas aeruginosa, 22.72%* was Escherichia *coli, 4.54% was Proteus mirabilis* (4.54%)

Bansal et al isolated 9% of the total fungal isolates. Among the Candida sps, predominant were *C.tropicalis, C.albicans* followed by Aspergillus *flavus and Aspergillus niger*. Chellan G et al isolated *C.parapsilosis , Trichosporan asahii, C.albicans* and *Aspergillus* species. Heald AH et al, Echkard M et al isolated *C.parapsilosis, C.guillermondi, C.krusei, C.tropicalis, C.kefyr and C.glabrata* .[99,100]In our study *Aspergillus flavus* (0.7%) is isolated.

CONCLUSION

This study has shown that diabetic foot ulcers harbour polymicrobial infection with aerobes, anaerobes and fungal organisms. Culture methods are still economical and gold standard in Microbiology. Isolation of these organisms and their antibiotic susceptibility should be determined to start appropriate antibiotic therapy as early as possible, to overcome the morbidity and mortality associated with the disease.

Methicillin resistant *Staphylococcus aureus* can complicate the already deteriorating ulcer and also increase the cost of therapy. *Klebsiella species* and *Pseudomonas aeruginosa* were resistant to most of the antibiotics used. Thus it is of utmost importance to screen all elderly patients for diabetes and educate them about foot care. Early identification of the risk factors and timely institution of appropriate treatment is indispensable to avoid amputations.

SUMMARY

The present study was conducted in the Department of Microbiology, J.N. medical college, Belgaum for a period of one year from January 2011 to December 2011.

The samples were collected from surgery OPD & from surgery ward at KLE Dr.Prabhakar Kore hospital & medical research centre, Belgaum.

- A total of 75 samples were collected.
- Our cases ranged from 25- 95 years with maximum number of cases in the age group of 58- 67 years.
- Male to Female ratio observed in our study is 3.6: 1.
- The mean duration of diabetes in our patients was 11 years. The maximum number of cases (48%) had diabetes for 10- 14 years in this study.
- Among the total study population, 96% had type 2 diabetes, whereas 4% had type 1 diabetes.
- In the present study, the maximum aerobes per case was isolated from Grade II ulcers and maximum number of anaerobes are from grade III ulcers.
- 75 samples yielded 139 isolates of which 57.34% were polymicrobial in nature and 46.27% were monomicrobial.
- Aerobes constituted for 76.98% of isolates and anerobes constituted 22.30% of isolates and fungus 0.71% in this study. In the present study, *Staphylococcus aureus* was the predominant aerobic isolate (22.01%). Other Gram positive cocci isolated was *Enterococci sps* (1.83%), *Coagulase negative Staphylococcus* sps (1.83%), *Streptococcus pyogenes* (0.93%).

- Among GNB, *Klebsiella pneumonia* was the commonest isolate accounting for 21.49% followed by *Pseudomonas aeruginosa* (14.96%). Other isolates in the present study were *Escherichia coli (*12.14%), *Proteus mirabilis* (8.14%), *Proteus vulgaris* (3.73%), *Acinetobacter sps*(1.87%), *Citrobacter sps*(0.93%).
- Among anaerobes *Bacteroides fragilis* was the common isolate accounting for 44.45%.
- Among the gram positive aerobes *Staphylococcus saccharolyticus* was the predominant (46.15%) isolate.
- MRSA accounted for 29.41% in the present study.
- ESBL accounted for 32.35% in the present study

ANNEXURE - I

PROFORMA

TOPIC: A CROSS SECTIONAL STUDY TO KNOW THE BACTERIAL AND FUNGAL PATHOGENS CAUSING DIABETIC FOOT ULCER

Name: Dr._____

Case no: IP/OP No:

Name: unit/ward

Age: Date:

Sex:

Address:

Chief complaints:

Past H/O:

Treatment H/O:

General Examination:

Systemic examination:

CVS CNS RS P/A

Clinical diagnosis:

Blood sugar: RBS: FBS:

Microbiological investigations:

Macroscopic examination:

Gram stain:

Culture:

Nutrient agar:

Blood agar:

Mac.Conkey agar:

Catalase :

Oxidase:

Motility:

A.Gram positive cocci:

Coagulase:

Slide coagulase:

Tube coagulase:

Antibiogram:

Ampicillin : Erythromycin:

Gentamycin: Amoxyclav:

Ciprofloxacin: Ceftazidime:

Methicillin:

Indole -citrate

H_2S: Mannitol fermentation

Urease -TSI media

Antibiogram:

Amikacin: Pipercillin:

Cefotaxime: Amoxyclav:

Ciprofloxacin: Levofloxacin:

Microaerophilic culture:

5% Blood agar:

Gram stain:

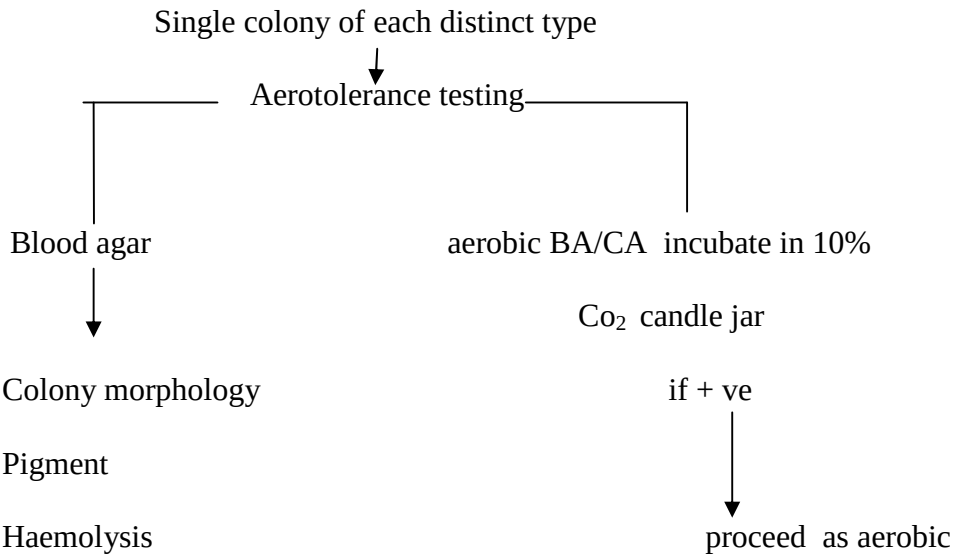
ANAEROBIC CULTURE

Growth on blood agar supplemented

with haemin & vitamin K:

KVLB:

BBE:



Antibiotic identification disc SPS disc

Gram stain of individual colony

Spot indole test

Catalase test

Nitrate test

Susceptibility to Vancomycin

Kanamycin

Colistin

FUNGAL CULTURE:

Wet mount:

Gram stain:

SDA:

Gram staining from colony:

Germ tube test:

ANNEXURE - II

CONSENT FOR PARTICIPATION IN RESEARCH

We are requesting you to enroll yourself in study titled **A CROSS SECTIONAL STUDY TO KNOW THE BACTERIAL AND FUNGAL PATHOGENS IN DIABETIC FOOT ULCER** conducted by **Dr._____,** postgraduate student in microbiology under the guidance of **Dr._____**. at J.N.M.C, Belgaum under KLE Academy of Higher education and Research centre ,Belgaum.

You have been requested to participate in research because you are into the study group. During the study you will be asked some questions and you are supposed to answer to the best of your knowledge.

Your participation in research is voluntary. Your decision whether or not to participate in the study will not affect your relationship with J.N.M.C. If you decide to participate you are free to withdraw at any time.

The purpose of research is to isolate and identify the aerobic anaerobic and fungal pathogens from clinically diagnosed cases of diabetic foot ulcer and to carry out antibiotic susceptibility for aerobic isolates.

PROCEDURE INVOLVED

Microbiological study of the tissue obtained from diabetic foot ulcer will be done to detect the aerobic, anaerobic and fungal pathogens causing diabetic foot ulcer.

RISKS AND BENEFITS

There are no extra risks involved and benefits are to be evaluated.

PRIVACY AND CONFIDENTIALITY

The only people to know that you are a research subject are members of the research team. No information about you or provided by you during research will be disclosed to others without your written permission, except:

1. In emergency to protect your rights and welfare.
2. If required by law.

AUTHORIZATION TO PUBLISH RESULTS

When the results of the research are published or discussed, in a conference, no information will be displayed that would disclose your identity. Any information that is obtained in connection with this study and that can be identified with you will remain confidential.

FINANCIAL INCENTIVES FOR PARTICIPATION

You will not be paid/offered any free gifts for participating in the research. You will not be reimbursed for expenses.

I undersigned _____ have been explained in my vernacular language about the study and participation in the study is voluntary. If I want, I can withdraw at any time. Also I have been given enough time to clear my doubts and rights as study participant.

In case you have any questions about your rights as a study participant you can contact **Dr.AS. Godhi (0831-2471350).**

CONSENT STATEMENT

Signature or left thumb print of participant or legally authorized representative

Participant s name _____ Signature _____

Witness name _____ Signature _____

Experimenters name _____ Signature _____

Date:

Place:

ANNEXURE - III

KEY TO MASTER CHART

- S.aureus - Staphylococcus aureus
- K.pneumoniae - Klebsiella pneumonia
- Ecoli - Escherichia coli
- P.aeruginosa - Pseudomonas aeruginosa
- E - Erythromycin
- Cf - Ciprofloxacin
- Cot - Cotrimoxazole
- A - Amoxicillin
- Ac - Amoxyclav
- M - Methicillin
- Ak - Amikacin
- Caz - Ceftazidime
- Pc - Pipercillin
- Pt - Pipercillin\ tazobactum
- Le -Levofloxacin
- I -Imipenam
- V -Vancomycin
- Ao - Aztreonam
- Te -Teicoplanin
- Lz -Linezolid
- P.intermedia - Prevotella intermedia
- P.loeschii - Prevotela loeschii
- B.fragilis - Bacteroides fragilis
- S.saccharolyticus - S. saccharolyticus
- S.assacharolytica - Schleiferalla asaccharolytica
- M.micros - Micromonas micros
- F.magna - Finegolda magna

MASTER CHART

Sl No.	I. P. No.	Age	Sex	Type of Diabetes	Duration of Diabetes	Grade of Ulcer	Aerobes	Antibiogram of Aerobes isolated																Anaerobes
								E	Cf	Cot	Amx	AC	M	Ak	CAZ	Pc	Le	Pt	I	Ao	V	Lz	Te	
1	396434	50	F	II	8	II	Citrobacter sps	R	R	R		R		R		R	R	R	S					
2	396167	54	M	II	19	II	S.aureus	R	R	S	S	S	S											
3	394185	75	M	III	5	I	S.aureus	R	R	S	R		S											
							P.aeruginosa		R	R	R			R	S		R							
4	1469504	56	M	I	10	II	S.aureus	S	S	S	S	S	S											
5	398498	56	M	II	10	II	K.pneumoniae		S	S				S	S		S							
							P.aeruginosa		S					S	S		S	S						P.intermedia
6	405442	65	M	II	5	II	MRSA	R	I	R	R		R											
							E.coli		R					S	S	R		S						
7	404206	60	M	II	8	III	P.aeruginosa							S	S	S	S	S						
							K.pneumoniae							R	R	R	R		S	S				
8	404976	58	F	II	10	III	A.baumanii		R					R	R	S	R	S						S.saccharolyticus
9	410723	48	M	II	10	III	A.baumanii		S					S	S	S	S	S						S.assacharolytica
10	411502	54	M	II	10	III	S.aureus	R	R	S	S	S	S											M.micros
11	411405	40	M	II	5	III	P.aerug		R					R	R	R	R	R	S	R				Prevotella.sps
12	423234	52	M	II	8	II	MRSA	R	R	S	R	R	R											
13	410803	55	M	II	4	I	K.pneumoniae		R					R	R	R	R	S	S	S				
14	411662	60	F	II	12	III	S.aureus	S	S	S	S	S	S											S.assacharolytica
15	408241	50	M	II	10	III	P.aeruginosa		R					S	S	S	R	S						
16	413343	52	M	II	5	III	MRSA	R	S	S	R	R	R											
							K.pneumoniae		S	S				S	S	S	S	S						S.saccharolyticus
17	412509	56	M	II	10	III	S.aureus	S	R	S	S	S	S											S.saccharolyticus
							K.pneumoniae		R	R				R	R	S	R							
18	413416	64	M	II	10	III	S.aureus	S	S	S	S	S	S											Prev.intermedia
							K.pneumoniae		S	S				S	S	S	S							
19	416362	59	F	II	20	III	S.aureus	S	S	R	S	S	S											S.saccharolyticus
							P.aeruginosa		S					S	S	S	S			S				
20	417599	58	F	II	11	III	MRSA	R	R	R	R	R	R	R							S	S	S	
							E.coli		R					R	R	R	R	S	S					
21	418582	55	F	II	5	II	S.aureus	S	S	S	S	S	S											P.loeschii
22	416007	60	M	II	10	II	S.aureus		S	S	S	S	S											S.assacharolytica
							K.pneumoniae	S	S	S	S	S	S	S	S	S	S	S	S					
23	423349	60	F	II	11	III	E.coli							S	R	R	R	R	S					Bilophila sps
							K.pneumoniae							R	R	R	R	R	S	R				
24	423801	68	M	II	10	III	S.aureus	S	R	S	S	S	S											
25	423966	50	F	II	11	III	K.pneumoniae		R					R	R	R	R	S	R					B.fragilis
							P.aeruginosa		R					R	R	R	R	R	S	R				
26	424190	70	M	II	10	III	P.vulagaris		I					S	R	S	R	S						B.fragilis
							E.coli							S	R	S	R	R						
27	427952	56	M	II	10	II	P.vulgaris			S				S	S	S	S	S						B.fragilis
28	414998	45	M	I	10	III	S.aureus	S	S	S	S	S	S											Prophyromonas sps
29	427135	65	M	II	10	II	E.coli		R	S				S	S	S	S							P.intermedia
30	429106	75	M	II	5	II	K.pneumoniae		R	R				R	R	R	R		S	S				
31	422704	70	M	II	8	II	K.pneumoniae		S					S	R	R	R	R						
32	432664	65	M	II	7	I	P.aeruginosa		R					R	S	S	R	R						
33	425670	45	M	II	4	II	E.coli/K.pneumoniae																	F.magna
34	425813	59	F	II	10	II	P.vulgaris							S	S	S	S	S	S					B.fragilis
35	430123	65	M	II	5	II	K.pneumoniae																	
36	433425	70	M	II	5	I	S.aureus	R	R	S	S	S	S											
37	434665	50	M	II	10	II	MRSA	R	R	R	R	R	R											
							K.pneumoniae		R					R	R	R	S	R						
38	433425	70	M	II	5	II	P.mirabilis		R					R	R	R	R	S	S	R				B.fragilis
							P.aeruginosa		R					R	R	R	R	S	S	S				
~~39~~	~~434980~~	~~50~~	~~M~~	~~II~~	~~10~~	~~II~~	~~P.mirabilis~~		~~R~~					~~R~~	~~R~~	~~R~~	~~R~~	~~S~~	~~S~~	~~R~~				
							K.pneumoniae		R					R	R	R	R	S	S	R				P.loeschii
40	439165	30	M	I	14	III	S.aureus	R	S	R	R	R	R											
41	443863	95	F	II	1	II	S.aureus	S	S	S	S	S	S											
42	446150	50	M	II	10	I	S.aureus	R	S	S	R	R	R											
43	446321	61	M	II	5	I																		F.magna
44	447152	53	M	II	5	II	P.aeruginosa		S					S	S	S	S	S						
							CONS	R	S	R	S	S	S											
45	438417	46	M	II	15	III	P.aeruginosa		R					R	S	S	S	S						S.saccharolyticus
							Enterococcus	S	S	S	S	S									S			
46	453218	71	F	II	10	II	K.pneumoniae		S					S	S	S	S	S						
							E.coli		S					S	S	S	S	S						
47	444993	62	M	II	5	II	P.mirabilis		R					S	R	R	S	S						B.fragilis
							E.coli		R					S	S	S	S	S						
48	445407	48	M	II	4	II	P.aeruginosa		S					S	S	S	S	S						
49	443456	52	M	II	10	II	S.aureus	S	S	S	S	S	S											
							P.aeruginosa		S					S	S	S	S	S						B.fragilus
50	450759	28	F	I	4	I	S.aureus	S	S	S	S	S	S											
							K.pneumoniae		R	S				S	R	S	S							P.loeschii
51	451769	41	M	II	5	II	S.aureus	R	R	S	S	S	S											
							Enterococcus sps	S	S	S	S	S									S			
52	453511	62	M	II	15	III	S.aureus	S	S	S	S	S	S											
							Enterococcus sps	S	S	S	S	S									S			
53	453616	44	M	II	10	II	S.aureus	S	R	S	S	S	S											
54	451836	45	F	II	6	II	CONS	S	S	S	R	R	R											B.fragilis
55	454518	64	F	II	10	II	P.mirabilis	R	R	S	S	S	S											
							S.aureus		R					I	R	S	R	R						
56	451769	41	M	II	10	II	E.coli		R					S	R	R	R	R						
57	456532	62	M	II	10	III	K.pneumoniae		R					S	R	S	S	S						Prevotella sps
58	453977	46	M	II	3	I	K.pneumoniae		R					R	R	R	R	R	S	R				
59	469350	58	F	II	2	I	S.aureus	S	R	R	S	S	S											
60	461940	60	M	II	20	II	P.mirabilis		S					S	S	S	S	S						
							K.pneumoniae		S					S	S	S	S	S						
61	458536	60	M	II	2	I	E.coli		S					S	R	S	S	S						
62	456559	60	M	II	15	III	S.aureus	S	R	S	S	S	S											
							E.coli		R	R				S	S	S	R							
							P.vulgaris		S					S	S	S	S	S						
63	45616	44	M	II	10	II	K.pneumoniae		S					S	S	S	S	S						
64	475150	47	F	II	4	II	S.aureus	R	S	S	S	S	S											
65	458756	71	M	II	15	II	S.aureus	R	R	R	S	S	S											S.saccharolyticus
							E.coli		R	S				S	R	S	S							

66	457004	43	M	II	2	I	K.pneumoniae		R	R				S	R	R	R							
67	458840	56	M	II	5	II	P.mirabilis		S					S	R	S	S	S						S.assacharolytica
							k.pneumoniae		S					S	S	S	S	S						
68	458976	56	M	II	10	I	S.aureus		S	S	S	S	S	S										
							P.mirabilis			S	R			S	R	S	S							
69	419405	60	M	II	1	I	P.aeruginosa		R					R	R	S	R	S	S					
70	473019	52	F	II	10	II	P.aeruginosa		R					R	R	S	R	S	S	R				
71	472455	42	M	II	10	I	P.mirabilis		R					R	R	R	S							
72	472279	56	M	II	1	II	S.aureus	S	S	S	S	S	S											
73	464230	70	M	II	10	I	S.aureus	S	S	S	S	S	S											
74	485184	60	M	II	4	I	S.pyogenes	R	R	S	R	R												
							P.mirabilis		S					S	S	S	S	S						
75	485678	56	M	II	10	I	E.coli		R					S	S	R	R	S						
							P.aeruginosa		S					S	R	S	S	S						

Fungus
A.flavus