CHARACTERIZATION OF MICROBIAL AGENTS IN DIABETIC FOOT INFECTION AND ITS CORRELATION WITH CHRONIC SUBCLINICAL INFLAMMATION

A DISSERTATION SUBMITTED TO THE DEPARTMENT OF MICROBIOLOGY, UNIVERSITY OF DHAKA FOR PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PHILOSOPHY

IN

MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY
UNIVERSITY OF DHAKA
DHAKA-1000
SEPTEMBER, 2014

SUBMITTED BY
EXAMINATION ROLL NO. 03
REGISTRATION NO. 93
SESSION: 2010 - 2011

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M.PHIL.THESIS

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Dedicated to……

My beloved Parents

Certification

It is hereby certified that student bearing **Roll no: 03, Registration no: 93** has carried out the research work entitled "**Characterization of Microbial Agents in Diabetic Foot Infection and its Correlation with Chronic Subclinical Inflammation**" for the partial fulfillment of her **Master of Philosophy** Degree in Microbiology from the University of Dhaka, Bangladesh, under our academic supervision in the Microbial genetics and Bioinformatics Laboratory, Department of Microbiology, University of Dhaka and Bangladesh University of Health Sciences, Darus Salam, Mirpur-Dhaka.

…….

Dr. Md. Anwar Hossain, PhD
Professor
Department of Microbiology
University of Dhaka
Dhaka-1000, Bangladesh

…….

Prof. Dr. Shah Md. Zahurul Haque Asna
Department of Microbiology
Bangladesh University of Health Sciences
Darus Salam, Mirpur
Dhaka-1216, Bangladesh

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Author

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Deceleration

I hereby declare that the whole work submitted as a thesis entitled **"Characterization of Microbial Agents in Diabetic Foot Infections and its Correlation with Chronic Subclinical Inflammation" i**n the Department of Microbiology, University of Dhaka, for partial fulfillment of the requirements for the degree of Master of Philosophy in Microbiology under supervision of **Dr. Md Anwar Hossain , PhD, Professor** , Department of Microbiology, University of Dhaka. and **Professor Dr. Shah Md. Zahurul Haque Asna**, Department of Microbiology Bangladesh University of Health Sciences , Darus Salam, Mirpur-Dhaka.

I, further declare that this thesis or of its part has not been currently submitted for the award of any Degree or Diploma anywhere.

Mousumi Karmaker

Date: 3.9.2014

Abstract

Diabetes mellitus **(DM)** is a metabolic disorder with chronic physiological complication increased risk of developing **Diabetic Foot Infection (DFI)** by 25% in diabetics' patients. The pathophysiological differences between DFI and non-DFI patients may alter microbial compositions in infections. The present study aims at culture based comparative analysis of microbes' colonized in DFI and non-DFI patients in Bangladesh and explore their correlation with chronic subclinical inflammation (CSI). Pus specimens were collected from 67 DFI and 10 non-DFI patients. Most of them were from rural area; belong to lower middle income group and age between 28-75 years. *Enterococcus* spp. (9%), and *Klebsiella* spp. (8%) occurred only in DFI and the other predominant bacteria in DFI over non-DFI patients were *Enterobacter* spp. (22%) and *Bacillus* spp. (12%). In contrast, non-DFI patient's samples predominated *Pseudomonas* spp. (29%) and *Citrobacter* spp. (29%). In both cases, *Staphylococcus* spp. (13%) and *Acinetobacter* spp. (10%) contained same percentages. The statistical calculation (Comparative bacterial significance analysis: at 90% confidence level) revealed that the rate of occurrence of three organisms; *Enterococcus* spp. $|Z|=2.2125$, *Klebsiella* spp. $|Z|= 1.732$, *Bacillus* spp. $|Z| =1.9034$ is significance in DFI patients compare to non-DFI patients. However in non-DFI patients rate of occurrence of *Citrobacter* spp. $|Z|=14.1901$ were significance. Most of the isolates from DFI patients were commonly resistant to cephalosporins (ceftazidime, ceftriaxone, cefuroxime) and monobactam (azteronam) groups of antibiotics. Plasmid profile analysis of the isolates revealed that most of the multidrug resistant (MDR) isolates were plasmid free implicating chromosome mediated MDR properties. A plasmid free, *P. studzeri* isolated from DFI showed resistant to more than 12 groups of antibiotics. DFI patients had comparatively higher C-reactive protein (CRP) values than non-DFI patients; and there were positive correlation with MDR with CRP values. A significant correlation ($p<0.01$) were observed between high serum CRP and infection with MDR organism in DFI patients. In conclusion, this investigation showed that there is a clear demarcation of resistant microbiomes composition between DFI and Non-DFI patients' samples and a positive correlation of CRP values and resistant microbiomes in DFI patients. These results have implication the need for an alternate treatment of DFI patients in Bangladesh.

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Abbreviations

%	Percent
e. g.,	Examplia gratia
et al	And others/ *et alli*
bp	Base pair
kbp	Kilo base pair
kb	Kilo base
mm	Millimeter
g	Gram
µg	Microgram
ml	Milliliter
µl	Microliter
cfu	Colony Forming Unit
min	Minute
sec	Second
°C	Degree Centrigrade
rpm	Rotation per minute
DMC	Dhaka Medical College Hospital
FCH	Foot Care Hospital
DM	Diabetes mellitus
T2DM	Type 2 Diabetes mellitus
T1DM	Type 1 Diabetes mellitus
DFI	Diabetic foot infection
Non-DFI	Non diabetic foot infection
MDR	Multi drug resistant
MH	Muller Hinton
MAC	Mac Conkey
NA	Nutrient Agar
Cefo	Cefotaxime
Cip	Ciprofloxacin
Swab	Swab Stick

Abbreviations

NIDDM	Non-insulin dependent Diabetes Mellitus
IDDM	Insulin Dependent Diabetes Mellitus
GDM	Gestational Diabetes Mellitus
SCI	Sub Clinical Inflammation
GLP	Glucagon Like Peptide
GIP	Glucose-dependent insulin tropic peptide
Spp.	Species
MRSA	Methicillin-resistant *Staphylococcus aureus*
CRP	C reactive Protein
CIT	Citrate
VP	Voges Proskaur
MR	Methyl Red
KIG	Kliger's Iron Agar
H_2S	Hydrogen Sulfide
CAT	Catalase
BIS	Bile esculin
OX	Oxidase
OD	Optical density
PRR	Pattern recognition receptor
TSB	Trypticase soya broth
TCRVB	Total ciprofloxacin resistant viable bacterial count
TCERVB	Total cefotaxime resistant viable bacterial count
TC+CERVB	Total ciprofloxacin + cefotaxime resistant viable bacterial count

Chapter 1

Introduction and Literature Review

1.0 Introduction:

1.1 General introduction:

Diabetes mellitus **(DM)** – the most common non-communicable disease is now become a major health problem causing 5th leading toll of death and increasing globally at an alarming rate (Ridker *et al.*, 2000). It has been declared as an epidemic in developing countries including Bangladesh and there is a strong evidence that Bangladeshi population have a greater susceptibility to DM (Hussain *et al.*, 2005). About 347 million people are suffering from DM worldwide and predicted to be doubled by the year 2025 (Wild *et al*.,2004). DM is a metabolic disorder in which there is an increased level of blood glucose because of insulin deficiency leading much of complications and significant morbidity and mortality (Johar *et al*., 2008). The prevalence of DM in Bangladesh is increasing rapidly leading to complications of chronic diabetes. Main complications associated with DM are cardiovascular disease, retinopathy, nephropathy, neurological, peripheral vascular diseases and infections. Sensory neuropathy, atherosclerotic vascular disease and uncontrolled hyperglycemia are the favoring factors for development of skin and soft tissue infections. Infection can affect any part of the body. But the most common area affected in skin is feet.

Diabetic foot infection (DFI) is one of its most serious complications of DM patients. Every 30 seconds a leg is lost due to diabetes somewhere in the world. DM affects micro vessels which limits the blood supply to superficial and deep structures. Local injuries and improper foot wear further compromise the blood supply in the lower extremities. Non-healing foot ulcer is common in clinical practice. It increase the hospitalization and amputation which in turn results in physical disability of patients with long term devastating consequences in clinical, social, psychological and economic terms (Shaw *et al.*, 2010). A substantial and increasing proportion of the elderly population is at risk of micro and macro vascular complications, including DFI. Diabetic patients have a lifetime risk as high as 25% for developing foot infection (Singh *et al.*, 2005).The exact pathophysiology of DFI is unsettled, but it is now recognized that autonomic neuropathy (with consequent decrease in peripheral circulation), decreased immunity and congenital biochemical environment created by hyperglycemia (leading to secondary bacterial infections) interact to develop the moist gangrene which is the main reason of frequent amputation in such patients. (Lipsky *et al.*, 2004).

(Weigelt *et al*., 2009) showed that chronic subclinical inflammations (CSI) significantly associated with the development of acute infection in diabetic foot. CSI is now implicated with a number of macro and micro vascular complications of diabetes and its association with DFI may partly explain why this condition frequently consist with other systematic complications of DM. It is now also recognized that there is substantial genetic contribution in peripheral neuropathy as well as in other basic defects of diabetic foot which creates substantial heterogeneity from population to population. People with diabetes have infections that are more severe and take longer to cure than equivalent infections in other non-diabetic people (Armstrong *et al.*, 2007). The primary cause of DFI is microbial agents and their early diagnosis is essential to use the appropriate antimicrobial therapy. There are pathophysiological differences between DFI patients and non-DFI ones due to which organisms causing infections and CSI are more aggressive in DFI patients than in non-DFI ones. There are also differences in microbial population in foot lesions of DFI patients compared to that of non-DFI ones. Thus, it is important to investigate the types of microbial agents and their antibiogram in foot lesions of different populations. Common organisms reported from DFI are mainly *Staphylococcus* spp. and *Enterococcus* spp. arising from patients own body. Extensive tissue destruction and poor blood circulation are result of infection by *Pseudomonous* spp., *Enterococcus* spp., *Proteus* spp., *Acinetobacter* spp. group of bacteria (Chincholikar *et al*., 2002). The microbial agents involved in DFI are usually multi drug resistant (MDR). Early diagnosis of microbial infection and its magnitude are essential to use the appropriate antimicrobial therapy (Frykberg *et al.*, 2000) . Once the infection is developed in DFI patients, it is difficult to treat because of impaired micro vascular circulation to the lower limb, which limits the access of phagocytic cells and poor delivery of antibiotics in the infected sites (Alavi *et al*., 2007). Besides pathophysiological conditions, demography, nutritional and immunological status of patients further complicated the composition of microbiomes associated with DFI. A thorough knowledge on the association of microbiomes with DFI patients is important for the containment of the infection. So far, no study has been reported in this issue on any Bangladeshi subjects. The study is expected to generate valuable information which will be helpful in the management and prevention of DFI in our population.

1.2 Literature Review

Diabetes mellitus, or simply Diabetes, is a group of metabolic diseases in which a person has high blood sugar, either because the pancreas does not produce enough insulin, or because cells do not respond to the insulin that is produced.

There are three main types of Diabetes mellitus (DM):

- Type1 Diabetes Mellitus
- Type2 Diabetes Mellitus
- Gestational Diabetes Mellitus

1.2.1. Type 1 Diabetes mellitus (T1DM)

Type 1 Diabetes mellitus (T1DM) known as insulin dependent DM is an auto-immune disease where the body's immune system destroys the insulin producing beta cells in the pancreas (Daneman *et al*., 2006). The pathophysiology T1DM is characterized by loss of the insulin-producing Beta cell of the Islets of Langerhans in the pancreas, leading to insulin deficiency. This type of DM is also known as juvenile-onset DM and accounts for 10-15% of all people with the disease. (Campbell *et al*., 2011). The incidence of T1DM has been increasing by about 3% per year (Aanstoot *et al*., 2007).

1.2.2. Type 2 Diabetes mellitus (T2DM)

T2DM results from insulin resistance, a condition in which cells fail to use insulin properly, sometimes combined with an absolute insulin deficiency. This type of DM is non-insulin dependent Diabetes mellitus (NIDDM) or "adult-onset Diabetes" (Landon *et al*., 2010) . The defective responsiveness of body tissues to insulin is believed to involve the insulin receptor and reduced insulin sensitivity. This functional islet incompetence is the main quantitative determinant of hyperglycemia (Ferrannini *et al.*, 2005) and pancreatic a-cells hyper secrete glucagon, further promoting hepatic glucose production abnormalities in the incretion system (represented by the gut hormones, glucagon-like peptide 1 (GLP-1), and glucose-dependent insulin tropic peptide (GIP) are also found in T2DM. (Nauck *et al*., 2009). T2DM is increasing epidemic in Asia and more than 60% of the world's population with DM will come from Asia, because it remains the world's most populous region (Chan *et al.*, 2009) .

T2DM is now diagnosed as frequently as T1DM in teenagers in the United States (Gardner *et al*., 2011). T2DM patients in Bangladesh were more than 7 million in 2011.This number will expected to rise 11.1million by the year 2030 (Yamada *et al.*,2013).

1.2.3. Gestational Diabetes Mellitus (GDM)

GDM occurs in about 2%–5% of all pregnancies and may improve or disappear after delivery. Gestational Diabetes mellitus (GDM) resembles T2DM in several respects, involving a combination of relatively inadequate insulin secretion and responsiveness. During the late phase pregnancies, there is an increase in hormones such as cortisol, prolactin, progesterone and human placental lactogen which leads to a state of relative insulin resistance, possibly via a post receptor defect in the cells.

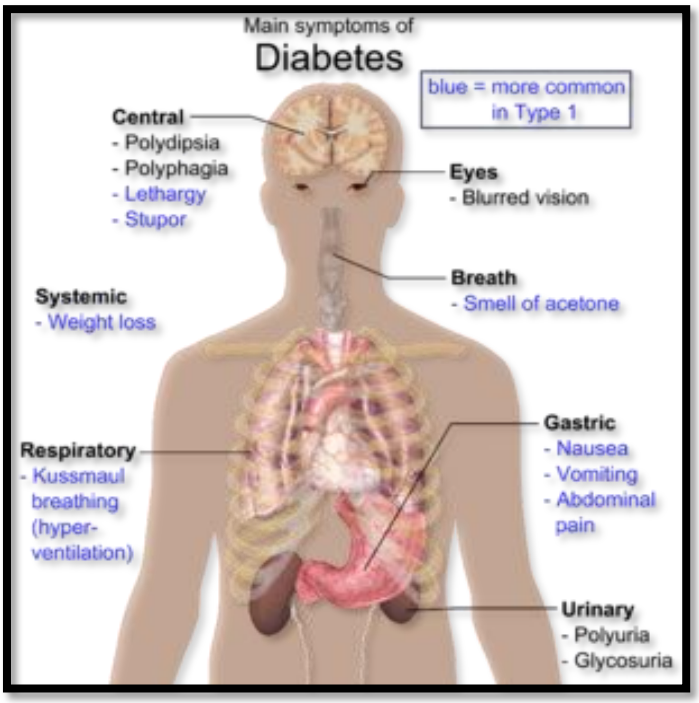


Figure1.0: Sign symptom of Diabetes mellitus. (Wikipedia)

1.2.4. Complications of Diabetes mellitus

All forms of DM increase the risk of long-term complications. These typically develop after many years (10–20). The major long-term complications relate to damage to blood vessels. DM doubles the threat of Cardio vascular disease mainly atherosclerosis, ischemic heart disease, stroke and peripheral vascular disease. DM also causes retinopathy and effects on the nervous system most usually causing numbness, tingling and pain in the feet. Together with vascular disease in the legs, neuropathy contributes to the risk of diabetes foot problem (Diabetes foot ulcer) that can be difficult to treat and occasionally require amputation (Pittet *et al.,* 1999).

1.2.5. Diabetic Foot infection (DFI):

Diabetic foot infections (DFI) are among the most serious and frequent complications encountered in patients with DM (Shea *et al*., 1999). Most DFI begin with a wound and once an infection occurs, the risk of hospitalization and amputation increases dramatically. (Young *et al.*, 1993) Foot infections are common in patients with DM and are associated with high morbidity and risk of lower extremity amputation. More than 85% of lower amputations in patients with DM are preceded by foot infection (Pecoraro *et al.*, 1990). DFI are classified as mild, moderate, or severe. Every year more than million DFI patients require foot amputations.

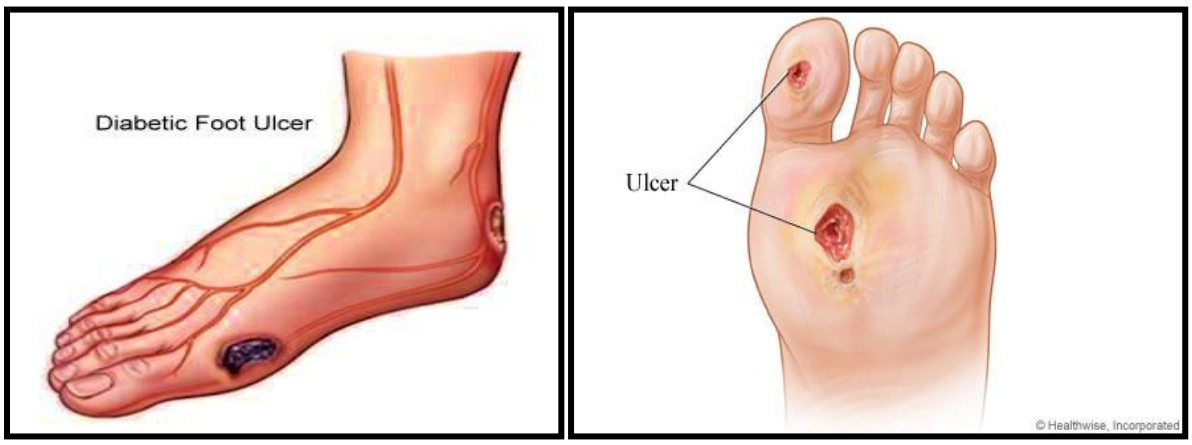


Fig 1.1: Diabetic patients with foot infection.(Eddy *et al*., 2010)

There are several well accepted predisposing factors that place patients with DM at high risk for a lower extremity amputation. The most common components in the causal pathway to limb loose include peripheral neuropathy, ulceration, infection and peripheral vascular disease (Armstrong *et al.*, 2007). Numerous factors related to DM can impair Wound healing, including hypoxia (inadequate oxygen delivered to the wound) infection, nutrition deficiencies, and the disease itself (Lavery *et al.*, 2006). Unstable blood sugar and hypoxia from poor circulation may impair the ability of white blood cells to destroy pathogenic bacteria increasing risk of infection (Stadelmann *et al.*, 1998).

1.2.6. Severity of Diabetic foot Infection (DFI)

1.2.6. a. Mild infection: The symptom of Mild foot infection is Presence of 2 or more manifestations of inflammation (purulence, or erythema, pain, tenderness, warmth or induration), but any cellulitis extends to 2 or less cm around the ulcer and infection is limited to the skin or superficial subcutaneous tissues ; no other local complications or systemic illness.

1.2.6. b. Moderate infection: In moderate infection patient is systemically well and metabolically stable but has 1 or more of the following characteristics: cellulitis extending greater than 2 cm, lymphangitic streaking, spread beneath the superficial fascia, deep tissue abscess, and involvement of muscle tendon, joint or bone.

1.2.6. c. Severe infection: In severe cases patient has systemic toxicity or metabolic instability (e.g. fever, rigors, tachycardia, hypotension, confusion, vomiting, leucocytosis, severe hyperglycaemia) (Lipsky *et al.*, 2004)

1.2.7. Risk Factor of developing DFI:

DM patients reduced sensations of feet and the nerves that take messages of sensation and pain from the feet are commonly affected. That will increase the risk of developing foot infection. DM is poorly controlled is one of the reasons for developing foot infection. DM causes of developing narrowing of the arteries (peripheral vascular disease). This can reduce the blood flow to various parts of the body. The arteries in the legs are quite commonly affected. This can cause a reduced blood supply (poor circulation) to the feet. Skin with a poor blood supply does not heal as well as normal and is more likely to be damaged.

Therefore, if patient get a minor cut or injury, it may take longer to heal and be prone to becoming worse and developing into an ulcer.

1.2.8. Causes of DFI:

- Several factors predispose diabetic patients to developing DFI, including neuropathy, vasculopathy and immunopathy. Peripheral neuropathy occurs early in the pathogenesis of diabetic foot complications and is considered the most prominent risk factor for DFI.
- DM patients with impaired protective sensation and altered pain response are vulnerable to trauma and extrinsic forces from ill-fitting shoe wear. Motor neuropathy causes muscle weakness and intrinsic muscle imbalance leading to digital deformities such as hammered or clawed toes. This results in elevated plantar pressure due to metatarsophalangeal joint instability.
- Autonomic dysfunction leads to changes in micro vascular blood flow and arteriolar-venous shunting, diminishing the effectiveness of perfusion and elevating skin temperatures. With the loss of sweat and oil gland function, the diabetic foot becomes dry and keratinized which cracks and fissures more easily, leading to a portal for infection.

1.2.9. Pathophysiology of DFI:

Patients with DM are particularly susceptible to foot infection primarily because of neuropathy, vascular insufficiency, and diminished neutrophil function. Peripheral neuropathy has a central role in the development of a foot infection and it occurs in about 30 to 50 % of patients with diabetes.

- With damage to the nervous system, a person with DM may not be able to feel his or her feet properly. Normal sweat secretion and oil production that lubricates the skin of the foot is impaired. These factors together can lead to abnormal pressure on the skin, bones, and joints of the foot during walking and can lead to breakdown of the skin of the foot. Sores may develop.
- Damage to blood vessels and impairment of the immune system from diabetes makes it difficult to heal these wounds. Bacterial infection of the skin, connective tissues, muscles, and bones can then occur. These infections can develop into severe

gangrene. Because of the poor blood flow in the infected site, antibiotics cannot get to the site of the infection easily. Often, the only treatment for this is amputation of the foot or leg. If the infection spreads to the bloodstream, this process can be life-threatening.

- DFI results from a complex interaction of a number of risk factors. Neuropathy (with alterations in motor, sensation, and autonomic functions) plays the central role and causes ulcerations due to trauma or excessive pressure in a deformed foot without protective sensibility (Lazarus *et al.*, 1994). Once the protective layer of skin is broken, deep tissues are exposed to bacterial colonization. Infection is facilitated by DM-related immunological deficits, especially in terms of neutrophils, and rapidly progresses to the deep tissues (Robert *et al*., 2006).
- The faulty healing response is additionally affected due to the wound hypoxia caused by the microvascular and macrovascular conditions within the DM patients (Yamasaki *et al.*, 2010;Kashiwagi *et al*., 2010) and resistance to infections which further leads to gangrenous ulcer with lower limb amputations (Gadepalli *et al.*, 2006).

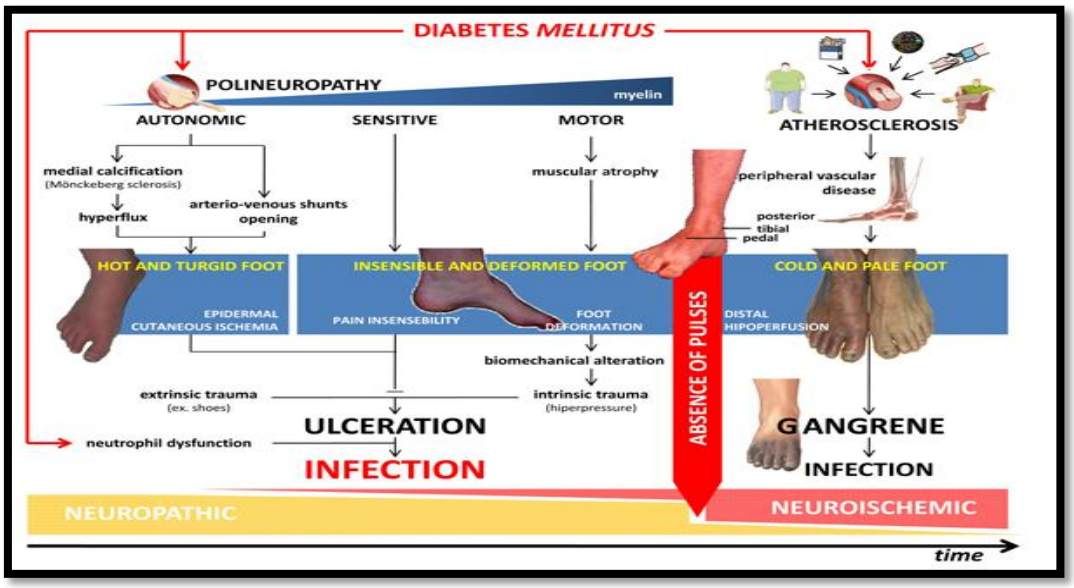


Figure 1.2: Pathophysiology of DFI (Mendes *et al*., 2012)

1.3 Common Microbial agent in DFI:

The most common pathogens in acute, previously untreated, polymicrobial infection were observed in 40% of the patients. Gram-negative bacteria were more commonly isolated compared with Gram-positive bacteria (Hefni *et al.*, 2013)

1.3.1. Gram positive Organisms

Most frequently found Gram positive Organisms were:

- *Staphylococcus aureus,*
- *Streptococcus pyogenes,*
- *Staphylococcus epidermidis*
- *Methicillin-resistant S. aureus* ,
- *Enterococcus* spp.
- *Bacillus* spp.

1.3.2 .Gram-negative organisms

Most common Gram-negative organisms were:

- *Pseudomonas* spp.
- *Klebsiella* spp.
- *Acinetobacter* spp.
- *Enterobacter* spp.
- *Escherichia coli*
- *Proteus* spp.
- *Citrobacter* spp.

1.3.1.1 Gram positive Organism:

Gram positive aerobes *Staphyloccoci* are more prevalent in DFI and shows multi drug resistance (MDR) phenotype. The most common pathogen isolated from the infected wounds was *S. aureus* in approximately 46% cases (Salahuddin *et al.*, 2013).The organisms that occur on foot infections are generally *S. aureus* and *Streptococcus pyogens* arising from the patients' own skin. Research has shown that 86% of ulcer for most research *S. aureus* and coagulase – negative staphylococci has been the predominant organisms isolated from chronic wounds (Howell *et al.*, 2005) Methicillin-resistant *Staphylococcus aureus* (MRSA) is a more common pathogen in patients who have been previously hospitalized or who have recently received antibiotic therapy. MRSA infection can also occur in the absence of risk factors because of the increasing prevalence of MRSA in the community (Dowd *et al.*, 2008).

Other aerobic bacteria or facultative anaerobic bacteria isolated from chronic wounds include *S. epidermidis* (Brook *et al.*, 1998). *S. epidermidis*, a non-pathogenic member of common cutaneous microbial flora, expresses few virulence factors under normal condition (Iwatsuki *et al.*, 2006). Coagulase-negative *Staphylococcus* spp., such as *S. epidermidis*, are responsible for the majority of chronic osteomyelitis associated with orthopedic implants. (Ciampolini *et al*.,2000). Studies with microbiological examination of bone samples have shown isolation rates of *S. epidermidis* between 10% and 50% (Senneville *et al.*, 2006). *S. aureus* and *Enterococcus* spp. were susceptible mostly to VA, LEV, and CIP with varying susceptibility to DO and 67% of *S. aureus* were methicillin-resistant (Esma *et al.*, 2012).

Most staphylococcus infections that are visible usually have a reddish, swollen, and tender area at the site of infection. Often the site secretes pus or has some crusty covering with drainage. Sites of infection can be small like a pimple or large like a carbuncle. Cellulitis often shows redness and swelling without pus, but impetigo shows a crusty weeping rash with an occasional blister. Scalded skin syndrome shows extensive skin redness with bullae (fluid- or pus-filled blisters). *S. aureus* strains first infect the skin and invade damaged skin Sometimes the infections are relatively limited but other times they may spread to other skin areas. Unfortunately, these bacteria can reach the bloodstream (bacteremia) and end up in many different body sites, causing infections (wound infections, abscesses) that may severely harm or kill the infected person.

S. aureus strains also produce enzymes and exotoxins that cause or increase the severity of certain diseases. Such diseases include food poisoning, septic shock, toxic shock syndrome, and scalded skin syndrome. *S. epidermidis* cause foot infections in people whose immune system is suppressed.

E. faecalis and *E. faecium* are most common, clinically relevant intestinal species previously classified as Group D streptococci: Possess a Group D specific cell wall carbohydrate (glycerol teichoic acid linked to cytoplasmic membrane) Non-motile Gram-positive cocci in pairs or short chains: Difficult to distinguish from *S. pneumonia.*

Enterococcus is a genus of lactic acid bacteria of the phylum farmicutes. *Enterococci* are gram positive cocci that often occur in pairs (diplococci) or short chains, and difficult to distinguish from streptococci on physical characteristics alone. Two species are common commensal organisms in the intestines of humans: *E. faecalis* (90-95%) and *E.faecium* (5-10%). In DFI patients *Enterococcus* spp. was found highly resistance to OX, P, AMC antibiotics. Broad range multidrug antibiotic resistance: Mediated by Plasmids those are "promiscuously" passage between bacteria. Rare clusters of infections occur with other species, including *E. casseliflavus*, *E gallinarum* and *E. raffinosus*. *Enterococcus* spp. colonizes and secretes fibrinolytic protein and carbohydrate factors which regulate adherence, and inhibit competitive bacteria (Sood *et al*., 2008).

Bacillus species are disseminated in the natural environment (source is mainly soil, grass) and some of these species act as an opportunistic pathogen. *Bacillus* is a gram positive, rod-shaped bacteria and a member of the phylum farmicutes. *Bacillus* species can be obligate aerobes (oxygen reliant), or facultative anaerobes (having the ability to be aerobic or anaerobic). *Bacillus* spp. is a common laboratory contaminants and a normal flora mostly in patients hospitalized for long time. Spores of *Bacillus* spp. are resistance to ultraviolet ray, disinfectants and sterilizing agents therefore they are difficult to destroy. Spore can remain viable for many years and are virulent. *Bacillus* species infections have been associated with immunosuppression, surgical wounds infection, burns, and parenteral drug abuse. They will test positive for the enzyme catalase when there has been oxygen used or present. Ubiquitous in nature, *Bacillus* includes both free-living (non-parasitic) and parasitic pathogenic species. Under stressful environmental conditions, the bacteria can produce oval endospores that are not true spores but which the bacteria can reduce themselves to and remain in a dormant state for very long periods. These characteristics originally defined the genus, but not all such

species are closely related, and many have been moved to other genera of firmicutes. Many species of *Bacillus* can produce copious amounts of enzymes which are made use of in different industries. *Bacillus circulans* causes fatal sepsis (infection in Blood) and also damage vital organ liver, heart, kidney and therefore can be considered as a major human threat (Turnbull *et al*., 1996).

1.3.2. Gram Negative Organism:

Among gram negative organism *P. aeruginosa* is another frequently identified organism in more than 40% of ulcers (Bowler *et al.*, 2001), Bacteriological study of DFI in an urban hospital of Dhaka City (Bangladesh) found gram negative organism was most frequently isolated (80%) bacteria. *Pseudomonas* (48%) and *Proteus* spp. (33%) was the most common Gram negative organisms isolates, and the other Gram negative isolates were *Klebsiella* spp. (28%), *E. coli* (14.7%), *Acinetobacter* spp. (6.6%), and *Citrobacter* spp. (5.3%) (Samir *et al*., 2009). Polymicrobial infection was observed in 39.1% of the patients. High prevalence of MDR pathogens were observed. 80% of the isolates belonging to *Enterobacteriaceae* were producing ESBL. The members of *Enterobacteriaceae* as well as *Pseudomonas* spp. and *Acinetobacter* spp. were found to be susceptible mainly to IPM, CIP and AK (Islam *et al*., 2012).

The organisms in the genus *Pseudomonas* are mostly free-living bacteria widely distributed in soil and water. For the most part they are found wherever organic matter is decomposing. These are morphologically resembled enteric bacilli and vibrios. However, biochemically they are quite different in their metabolism and composition of cytoplasmic membrane. They are strict aerobes and motile by peritrichous flagella. Although there are over 200 species of *Pseudomonas* only three are known to be pathogenic for man, *P. aruginosa* was found 25 % in DFI patients. When this organism is present in the body in small numbers, as is normal, the synthesis and release of toxins is insignificant.

*Pseudomona*s spp. becomes dominant organism in some part of the body, produced toxin and that causes distinct pathologic change (Raja *et al*., 2007). Some of these toxins are:

- **Pyocyanin** - water soluble bluish-green pigment, which interferes with the terminal electron transfer system by complexing with flavoproteins.
- **Lecithinase** - an enzyme which degrades lecithin, a phospholipid. Lecithin occurs in cell membranes of various body cell types, including RBC's. The result of lecithinase action is cell lysis.
- **Collagenase** - an enzyme which degrades collagen, a scleroprotein in connective tissue of the body (especially in tendons, hair, nails). Collagen is a fibrous protein which is insoluble in water and resistant to enzyme attack because of its three-dimensional configuration.
- **Lipase** - an enzyme which splits fatty acids from lipids leaving glycerol.
- **Hemolysin** - lysis red blood cells.
- **Fluorescein** - a water soluble greenish-yellow pigment which fluoresces under UV light.

Klebsiella is well known to most clinicians as a cause of community-acquired bacterial pneumonia, *Klebsiella* spp. primarily attack immune compromised individuals who are hospitalized and suffer from severe underlying diseases such as DM or chronic pulmonary obstruction. The genus is defined as containing gram-negative, non motile, usually encapsulated rod-shaped bacteria of the family *Enterobacteriaceae*, which produce lysine decarboxylase but not ornithine decarboxylase and are generally positive in the Voges-Proskauer test (Richard *et al*., 1998). *Klebsiella* species are usually identified and differentiated according to their biochemical reactions. and was found respectively 20% and 15% *Klebsiella pneumoniae* species in DFI patients (Sivaraman *et al*., 2005).

Acinetobacter is a genus of gram negative bacteria belonging to the wider class of gamma proteobacter. *Acinetobacter* species are non-motile, oxidase negative. (Manikal *et al*., 2011) reveled *Acinetobacter* are a key source of infection in debilitated patients in the hospital, in particular the species *Acinetobacter boumannai*. *Acinetobacter boumannai* is an important nosocomial pathogen. (Abbo *et al.*, 2005) revealed that 88% MDR *Acinetobacter boumannai* were hospital acquired. *Acinetobacter baumannii* (33%) was isolated from DFI patients, and

among them 26% were found resistance to Carbapenem (IPM) groups of antibiotics in the study of (Rasha *et al.*, 2014).

Enterobacter is a genus of common gram negative, facultatively anaerobic, rod-shaped, non-spore-forming bacteria of the family *Enterobacteriaceae*. Several strains of these bacteria are pathogenic and cause opportunistic infections in immune compromised (usually hospitalized) hosts and in those who are on mechanical ventilation. The urinary and respiratory tracts are the most common sites of infection (Cabral *et al*., 2010). The genus *Enterobacter* is a member of the coliform group of bacteria. It does not belong to the fecal coliforms (or thermotolerant coliforms) group of bacteria, as does *Escherichia coli,* because it is incapable of growth at 44.5°C in the presence of bile salts.

Proteus **spp.** is a genus of gram-negative proteobacteria. Proteus bacilli are widely distributed in nature as saprophytes, being found in decomposing animal matter, in sewage, in manure soil, and in human .They are opportunistic pathogens, commonly responsible for urinary and septic infections, often nosocomial. Three species *P. vulgaris, P. mirabilis* and *P. penneri* are opportunistic human pathogen. *P. mirabilis* causes wound and urinary tract infections. (Raja *et al*., 2007) was found 28% *Proteus spp* in DFI patients. Most strains of *P. mirabilis* are sensitive to penicillin (AMP,P) and cephalosporin (CAZ,CRO,CXM). *P. vulgaris* is not sensitive to these antibiotics. However, this organism is isolated less often in the laboratory and usually only targets immunosuppressed individuals (Adebayo *et al*., 2009). *P. vulgaris* occurs naturally in the intestines of humans and a wide variety of animals, and in manure, soil, and polluted waters. *P. mirabilis*, once attached to the urinary tract, infects the kidney more commonly than *E. coli*. *P. mirabilis* is often found as a free-living organism in soil and water. About 10–15% of Kidney stones are caused by alkalization of the urine by the action of the urease enzyme (which splits urea into ammonia and carbon dioxide) of *Proteus* spp.

1.4. Association of Foot infection & Subclinical Inflammation:

Subclinical inflammation (SCI) represents a risk factor of T2DM and several DM complications, but data on Diabetic neuropathies are scarce. SCI is associated with DM polyneuropathy and neuropathic impairments. This association appears rather specific because only certain immune mediators and impairments are involved (Weigelt *et al.*, 2009).

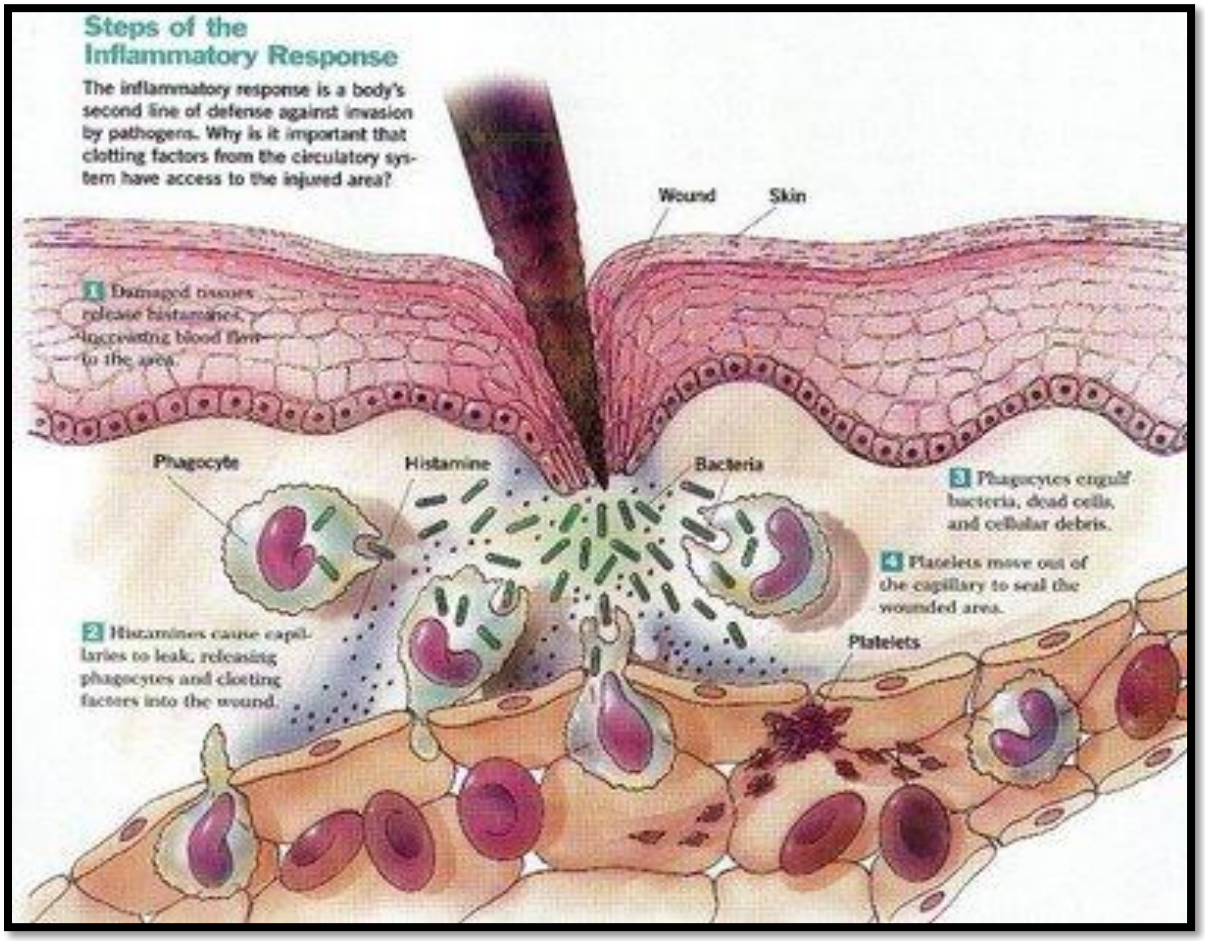


Figure1.3: Steps of Inflammatory response of foot infection (Jhonson *et al.*, 2014).

Diabetic foot is characterized by definite inflammatory reactions one of DM complications. Diabetic foot syndrome becomes more and more important as a major diabetes complication. The lifetime risk of a DM patient for development of a chronic foot wound has been estimated to reach 15–25% (Singh *et al*., 2005) and, despite considerable international efforts, foot ulcers continue to be responsible for a high number of lower-limb amputations that are associated with a substantial decrease in quality of life and increased risk of mortality.

The major risk factors for foot ulcer are Diabetes poly neuropathy and peripheral arterial disease (Paton *et al.*, 2014). Interestingly, data on the relevance of systemic inflammation are very scarce in this context, although low-grade immune activation represents an important risk factor not only for the development of T2DM but also for several macro vascular and micro vascular complications (neuropathy and nephropathy)(Burtey *et al*., 2008). The status of the immune system may be relevant at several stages in the development of chronic wounds. Immune activation may precede the incidence of a DFI in the same way that it precedes the manifestation of T2DM and coronary heart disease (Shai *et al.*, 2005).

Because pro and anti-inflammatory processes are crucial in the different phases of wound healing, it is conceivable that disturbances of the immune system interfere with tissue homeostasis and wound healing after the manifestation of ulcers and lead to the chronic, non-healing wounds that are characteristic of Diabetic foot syndrome. Given the surprising paucity of data on the role of systemic inflammation in DFI, we evaluated the association between foot ulcers and immune status in a cross-sectional study in DM patients with and without foot ulcers by measuring a range of immune mediators (acute-phase proteins, cytokines, and chemokines) representing different aspects of the immune system.

1.5. Subclinical Inflammation & C-reactive protein:

C-reactive protein (CRP) is a protein found in the blood, the levels of which rise in response to inflammation (i.e. CRP is an acute phase protein).Its physiological role is to bind to phosphocholine expressed on the surface of dead or dying cells (and some types of bacteria) in order to activate the complement system via the C1Q complex (Szalai *et al.*, 2000). CRP is synthesized by the liver in response to factors released by macrophages and fat cells (adiposites). CRP was the first pattern recognition receptor (PRR) to be identified. CRP levels were considered to be risk factors for amputation (Ahmed *et al.*, 2012).

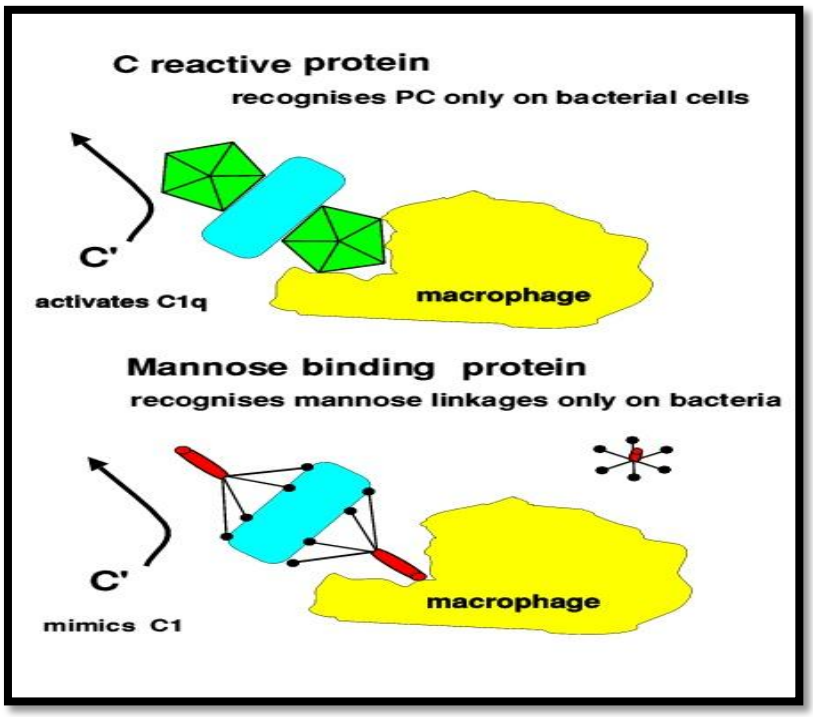


Figure1.4: Activation of C reactive protein in response to bacterial infection (Mehaboob *et al*., 2012)

CRP is a protein found in the blood that indicates "inflammation". It is produced in the liver and during infection, and with some forms of cancer and inflammatory diseases (rheumatoid arthritis, lupus, foot infection), it can be elevated in a blood test. CRP can also be elevated when there is inflammation in the arteries of the heart and is a "marker" for coronary artery disease.

1.5.1 Co-relation of Serum C-reactive protein concentration with Diabetic Neuropathy, foot ulcer and Bacteria Growth:

Diabetic foot is characterized by definite inflammatory reaction. DM and its complications as foot ulcers may develop as a result of peripheral neuropathy and ischemia. (Herder *et al.*, 2009) showed an association between that SCI with Diabetic polyneuropathy and neuropathic impairments. This association appears rather specific because only certain immune mediators and impairments are involved. They found an association between high level of CRP and IL-6 (interleukin-6). (Ammal *et al.*, 2012) found a strong correlation between the levels of inflammatory marker CRP level with DFI and DM subjects with no foot ulcer. They measured CRP levels by enzyme-linked immune sorbent assay (ELISA) in the serum samples from 31 patients with DFI and compared to 20 DM individuals with no foot ulcers and found CRP levels were significantly higher in the serum of the patients with DFI as compared with DM subjects with no foot ulcers. (Shler *et al.*,2012) found a correlation between serum CRP level in DFI patients with gram negative bacterial Isolates. Some studies showed that among 90 DFI patients 41% were infected with gram negative organism and their CRP level was found significantly higher than those who were infected with gram positive organism; and showed further gram negative bacteria (*E. coli, Pseudomonas* spp, *Enterobacter clocaea, Citrobacter frundi, Acinetobacter baumanii*) were most commnly related with elevation of serum CRP in DFI patients (Abe *et al.*, 2010; Shler *et al.*, 2012).

1.6. Aims & Objectives:

Diabetes mellitus (DM) a metabolic disorder and become epidemic all over the world. The situation of Diabetic in Bangladesh is getting worse. DFI is the notorious complication of DM, occurs about 25% diabetic populations at advance chronic condition of the disease. The infection associated with resistant polymicrobes. Furthermore, besides pathophysiological conditions, demography, nutritional and immunological status of patients further complicated the composition of the microbiomes associated with DFI. A thorough knowledge on the association of microbiomes and their resistance properties with DFI patients is important for the containment of the infection. The general aim of the present study is to compare the microbiomes present in DFI and Non-DFI patients, characterization of the microbial agents in DFI and to explore their association with CSI. The investigation has direct implication in treatment of DFI patients.

The specific objectives of the study are as follows:

- To investigate relative frequency of bacteriomes associated in pus samples from DFI as well as non-DFI patients and their statistical significance;
- To investigate the association of CSI with the major types of microbes present in DFI and Non-DFI patients, and the correlation among CRP, microbiomes and resistant pattern in DFI patients; and
- To investigate the antibiogram of bacteriomes associated with DFI and Non-DFI;

Chapter 2

Materials and Methods

2.0. Materials and Methods

This investigation focused on bacteriomes associated with DFI and non-DFI patients. And also originate a correlation of organism growth with CSI (serum CRP level). The overall study design of the investigation is depicted in figure 2. All media compositions, chemicals, reagents and apparatus used in this study are given in the (Appendix I & II).

Work Flow:

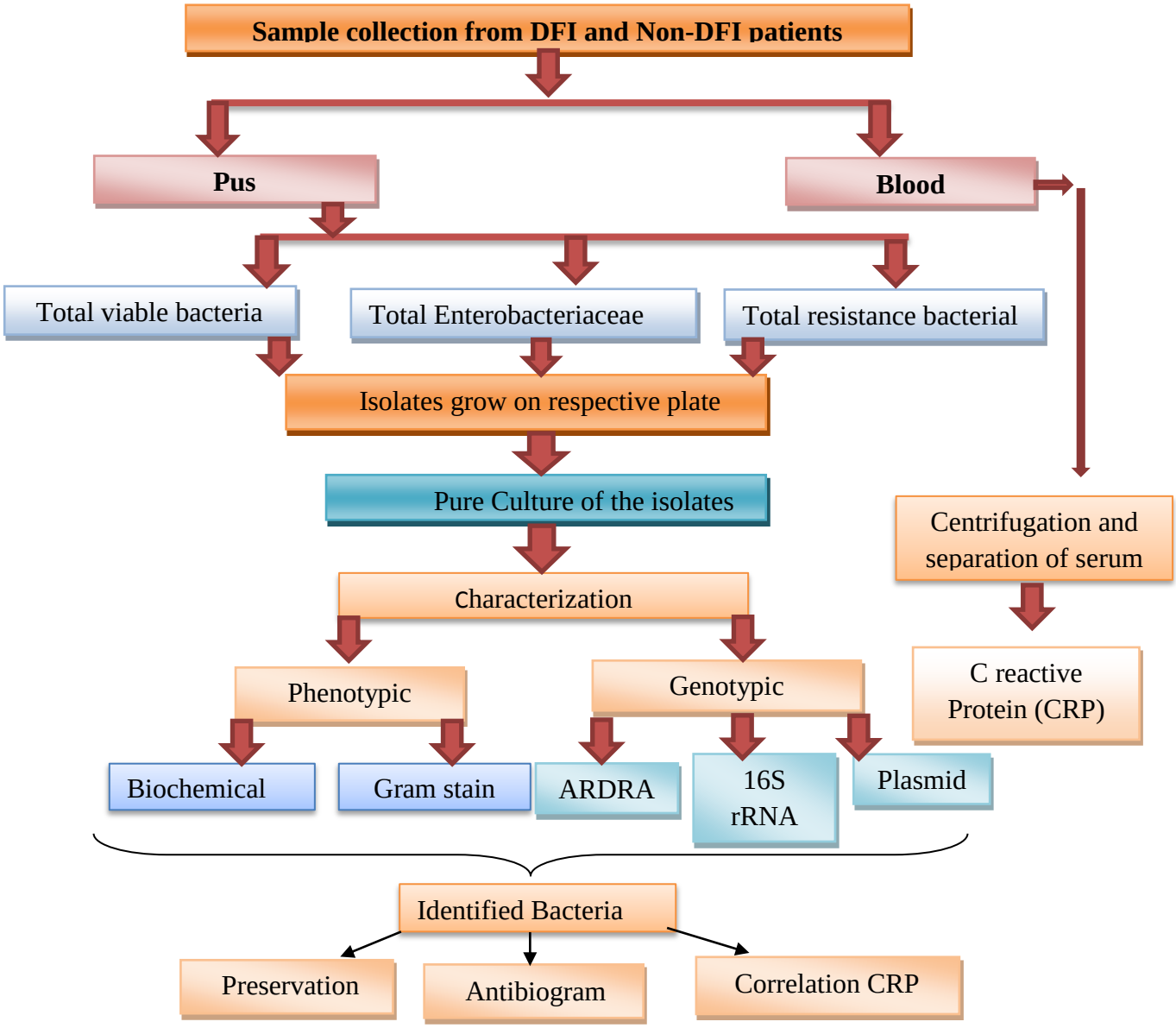


Figure 2.0: Diagrammatic presentation of workflow of investigation.

2.1. Study place and period:

Pus specimens were collected from the infected site of foot wounds of 67 DFI and 10 non-DFI patients from the Diabetic Foot Care Hospital (DFCH) and Dhaka Medical College Hospital (DMCH) respectively. The samples were collected from August, 2012 to January, 2014.

Ethical approval

This study was reviewed by ethics committee of the Diabetic Association of Bangladesh (BADAS) **(reference number BADAS-ERC/EC/14/00137: Appendix III)**

2.1.1. Study Subjects:

2.1.1. a. *Diabetic Patients with infected foot lesion.*
The study was conducted with DM patients, having infected foot lesion, admitted in DFCH. A total of 67 pus samples were collected from foot lesions of these patients.

2.1.1. b. *Non- diabetic patients with infected foot lesion*:
These study subjects were non-diabetic but they were admitted in DMCH with Foot infection. These patients were used as control. Status of Diabetic and non-Diabetic was determined by estimation of Blood sugar level +HbA_1C of the patients.

2.2 Patients History taking:
A questionnaire was used for collection of data by face-to-face interview. Data on age, sex, livelihood, occupational status, clinical features, type of water used, socio-economic status, use of shoes, type of antibiotic used, etc. were collected. The details of questionnaire are given in Appendix II.

2.3. Collection and transportation of Specimen:

Pus was collected from infected foot lesion and blood from anti-cubital vein.

2.3.1. *Collection of pus specimen*:

Pus samples from the infected foot lesions were collected aseptically by using sterile cotton swab. To avoid the chance of contaminations, at first, foot lesion and tissue debris were thoroughly cleaned with sterile normal saline followed by cleaning of the lesion with gently rubbing by 70% alcohol. Thereafter the lesion was gently compressed and the expressed pus from inside was collected with sterile swab stick.

Sterile cotton swab sticks were moistened with sterile normal saline before collecting the specimens. The swab sticks were extended deeply into the depth of the lesion avoiding touching of surrounding skin area around the wound. When copious volume of pus existed, samples were collected aseptically by needle aspiration to avoid major exogenous contaminations. Pus samples from all DFI and non-DFI patients were collected following same procedure

(a) Diabetic Sample collection **(b) Non Diabetic sample collection**

Figure 2.1: Collection of Pus specimen from (a) DFI & (b) Non-DFI patients.

2.3.2 *Transportation of Pus*

After collection of pus samples, the swab sticks were immersed in sterile transport container comprising sterile transport medium (20 ml sterile tube contained 10ml peptone water). The samples were properly labeled and transported without any delay, to the laboratory of Microbiology Department, University of Dhaka, in an insulated ice box.

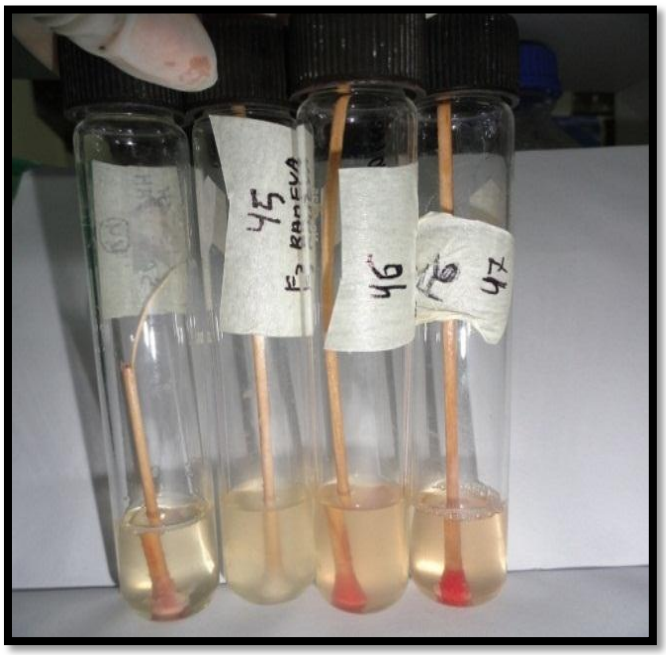


Figure 2.2 Transportation of pus specimen in (20 ml sterile tube contained 10 ml peptone water)

2.3.3 *Collection of Blood*

Under all aseptic precautions (cleaning the area with povidone iodine followed by adequate rubbing by alcohol pad containing 60% isopropyl) 5 ml of venous blood was collected from median ante-cubital or appropriate veins by gentle suction. The blood samples were allowed to clot at 15-24 °C. The clotting time was minimally 30 minutes and maximally one hour.

2.3.4 Blood sample transportation:

Immediately after collection of the blood sample with proper labeling blood was carried to the laboratory.

(a) Blood samples collection **(b) Serum separation**

Figure: 2.3: Procedure of (a) Blood samples collection (b) Serum separation

2.4 Serum Preparation and storage:

For serum preparation, blood sample was spin for 10 minutes at 1500 g. After centrifugation, the tubes were inspected carefully in order to recognize possible hemolysis. The serum was promptly separated from clot or cells and transferred to a clean eppendorf tube and was carefully marked with sticker or other method with identification code. The serum was immediately frozen at -20°C.

2.5. Determination of total viable, *Enterobacteriaceae* and resistant bacteria count

2.5.1. *Inoculation and pre-enrichment*

Each pus sample collected with cotton swab was inoculated into a 20 ml test tube containing 10 ml of peptone water as pre-enrichment medium. Sample tubes containing pus swabs (67 diabetic and 10 non-diabetic foot infected) within peptone water was incubated in a rotary shaker (Thermo Forma, USA) at 120 rpm at 37˚C for 1 hour.

2.5.2. *Total bacterial count*

Collected pus sample were serially diluted up to 10^{-4} with sterile normal saline (0.85%). To determine total viable count (TVC), *Enterobecteriaceae* count (EC) and resistant bacteria count (RBC) 100 µl serial diluted pus samples were spread by spread plate techniques on Nutrient Agar (NA) plate, Mac Conkey Agar (MAC) plate and NA supplemented with ciprofloxacin (CIP, 0.016 µg/ml) or cefotaxime (CEF, 1.0 µg/ml) and both ciprofloxacin & cefotaxime. All plates were incubated at 37˚C overnight and suspected colonies were purified on the same media plate and were preserved in 20% glycerol broth for further investigation.

2.5.2.1 *Total Viable Bacterial Count (TVC)*

Total viable bacterial count was done using commercially available NA media (Oxoid, UK), which was prepared according to manufacturer instruction. The solidified NA plate was placed in an incubator at inverted position and was incubated for 24 hours at 37^0C temperature to check for any possible contamination. 100 µl sample from each of the 10^{-2} and 10^{-4} dilutions as well as from the original pus sample was spread onto the sterile NA plates using sterile spreader. Following incubation at 37^0C for 18-24 hours, appearance of individual colony on each plate was enumerated and recorded.

2.5.2.2 *Total Enterobacteriaceae Count (TEC)*

MAC (Oxoid, No. 3) was used as a selective culture medium for the detection and isolation of gram negative *Enterobacteriaceae* from pus samples. Sample dilution and inoculation of the sample was done in a similar manner as described in above section.

2.5.2.3 *Total Antibiotic Resistant Count (TARC)*

Ciprofloxacin (CIP) and Cefotaxime (CEF) antibiotics were supplemented within NA media to determine total viable resistant bacterial count. Five types of plates were prepared for total viable bacterial count and total resistant bacterial count. These plates were as is mentioned in the following table:

Table2.0: Design of total viable bacterial count and total resistance bacterial count

Total Count	Antibiotic used working volume (µg/ml)	Media used	Comments
Total viable bacterial count	No antibiotic	NA	Total viable bacterial count (TVC)
Total viable Enterobacteriaceae count	No antibiotic	MAC	Total viable Enterobacteriaceae count (TEC)
Total viable resistance count	Ciprofloxacin(0.016 µg/ml)	NA	Total ciprofloxacin resistant viable bacterial count (TCRVB)
	Cefotaxime (1µg/ml)	NA	Total cefotaxime resistant viable bacterial count (TCERVB)
	Ciprofloxacin (0.016 µg/ml)+Cefotaxime(1µg/ml)	NA	Total ciprofloxacin + cefotaxime resistant viable bacterial count (TC+CERVB)

2.6. Sample dilution and inoculation

The prepared plates were fresh and dry. 85% sterile normal saline containing test tubes were used to perform serial dilution up to 10^{-4} dilution factor. Spread plate technique was adopted to find out the viable count of organism in the samples. 100µl diluted samples were spread on each and every plates of the nine media type using spread plate technique. Here, a mentionable point is that, the antibiotic supplemented media would show the count of antibiotic resistant organism in to per milliliter of sample. The counts of organism at dilution factor 10^{-4} were found to be the most favorable to determine the viable organism count per milliliter or colony forming unit per milliliter (cfu/ml).

2.7. Preparation of stock solution of antibiotics

Stock solutions of CEF and CIP at concentration of 1mg/ L were prepared using the guideline described above. For the preparation of antibiotic stock solution, powdered forms of antibiotics (Wake, UK) were used. Antibiotic powders were dissolved in suitable solvents and then diluted up to the required volume with sterile distilled water. From the stock solution, desired working concentrations were obtained further to carry out the work.

2.8. Preparation of media supplemented with antibiotics and sample inoculation

NA (Oxoid, UK) media were supplemented with CEF, CIP (stock solution 10 mg/ml) to obtain a final concentration of 1µg/ml for CEF and 0.16 µg/ml for CIP. Different combination of antibiotic and media is used to prepare plate. Antibiotics at stated concentrations (Table2.0) were added to the molten NA media at about 55^0C. The antibiotic supplemented agar plates were incubated for 18 hours at 37^0C in an incubator to check possible contamination. Then 100µlsample from each of the 10^{-2} and 10^{-4}dilutionsas well as from the original wastewater was spread onto the respective media (Table2.0) using sterile spreader. Following incubation at 37^0C for 18-24 hours, appearance of individual colony on each plate was enumerated and recorded for determination of NA, MAC, Total CIP resistance viable bacterial count (TCRVB), Total CEF resistance viable bacterial count (TCERVB), Total CIP + CEF resistance viable bacterial count (TC+CERVB).

2.9. Isolation and Preservation of Isolates:

Pus samples were diluted with normal saline up to 10^{-4} fold. The diluted samples were then spread onto NA and MAC plate containing antibiotic CEF, CIP and both CIP & CEF for isolation of isolates**.** The plates were inoculated and incubated at 37ºC overnight.

2.9.1. *Preservation of the isolates*

The single colony of isolates was streaked aseptically onto NA plate. NA Slant had been prepared into 3 ml vial for each isolate. The single colony from NA plate inoculated into Agar slant with a sterile loop and incubated for 18 hours at 37ºC.The culture in slant had been dipped into paraffin oil and stored at room temperature. These stocks of isolates had been used for regular experiments. Additionally, the single colony from NA plate was scrapped out with sterile loop and suspended thoroughly into nutrient broth containing 25% glycerol into sterile eppendorf tube and kept into -20^0C. Duplicate sets of eppendorf tubes were prepared for each isolates store at -80^oC.

2.10. Morphological and biochemical identification of the isolates

2.10.1 *Morphological and cultural properties*

The preserved isolates were streaked onto selective Agar plate and incubated overnight at 37^o C. Colonies were selected as pure culture on the basis of culture and biochemical properties.

2.10.1.1. *Microscopic Examination of the isolates*

Microscopic studies gram staining was performed according to the methods as described in "in Bergey's Manual of Determinative Bacteriology (Williams e*t al.,* 1994). The morphological characteristics (shape, size, edge, elevation, form and opacity) on NA plates were recorded.

2.10.1.2. *Biochemical identification of the isolates*

Some biochemical tests were performed according to the method described in "Bergey's Manual of Determinative Bacteriology (Bergey and John, 1994)". These biochemical tests were:

2.10.1.2. *a. Kliger's Iron Agar (KIA) test*

This test was done to differentiate among members of *Enterobacteriaceae* and distinguish between the *Enterobacteriaceae* and other groups of intestinal bacilli. KIA slant was inoculated with test organisms and incubated at 37^0C for 24 hours. After incubation, the color change of butt & slant and presence of gas & H_2S were noted.

2.10.1.2. *b. Indole test*

This test was done to determine the ability of the organisms to break down the amino acid tryptophan to indole. Tubes with 2.8% bacteriological peptone with trypticase soya broth (TSB) were inoculated with a loopful of 24 hours of old culture of the test organism and incubated for 24-48 hours at 37ºC. After incubation few drops of kovac′s reagent was added and shaken vigorously. A red color in the reagent layer indicated the production of indole.

2.10.1.2. *c. Methyl red (MR) test*

This test was done to determine the ability of microorganisms to oxidize glucose with the production and stabilization of high concentrations of acid end products and also to differentiate between all glucose- oxidizing enteric organisms, particularly *Escherichia coli* and *Enterobacter* spp. MR-VP medium was inoculated with a loopful of 24 hours of old culture of the test organism and incubated for 24-48 hours at 37ºC. After incubation a few drops of methyl red solution were added in each tube. A distinct red color indicated methyl red positive and yellow color indicated methyl red negative.

2.10.1.2. *d.VogesProskaur (VP) test*

This test was done to differentiate further among enteric organisms such as *E. coli*, *Enterobacter* spp. and *Klebsiella pneumonia* on the basis of acid production. MR-VP medium was inoculated with a loopful of 24 hours of old culture of the test organism and was incubated for 24-48 hours at 37º C. After growth, 3 ml napthol solution was added to each of the test tubes followed by one ml of potassium hydroxide- creatine solution. The tubes were shaken vigorously for 2 minutes. Appearance of pink color indicated the positive result.

2.10.1.2. *e. Citrate utilization*

This test was done to differentiate among enteric organisms on the basis of their ability to utilize citrate as sole source of carbon for metabolism. Citrate medium was inoculated with 24-48 hours old test culture and was incubated at 37º C for 24 hours. After incubation the presence of growth and coloration of the medium observed.

2.10.1.2. f .*Oxidase test*

This test was done to determine the ability of bacteria to produce cytochrome oxidase. To determine this 2 or 3 drops of the p-amino di-methylaniline oxalate was added to the surface of growth of test organisms. Color change from pink to maroon within 10-30 seconds indicates positive result.

2.10.1.2. g. *Catalase test*

A pure colony was picked by a sterile loop and immersed in 2-3 ml of 3% hydrogen peroxide solution in a test tube. The production of bubble indicate the positive result i.e. catalase producing bacteria.

2.10.1.2. h. *Sugar Utilization (Lactose, Glucose, Sucrose)*

This test was done to determine the ability of microorganisms to ferment carbohydrate. The carbohydrate fermentation broth contain trypticase, Sodium chloride, Phenol red, distilled water and 0.5% to 1% desired carbohydrate (Lactose, Glucose, Sucrose).Aseptically inoculate each labeled carbohydrate broth with bacterial culture. Incubate the tubes at 18-24 hours at 37^{o}C. After inoculation into a particular sugar, sterilize the loop in order to avoid cross contamination of the tube with other sugars. Keep un inoculated sugar tubes as control tubes. Durham tubes must put into all tubes, the Durham tubes should be fully filled with broth.

Acid production: Changes the medium into yellow color- organism ferments the given carbohydrate and produce organic acids there by reducing the pH of the medium into acidic.

Acid and Gas production: Changes the medium into yellow color-organism ferments the given Carbohydrate and produce organic acids and gas. Gas production can be detected by the presence of small bubbles in the inverted durham tubes.

Absence of fermentation: The broth retains the red color. The organism cannot utilize carbohydrate but the organism continues to grow in the medium using other energy sources in the medium.

2.11. Antimicrobial Susceptibility Test

The antimicrobial susceptibility of all selected isolates had been tested through standardized Double- Disk Diffusion method known as Kirbey- Bauer (Barry and Thornsberry, 1985). It is a modification of Baur's method (Baur *et al*., 1966). Mueller-Hinton Agar (Oxoid Limited, England) Standard agar-disc-diffusion method and was used to perform antibiotic susceptibility assay using twenty one commercially available antibiotic discs (Oxoid) belonging to 12 groups. The inhibition zone sizes were interpreted by referring to zone diameter interpretive standards from National Committee for Clinical Laboratory Standards (NCCLS) 2000 (Table 2.2).The control organism used was *Escherichia coli* ATCC 25922 and *Staphylococcus* ATCC 25923.

2.11.1. *Inoculum Preparation*

According to the standard guideline described by the Clinical and Laboratory Standards Institute (CLSI) 0.5 optical density standard was prepared. The standard inoculums were prepared for each isolate as following described method:

- The preserved isolates were inoculated on NA plates and incubated for overnight at 37º C. At least 2-3 well isolated colonies were selected from NA plate and transferred into Tripticase Soy Broth (TSB) using sterile loop. Each tube of TSB containing 5ml media were incubated at 37º C after inoculation.
- The broth cultures were incubated at 37º C to achieve the 0.5 optical density (OD) (usually 2-6 hours)
- The turbidity of the actively growing broth culture was adjusted with sterile broth to obtain turbidity optically comparable to the point of the McFarland standards.

2.11.2 *Inoculation of test plates*

Mueller- Hinton plates (Appendix I) were inoculated with the working culture according to the following process:

- Within 15 minutes of adjusting the turbidity of test culture, a sterile cotton swab was dipped into the adjusted suspension. The swab was rotated several time and pressed firmly on the inside wall of the respected culture tube above the culture to remove the excess culture from the swab.
- The dried surface of a Mueller-Hinton (MH) agar plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times rotating the plate approximately 60 each time to ensure an even distribution of inoculums. As a final step the rim of the agar was swabbed. The procedure was done under laminar air flow to avoid contamination.
- The lid was left ajar for 3-5 minutes but no more than 15 minutes, to allow for any access surface moisture to be absorbed before applying the drug impregnated disks.

2.11.3. *Application of antibiotic disks to inoculated agar plates*

Sterile antimicrobial disks were dispensed onto the surface of the inoculated agar plate using sterile forceps. Each disk was pressed down individually to ensure complete contact with the agar surface. The disk placed in the agar surface was not closer than 24 mm from center to center. A total of 7 disks were placed on one 150 mm plate. The plates were inverted and placed in an incubator set to 35^{o}C within 15 minutes after the disks were applied.

2.11.4. *Reading plates and results interpretation*

After 16-18 hours of incubation, each plate was examined. The resulting zone of inhibition was uniformly circular with a confluent lawn of growth. The diameters of the zones of complete inhibition were measured, including the diameter of the disk. Zones are measured to the nearest whole millimeter. Faint growth of tiny colonies, which can be detected only with a magnifying lens at the edge of the zone of inhibited growth, was ignored. The sizes of zones of inhibition were interpreted by referring to zone diameter interpretive standards from NCCLs (2000).

Table 2.1: Antimicrobial agents, their disc concentrations and zone interpretative reference according to NCCL

Antimicrobial Groups	Antimicrobial agents	Disc conc.(µg)	Zone interpretation (diameter in mm)*		
			S	M	R
Aminoglycosides	Gentamycin (CN)	10	≥15	13-14	≤12
	Streptomycin (S)	10	≥15	12-14	≤11
Carbapenems (carboxypenems)	Imipenem (IMP)	10	≥16	14-15	≤13
Cephalosporin	Cefepime (FEP)	30	≥18	15-17	≤14
	Cefotaxime (CTX)	30	≥23	18-22	≤17
Chloramphenicol	Chloramphenicol (C)	30	≥18	13-17	≤12
Lincosamide(lincomycins)	Clindamycin (DA)	2	≥21	15-20	≤14
Monobactams	Aztreonam (ATM)	30	≥22	16-21	≤15
Nitrofurans	Nitrofurantoin (F)	300	≥17	15-16	≤14
Penicillins	Ampicillin (AM)	10	≥17	14-16	≤13
	Penicillin-G (P)	10	≥29		≤28
Polypeptides	Polymixin B (PB)	300	≥12	9-11	≤8
	Colistin	300	≥10	9-11	≤8
Rifamycin	Rifampicin (RD)	5	≥22	14-21	≤13
Quinolone(Fluoroquinolones)	Ciprofloxacin(CIP)	5	≥21	16-20	≤15
Tetracyclines	Doxycyclines(DO)	30	≥13	10-12	≤ 9
***S, M and R indicate sensitive, intermediate and resistance zone respectively.**					

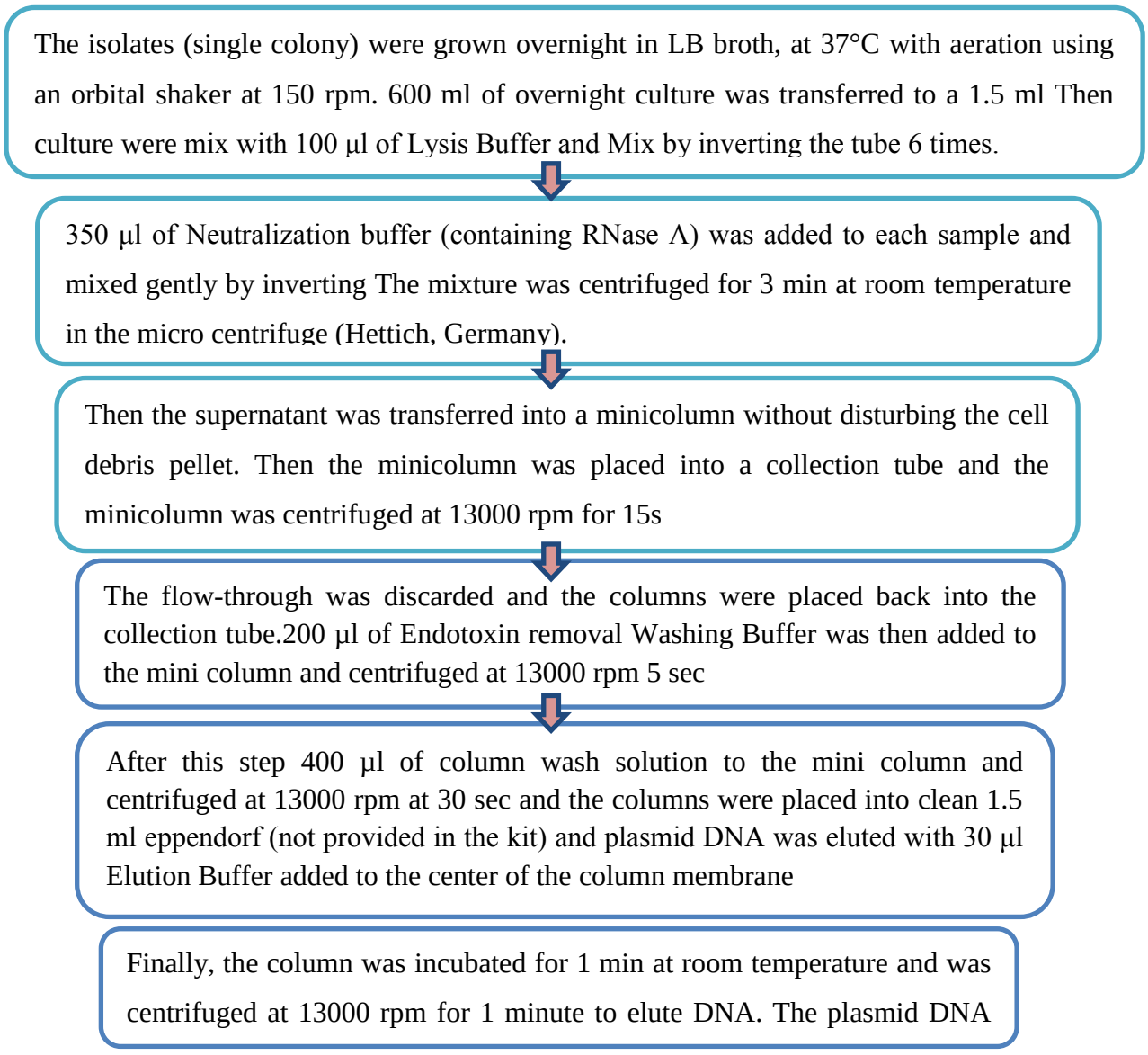
2.12. Molecular Characterization

2.12.1. *Plasmid profiling analysis*

2.12.1.1. *Extraction of plasmid DNA*

Plasmid DNA of 24 isolates was extracted by using Wizard plus SV Minipreps plasmid DNA Purification kit (Promega, USA) and by manual extraction procedure (Birnboim and Doly, 1979):

Extraction of plasmid DNA



2.12.1.2. *Separation of plasmid DNA by agarose gel electrophoresis*

Plasmid DNA was separated by horizontal electrophoresis in 0.8% agarose slab gels in a Tris-acetate EDTA (TAE) buffer at room temperature using 70 volt (50 mA) for 3 h. 30 µl of plasmid DNA solution was mixed with 5 µl of loading buffer (Appendix II) and was loaded into the individual well of the gel. The gel was stained with ethidium bromide for 30 min at room temperature. DNA bands were visualized and photograph was taken using UV trans illuminator (Gel Doc, Protein Simple, USA). The molecular weight of the unknown plasmid DNA was determined on the basis of its mobility through agarose gel and was compared to the mobility of the known molecular weight plasmids of Escherichia coli V517.

2.12.2. Extraction and Purification of Chromosomal DNA

Well isolated colonies from nutrient agar plate were grown overnight in 5 ml tripticase soy broth in test tube at 37ºC with aeration using shaking water bath set at 120 rpm

↓

1.0 ml culture was taken in an eppendorff tube (1.5 ml) and cells were collected by centrifugation for 10 min at 13,000 rpm using a centrifuge (Eppendorff, Germany).

↓

The cell pellets were washed with distilled water by re-centrifuging. Then 200µl PCR water was mixed.

↓

Then each eppendorf were kept at 100^0C boiling temperature for 10 minutes. After boiling the eppendorfs were kept in ice for 10 minutes

↓

The tubes were then centrifuged at 10,000 rpm for 10 minutes. The supernatant were collected into a fresh eppendorf tube (100-150 µl). DNA was measured as ng/ µl using Nanodrops (ThermoScientific, USA)

2.12.2.1. *Measurement of DNA concentration*

Extracted DNA was measured as ng/ µl using Nanodrops (ThermoScientific, USA). The ratio between the readings at 260 nm and 280 nm (OD260 /0D280) provides an estimate of the purity of the DNA. Pure DNA preparations have OD260/OD280 values of 1.8 (Maniatis *et al*., 1989).

2.12.3 Polymerase Chain Reaction (PCR)

2.12.3.1. *Preparation of reaction mixture*

The PCR reaction mixture was prepared by mixing the components at given volumes described in (Table 2.2). A master mix was prepared for all the isolates simultaneously using the amounts mentioned above. For individual test, separate primer set was used (Table 2.2). Finally the PCR tube containing reaction mixture was capped and centrifuged briefly to spin down the contents. The PCR tubes were then placed in a thermal cycler (Biometra, USA).

Table 2.2: Components of PCR reaction mixture

Component	Component Volume	Final Concentration
$MgCl_2$, 25 mM Solution	2.0–8.0 µl	1.0–4.0 mM
5X Colorless GoTaq® Flexi Buffer Or, 5XGreen GoTaq® Flexi Buffer	10 µl	1.0X
PCR Nucleotide Mix, 10 mM each	1µl	200 µM each
Upstream primer (27F)	5–50 pmol	0.1–1.0 µM
Downstream primer (1492R)	5–50 pmol	0.1–1.0 µM
GoTaq® DNA Polymerase, 5 u/µl	0.25 µl	1.25 u/50 µl
Template DNA	variable	<0.5 µg/50 µl
Nuclease-Free Water to a final volume of	**50 µl**	

2.12.3.2. *PCR conditions*

All the PCR tubes containing the appropriate mixtures were heated at 96^{O}C for 5 minutes in the thermal cycler to ensure the denaturation of all DNA templates. The PCR reaction was then continued with the following programed:

Segment – 1:	Initial heating at 96^{o}C for 5 min then continued with the denaturation at 94^{o}C, for 1 min 30 s
Segment – 2:	Annealing at55^{O}C for 1 min.
Segment – 3:	Extension at 72^{o}C for 1 min 30 s for a total of 35 cycles followed by a final extension of 10 min at 72^{o}C.

After this, PCR tubes were stored at -20º C until further analysis. The cycling profile for each primer- target combination was optimized accordingly.

2.12.3.3. *Post - PCR Detection of Amplified DNA by Electrophoretic Analysis*

The successful amplifications of the 16SrRNA genes were examined by resolving the PCR products in 1% agarose gel. Agarose (Sigma, USA) was dissolved in 1X Tris-acetate EDTA (TAE) buffer to give a final concentration of 1% agarose and was heated to dissolve in a microwave oven for about 2.5 - 3 minutes. When the temperature came down to 50º C, the gel was poured onto the gel tray (Labnet Gel XL Ultra V-2 Mini-Gel System, USA) already fixed with appropriate combs. Following solidification of the gel, it was submerged in 1X TAE buffer in a gel running tank. 15 µl l PCR products was mixed with 5 µl l of 1X gel loading dye and loaded into the slots of the gel with the aid of a micropipette. Electrophoresis was continued with 60 volts until DNA fragments were separated. The gel was then stained in staining solution (10 µl lehtidium bromide in 100 ml distilled water) for 15-30 minutes and was destained in distilled water for 15 minutes. The EtBr stained DNA bands were observed on a UV transilluminator (UVstar, USA). Photographs were taken using Gel Doc (AlphaImager, USA) system attached to a computer and bands were analyzed. The PCR products were further purified using a system such as Wizard® SV Gel and PCR Clean-Up System (Promega, USA) and was stored at –20°C.

2.12.4. Amplified ribosomal DNA Restriction analysis (ARDRA)

ARDRA is a rapid technique, which involves restriction digestion of amplified 16S rRNA gene with an appropriate restriction enzyme. The resulting restriction fragments are size separated by agarose gel electrophoresis, forming a characteristic restriction fragment length polymorphs (RFLP) (Kullenet *et al*., 1997).

Complete digestion of 16S rRNA gene product the isolates was done using the HhaI restriction enzyme. The restriction mixes (20μL of final volume) were carried out for 4 hours at 37°C. Each reaction tube contained 2 μL of 10X incubation buffer, 0.2 μL of bovine serum albumin, 6U of the restriction enzyme, 2.5 μL of distilled water and 15 μL of PCR product. The resulting digestion products were visualized under UV-light (LabNetTransilluminator, USA) at wavelength of 302 nm. After agarose gel electrophoresis using 1.5% agarose (w/v) gel running for 90 minutes at 75V and staining with ethidium bromide (0.5μg/mL). The restriction patterns were analyzed to cluster the isolates into identical banding patterns. The experimental controls used were:

a. Uncut experimental DNA,

b. Digestion of commercially supplied control DNA.

Two different size markers, 1Kb (Bioneer, South Korea) and 100 bp (Bioneer, South Korea) DNA ladders were used to analyze different restriction fragments.

2.12.5. Sequencing and Phylogenetic Analysis

The 16S rRNA gene PCR products of the isolates from each ARDRA groups were purified with the Wizard PCR SV Gel and PCR Clean-Up System kit (Promega,USA) and sequenced (ABI Prism 3130 Genetic Analyzer, USA) using forwards 27F and reverse 1492R primers. Briefly, the PCR products were separated through electrophoresis. Following electrophoresis, each DNA band was excised from the gel and was dissolved completely by incubation at 50–65°C in membrane binding solution (Appendix-II). Then equal volume of Membrane Binding Solution.

(Appendix-II) was added to the PCR amplicon. Dissolved gel mixtures were then transferred to the minicolume assembly and were centrifuge to bind the PCR amplicon. After discarding flow through, the column was reinserted into a collection tube. Amplified DNA bound to the spin column was then washed twice with 750 µl of Membrane Wash Solution (Appendix-II) and was eluted with 50 µl of nuclease-free water, added to the spin column. Finally, the column was centrifuged for 1 minute at room temperature and the pure amplified DNA was stored at –20°C for further analysis.

2.12.6. Sequence Alignment and Identification

Partial sequences, obtained using forward and reverse primers, were combined to full length sequences via the SeqMan Genome Assembler (DNA star, USA) and were compared to the GenBank database of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/GenBank) by means of the basic local alignment search tool (BLAST) to identify close phylogenetic relatives. Multiple sequence alignment of the retrieved reference sequences from National Center for Biotechnology Information was performed with the Clustal W (Larkin *et al.*, 2007) software and was exported to the Molecular Evolutionary Genetics Analysis (MEGA) program (Tamura *et al.*, 2007) for phylogenetic tree construction using the Neighbor joining algorithm and selecting 1000 bootstrap replication.

2.12.7 Construction of Phylogenetic Tree

Phylogenetic tree of the gene sequences of the isolates was constructed using the following bioinformaticsoftwares:

a. Reference sequences were download from

- NCBI: http://www.ncbi.nlm.nih.gov
- EMBL:http://www.ebi.ac.uk
-DDBJ:http://www.ddbj.nig.ac.jp

b. Acquired sequences were aligned, checked and trimmed by using

-ClustalW and GeneDoc
-MEGA 5

c. Phylogenetic tree was constructed by

-MEGA 5

Briefly, the multiple sequence alignment of the retrieved reference sequences from NCBI, EMBL or DDBJ and representative isolates' sequences were performed with the ClustalW (Larkin *et al*., 2007) software (Fig 2.4). Aligned sequences were exported to the GeneDoc software for sequence trimming and conserved region identification. Refined sequences were further exported to the Molecular Evolutionary Genetics Analysis (MEGA) (Tamura *et al*., 2007) software for phylogenetic tree construction using the Neighbor joining algorithm and selecting 1000 bootstrap replication. Further analysis of the genes was carried out using the Distance and Pattern analysis tool in the MEGA software.

Figure 2.4: Multiple sequence alignment using ClastalX [adapted fromEuropean Bioinformatics Institute (EBI)]

2.13. Analysis of blood samples for C-reactive protein

2.13.1. *Quantitative Determination of C-reactive protein (CRP) in serum:*

In acute phase, there was increased level of plasma protein, including CRP. CRP is one of the acute phase proteins, the serum or plasma levels of which rise during nonspecific response to infectious and noninfectious inflammatory response. CRP is synthesized in liver and is normally present as at race constituent of serum or plasma. CRP is the marker of CSI.

Total 30 Serum samples were select randomly from DFI, 10 non-DFI and 5 healthy control were used to measure CRP by using **Cardio** Phase **hs**CRP Reagent (Siemens Health care Diagnostics products GmbH, Germany) . The reagent consists of suspension of polystyrene particles coated with mouse monoclonal antibodies (<0.016g/L) to CRP. **(BN * II /BN prospect System)** Polystyrene particles coated with mono clonal antibodies specific to human CRP are aggregated when mixed with sample containing CRP. These aggregates scatter a beam of light passed through the sample. The intensity of the scattered light is proportional to the concentration of the relevant protein in the sample. The result is evaluated by comparison with a standard of known concentration.

Chapter 3

Results

3.0 Results:

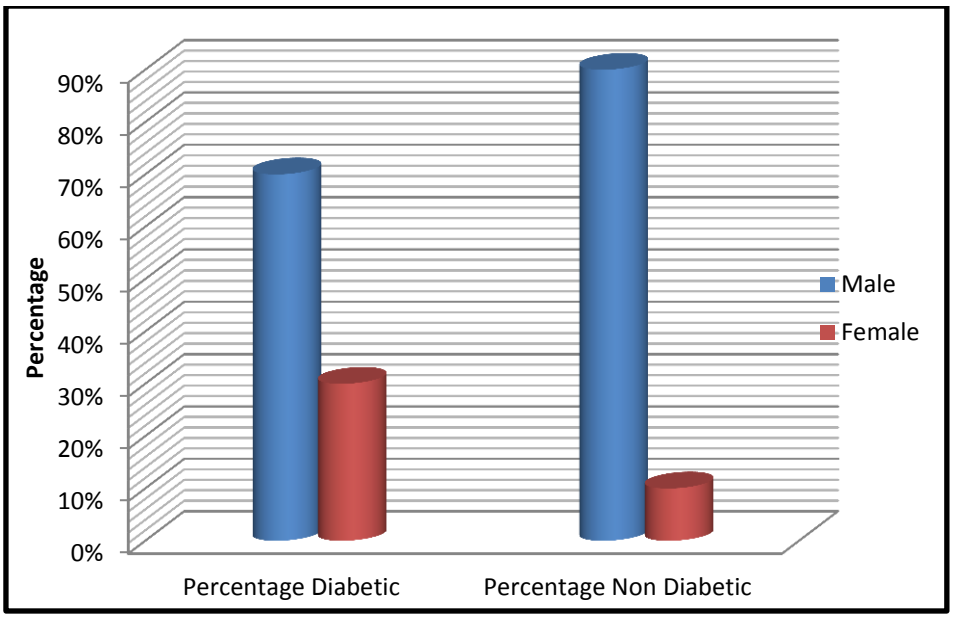
Diabetes mellitus (DM) is a metabolic disease and if not managed properly it leads to serious health complication, one of which Diabetic foot infections (DFI). The chronic DM and DFI difficult further the treatment by establishing DFI associated multidrug resistant (MDR) bacteriomes in infectious site of the patients. Appropriate knowledge on DFI associated bacteriomes and their resistance patters is extremely important. This study has described the demography and bacteriomes associated with the DFI of Bangladesh DM patients and the bacterial association was compared with Non-DFI.

3.1 Demographical data of diabetic and non-diabetic patients

This study was conducted on 67 patients admitted with DFI in Foot Care Hospital (FCH), Wari, Dhaka and 10 Non-DFI admitted with foot infection in Dhaka Medical College Hospital (DMCH). A questioner based survey has done for understanding the demography of the patients.

3.1.1 *Categorization of patients according to sex*

This study was conducted on 67 DFI patients and 10 non-DFI patients. Among DFI patients, 47(70%) were males and 20(30%) were females (Figure 3.1 a, b). Out of 10 Non-DFI patients, 9 (90%) were males. Males were the predominant in the study subjects in both DFI and non-DFI patients.

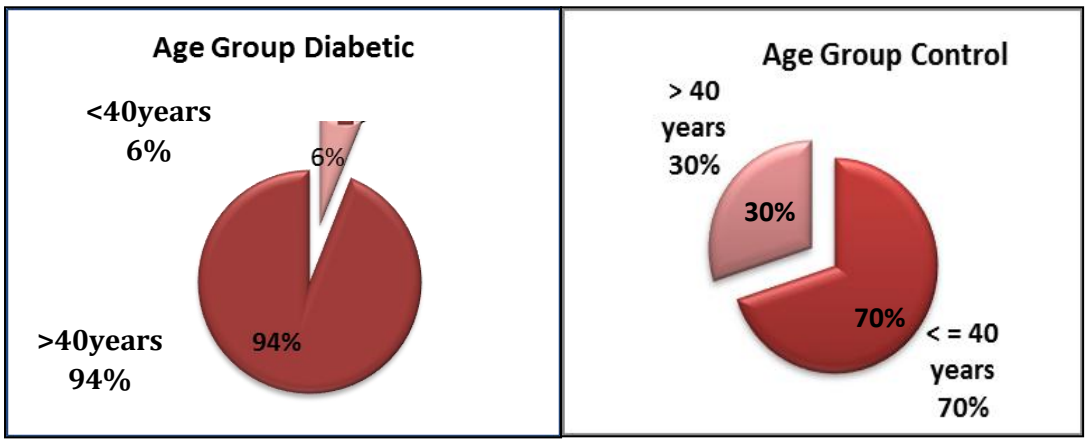


(a) **Diabetic** **(b)Non-Diabetic (Control)**

Figure 3.1: Diagrammatic representation of sex (male and female) among the Foot infected patients.

3.1.2 *Categorization of patients according to age*

The age of the Diabetic study subjects ranged between 28 to 75 years with a mean age being 56.25 years. It had been observed that only 4 (6%) DFI patients belonged to ≤ 40 years of age group, 63 (94%) subjects were belonged to > 40 years of age group. Among non-DFI patients, 7 (70%) belonged to ≤ 40 years of age group, 3 (30%) subjects were belonged to > 40 years of age. (Figure 3.2. a, b)



(a) Diabetic **(b) Non-Diabetic (Control)**

Figure 3.2: Diagrammatic representation of age group among the Foot infection patients.

3.1.3 *Categorization of patients according to Occupation:*

The categories of occupation of 67 DFI patients were divided into 7 groups (Service, Business, farmer, Teacher, Service-Ret, House Wife and daily Worker) (Figure 3.3), where it was observed that, 33% of total patients were businessman and the second prevalent patient group was housewife (21% of total patients) followed by service (retired) 13%, daily worker 15%, service 7%, teacher 4%, and farmer 6% (Figure 3.3). In case of non-DFI patients (designated as Control group), about 80% of study subjects were daily worker and 20% were farmer.

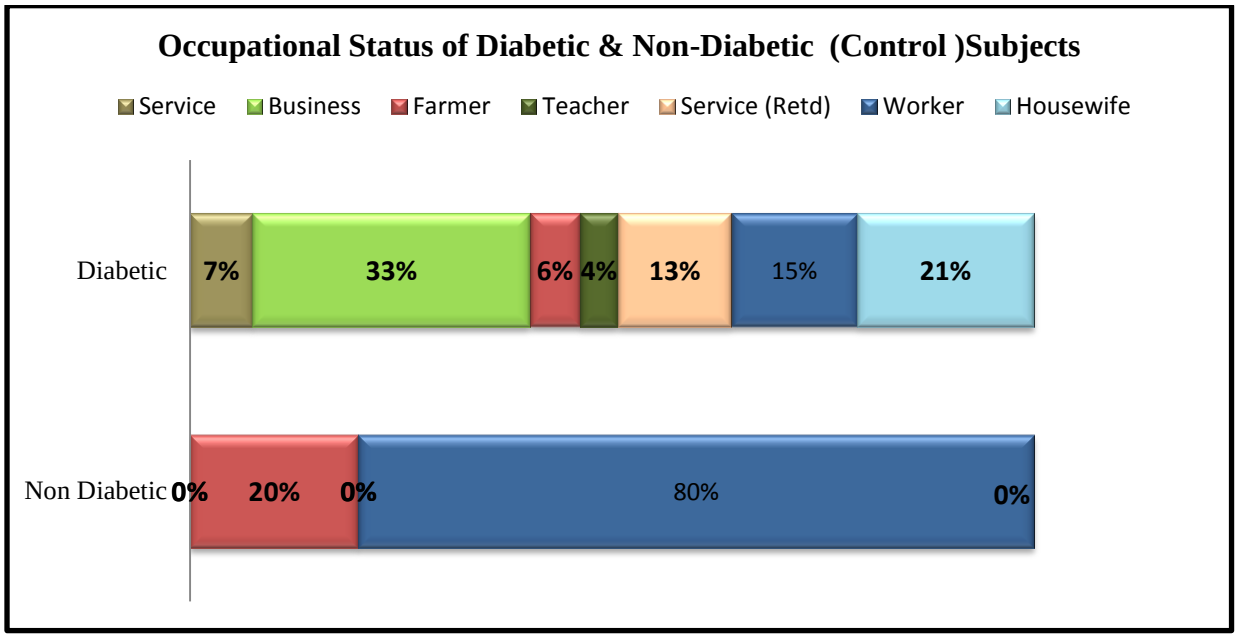


Figure 3.3: Diagrammatic representation of occupation among the Foot infection patients.

3.1.4 *Categorization of patients according to socio-economic status*

For categorization of socio-economic status of 67 DFI and 10 non DFI patients, World Bank index (Gross National Income (GNI) per capita and the calculation of World Bank (WB), 2006 was used and where it was observed that 69% of total DFI patients and 90% of total non- DFI patients were from Lower middle income (BDT 5,361-21,270) group (Figure3.4).

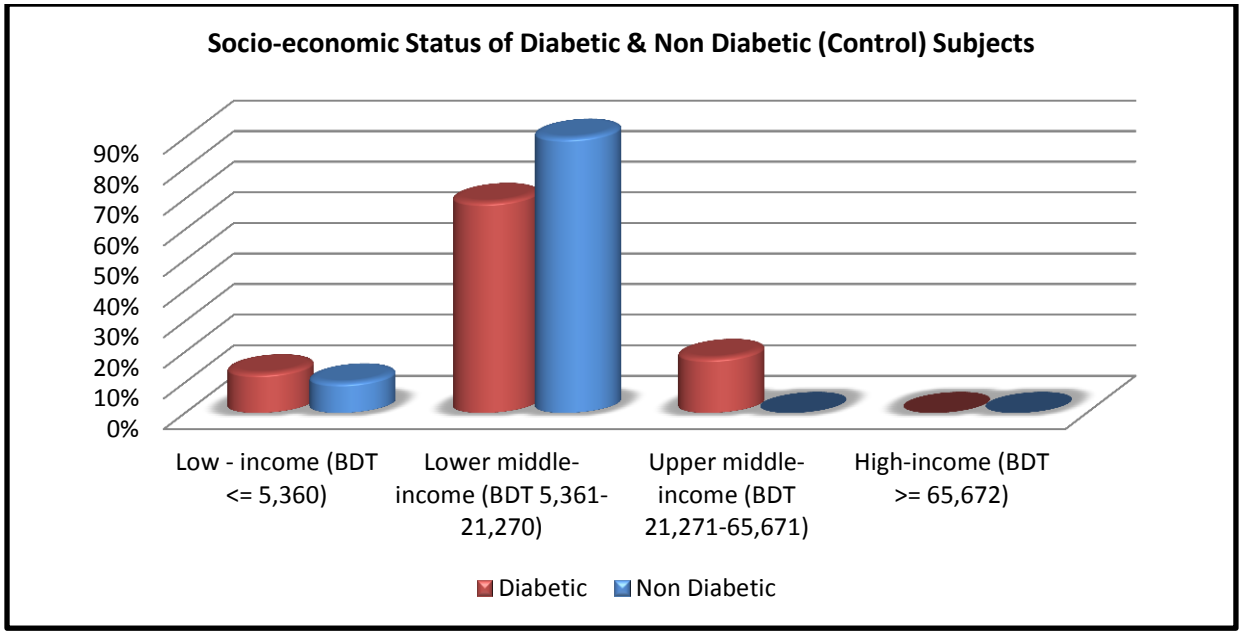
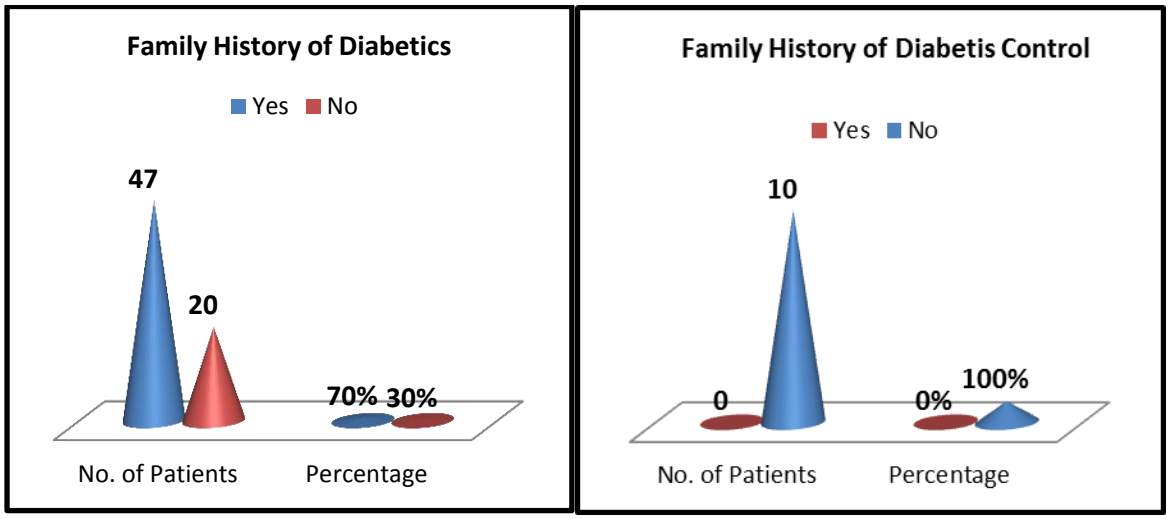


Figure 3.4: Diagrammatic representation of socio economic status among the Foot infection patients.

3.1.5 *Categorization of patients according to Family History of diabetes:*

Of the total 67 DFI patients 47 (70%) patients had family history DM and 20(30%) patients had no history of DM (Figure 3.5). Among non-DFI (control) patients there is no history of DM in their family.



(a) Diabetic **(b) Non-Diabetic (control)**

Figure 3.5: Diagrammatic representation of Family History of DM among the foot infected patients.

3.1.6 *Categorization of patients according to Life style:*

The following (Figure 3.6) is representing that out of 67 DFI patients, 49 (73%) patients came from rural areas and 18 (27%) were from urban areas. In non-DFI patients 9 (90%) were from rural area and 1(10%) were from urban area.

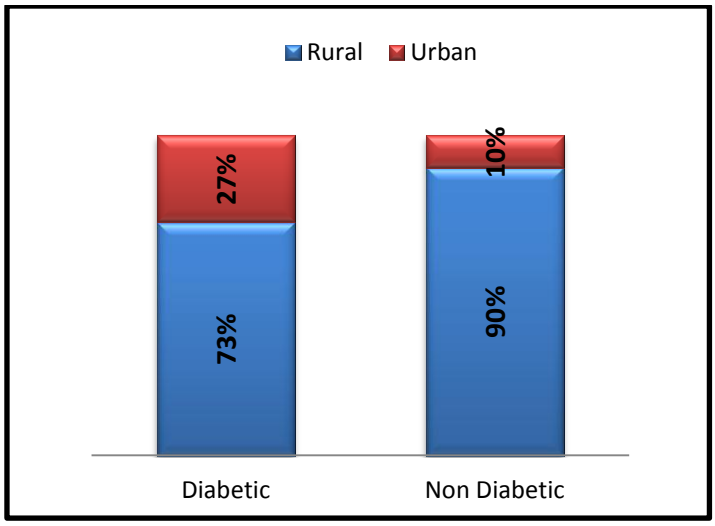
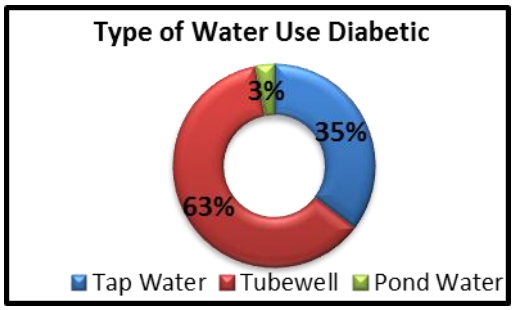


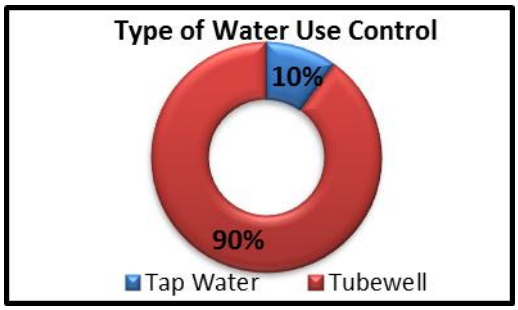
Figure 3.6: Diagrammatic representation of Life Style among the Foot infection patients.

3.1.7. *Categorization of patients according to types of water they use:*

Of the total 67 DFI patients it had been observed that 63% patients were using tube well water, 35% patients were using tap water and only 3% were using pond water regarding their daily activities (Figure 3.7 a, b). In non-DFI patients 90% were using Tube well water and only 10% were using Tap water. Here, it can be said that in both study groups most of the patients were using either tube well water.



(a) Diabetic



(b) Non-Diabetic (Control)

Figure 3.7: Diagrammatic representation of type of water uses the Foot infection patients.

3.2 Isolation and Identification of bacteria from pus samples

3.2.1 *Microbiological analysis of pus samples:*

Microbiological analyses of pus samples from DFI patients (30 samples) and non-DFI patients (10 samples) were carried out randomly to determine total viable bacterial count (TVBC), total *Enterobacteriaceae* count (TEC) and total antibiotic resistant count (TARC) using the antibiotic supplements, CIP (0.16 µg/ml.), CEF (1 µg/ml.) and both the antibiotic supplements within the NA and MAC agar media. Bacterial Load (TVBC, TEC, TARC) in DFI were higher than non-DFI samples (Table 3.1)

(a)

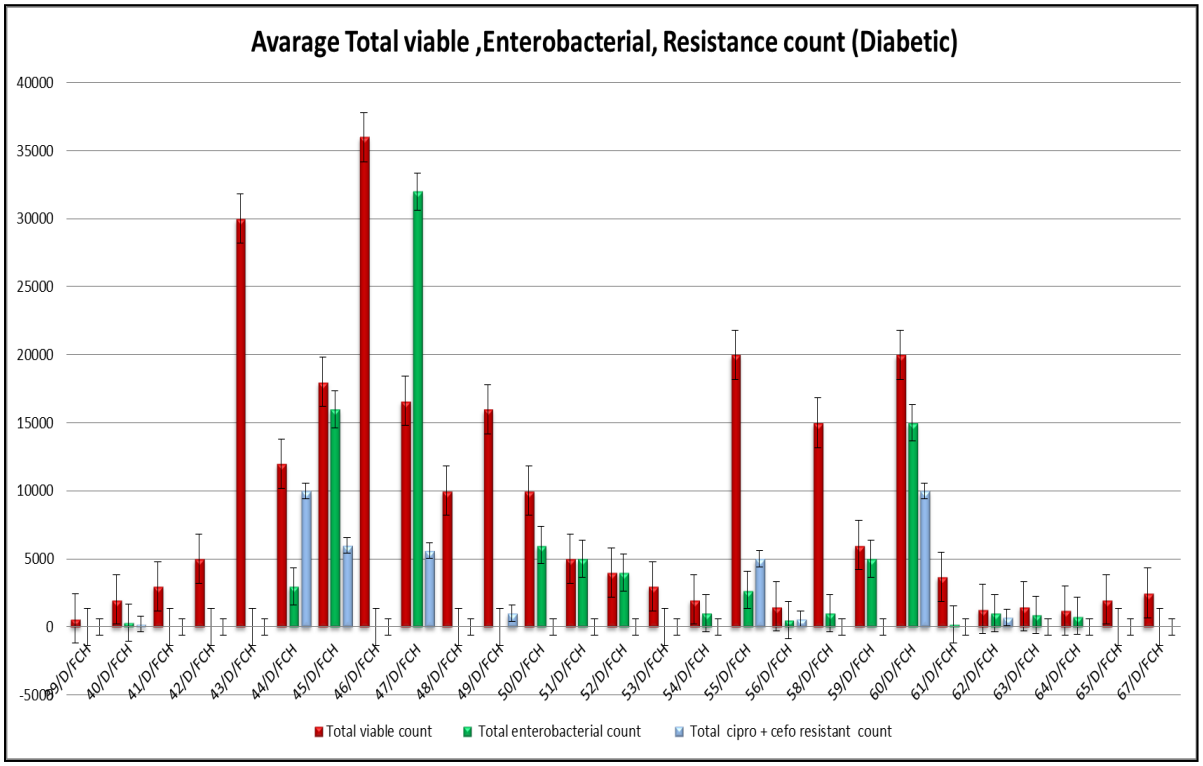


Figure 3.8: Total viable bacterial, Enterobacteriaceae and resistance count of DFI patient's pus samples. Total count and total resistance count of DFI patients pus samples were taken from NA and NA with antibiotics Ciprfloxacin(CIP) and cefotaxime(CEF) plates after overnight incubation at 37° C, respectively. Error bars have been made by using standard deviation. Standard deviation is calculated from the mean of duplicate results.

(b)

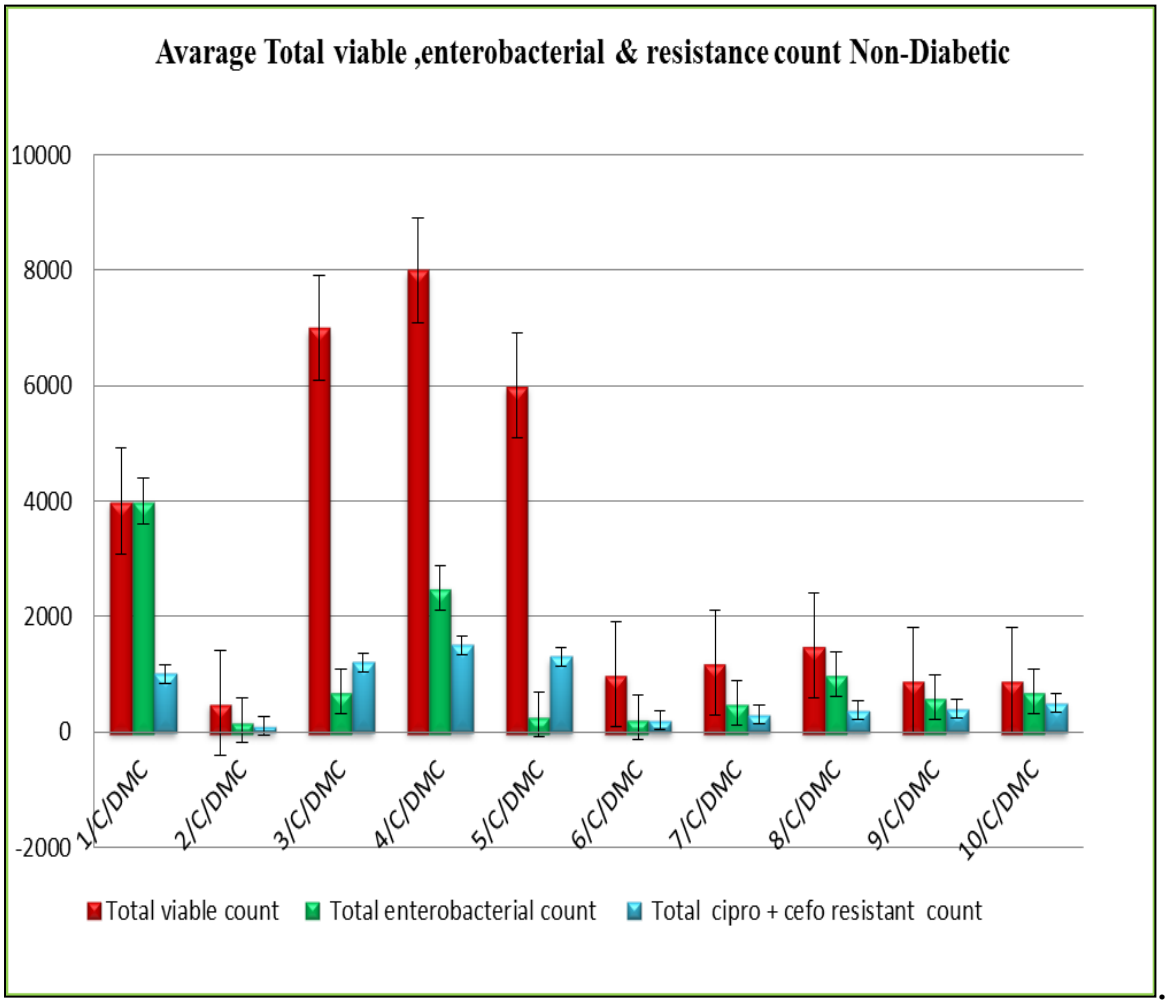


Figure: 3.9: Total viable bacterial count, and total resistance count of non-DFI patients pus samples were taken from NA and NA with antibiotics Ciprfloxacin(CIP) and cefotaxime(CEF) plates after overnight incubation at 37° C, respectively. Error bars have been made by using standard deviation. Standard deviation is calculated from the mean of duplicate results.

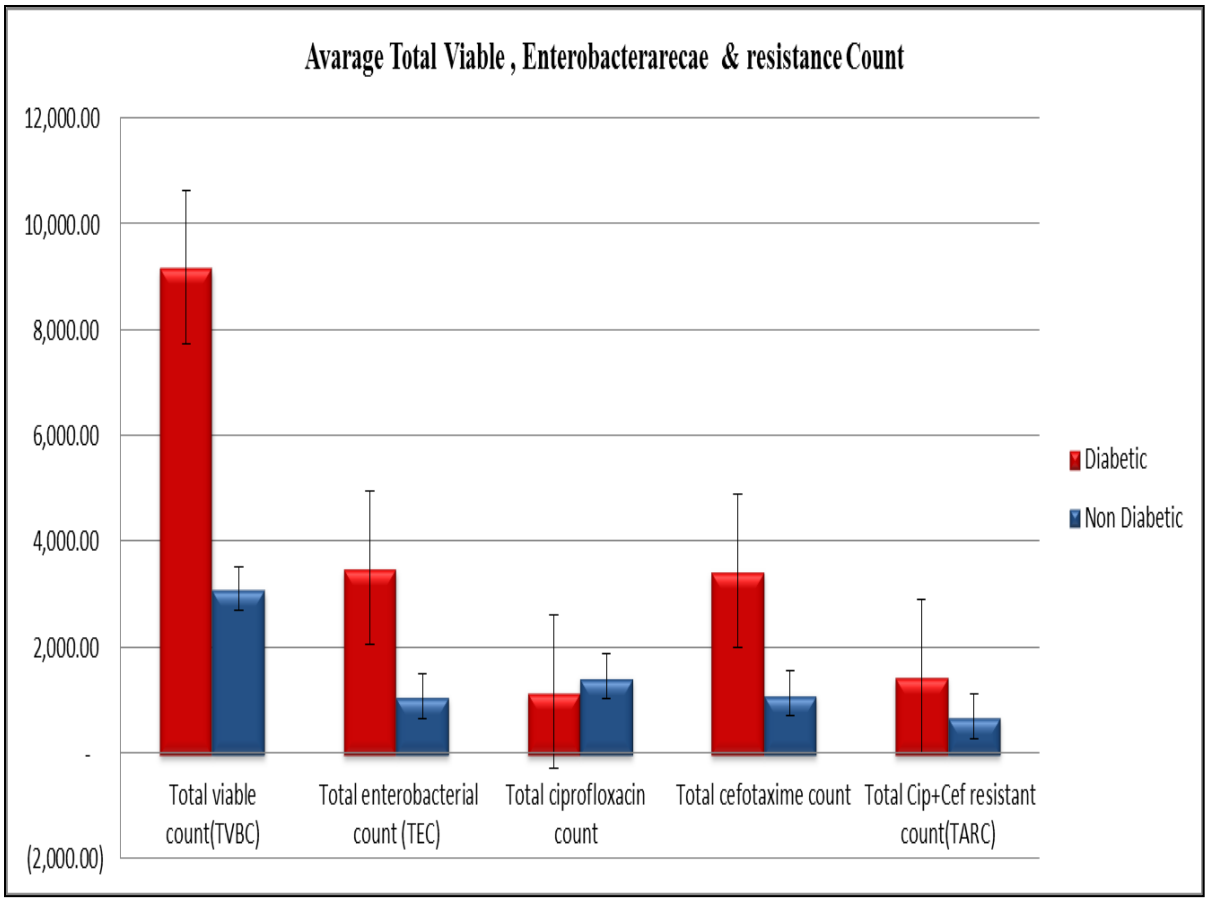


Figure: 3.10: Average total count and total resistance count of DFI & non-DFI pus samples were taken from NA and NA with antibiotics Ciprfloxacin (CIP)+cefotaxime (CEF) plates after overnight incubation at 37° C, respectively. Error bars have been made by using standard deviation. Standard deviation is calculated from the mean of duplicate results.

Table 3.2: Avarage total Viable (TVBC), Enterobacterecae (TEC) and resistance (TRC) bacterial count.

Count	Patients	Average ± SD CFU/ml
TVBC	Diabetic	9181 ± 9414
	Non Diabetic	3101 ± 2895
TEC	Diabetic	3496 ± 7065
	Non Diabetic	1076 ± 1223
TRC (Cip)	Diabetic	1162 ± 3477
	Non Diabetic	1440 ± 1427
TRC (Cefo)	Diabetic	3437 ± 7571
	Non Diabetic	1120 ± 1343
TRC(Cip+Cefo)	Diabetic	1448 ± 3023
	Non Diabetic	688 ± 510

Figure 3.10 and table 3.1 is representing Microbiological analyses of pus samples from DFI (30 samples) and non-DFI patients (10 samples) were carried out to determine total viable bacterial count (TVBC), total *Enterobacteriaceae* count (TEC), Total CIP resistance count, Total CEF resistance count and total both CIP+ CEF resistant count (TARC) using the antibiotic supplements, CIP (0.16 µg/ml.), CEF (1 µg/ml.) and both the antibiotic supplements within the NA and MAC media.

In DFI patients the average total TVBC, the mean ± SD cfu/ml ($9.18x10^3 \pm 9.41x10^3$cfu/ml) were found significantly higher compared to non-DFI patient samples ($3.50x10^3 \pm 2.89x10^3$cfu/ml, $p<0.01$). (Table 3.2) For the average total TEC, the mean ± SD cfu/ml of DFI were aslo found significantly higher ($3.10x10^3 \pm 7.07x10^3$cfu/ml) compared to total average TEC of non-DFI ($1.08x10^3 \pm 1.20x10^3$ cfu/ml, $p<0.01$). The average total CEF count Mean ± SD cfu/ml of DFI pus samples ($3.40\ x10^3 \pm 7.89x10^3$ cfu/ml) were found significantly higher compared to average total CEF count Mean ± SD cfu/ml of non-DFI pus samples ($1.12\ x10^3 \pm 1.34\ x10^3$ cfu/ml, $p<0.01$). However, average total CIP resistance count Mean ± SD cfu/ml of non-DFI pus samples was found higher ($1.4\ x10^3 \pm 1.2x10^3$cfu/ml) than average total CIP resistance count Mean ± SD cfu/ml of DM patient's pus samples ($1.1\ x10^3 \pm 3.5x10^3$cfu/ml). In case of average TARC, the mean ± SD cfu/ml of DFI samples $1.45x10^3 \pm 3.02x10^3$cfu/ml was significantly higher compared to average resistance bacterial count of non-DFI ($6.88x10^2 \pm 5.10x10^2$cfu/ml, $p<0.01$).

3.2.2 Isolation of Bacteria from pus samples

Pus samples of DFI patients were collected from DFI & non-DFI patients from DMC showed that both 93.3% DFI & 71.4 % non-DFI samples were culture positive on NA plate. Isolates of DFI patients collected from FCH were denoted with "D/FCH" and isolates of Non-DFI patients collected from DMC were denoted with "C/DMC" (Table3.3).

A total of 213 bacteria were isolated on NA and MAC plates from 67 DFI pus samples whereas 131 bacteria were obtained from the 10 non-DFI specimens. For population composition analysis all the isolates belongs to group 1-10 (9 DFI and 7non-DFI isolates).

Morphological group 1 consists of 20 DFI and 14 non-DFI isolates. Group 2 consists of 54 DFI and 50 non-DFI isolates. Group 3, group 5 and 7 consists of 23, 16 and 35 isolates only of DFI patients respectively. Group 4 includes 36 DFI and 5 non-DFI isolates. In group 6 there were 15 isolates in both DFI & non-DFI isolates. Group 8 consists of 25 non-DFI and 5 DFI isolates. Group9 includes 6 DFI & 19 non-DFI isolates. Group 10 consists of only one non-DFI isolates (Table 3.3).

Isolates were further extensively characterized according to their phenotypes (colony morphology, gram staining, biochemical properties, sugar fermentations) and genotypes (16s rDNA) properties. The isolates were identified up to genus and species level following the standard guideline (Bergey's Manual of Systematic Bacteriology, Volume 1, 1984) and clustering software BioCluster 2.0 (www. microbialgen.du.ac.bd/BioCluster). The organism belongs to each group were- group 1 *Staphylococcus* spp., group 2 *Pseudomonous* spp., group 3 *Enterococcus* spp., group 4 *Enterobacter* spp ., group 5 *klebsiella* spp., group 6 *Acinetobacter* spp., group 7 *Bacillus* spp., group 8 *Citrobacter* spp., group 9, *Proteus* spp. and *Providentia* spp., and group 10 Alcaligens *fecalis*.

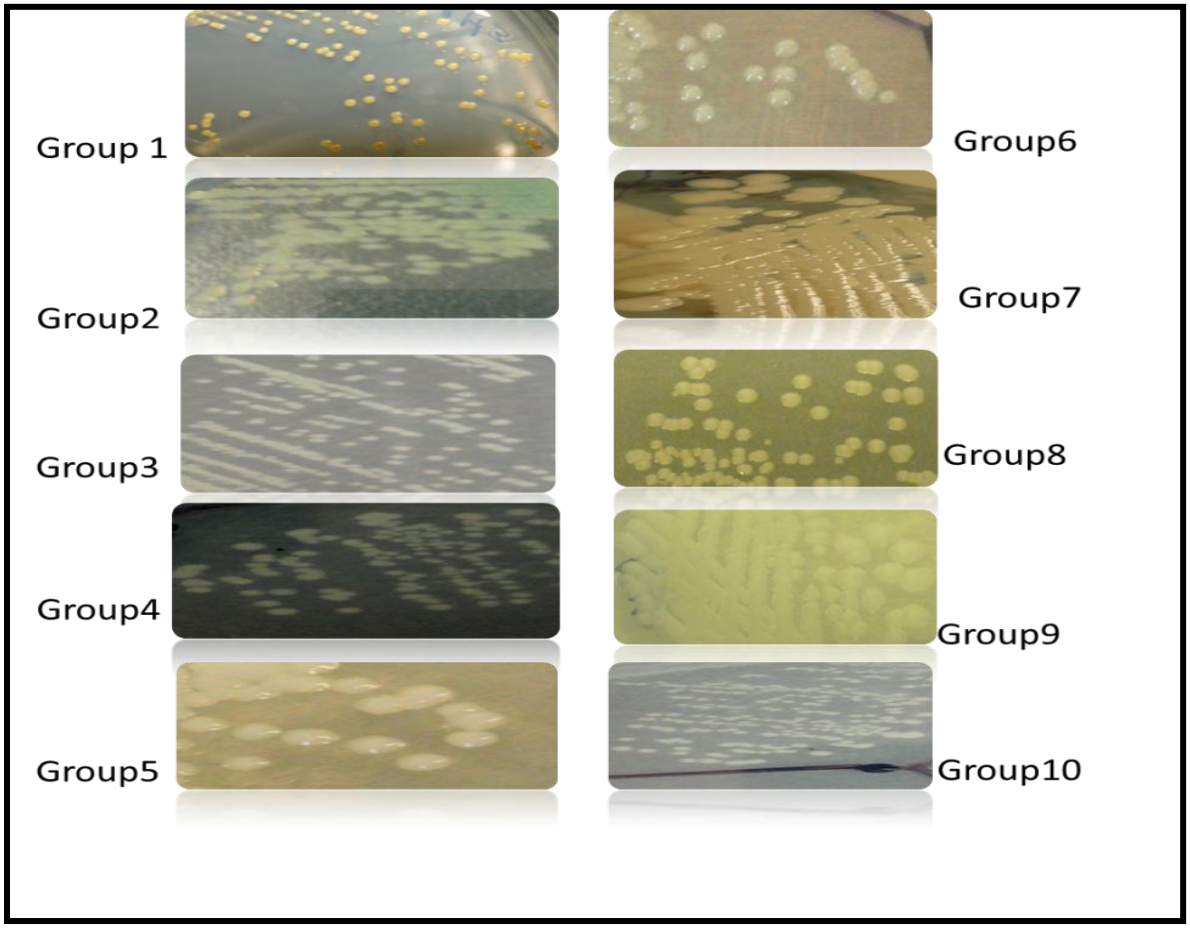


Figure 3.11: Colony characteristics of Representative isolates from 10 groups in NA plate after overnight incubation at 37° C.

3.3. Presumptive Identification of the Diabetic & Non Diabetic Isolates by Microscopic examinations & Biochemical tests:

From NA plate ten different morphological types (Figure 3.11) were obtained. Representative isolates were presumptively identified according to standard guideline (Bergey's Manual of Systematic Bacteriology, Volume 1, 1984)

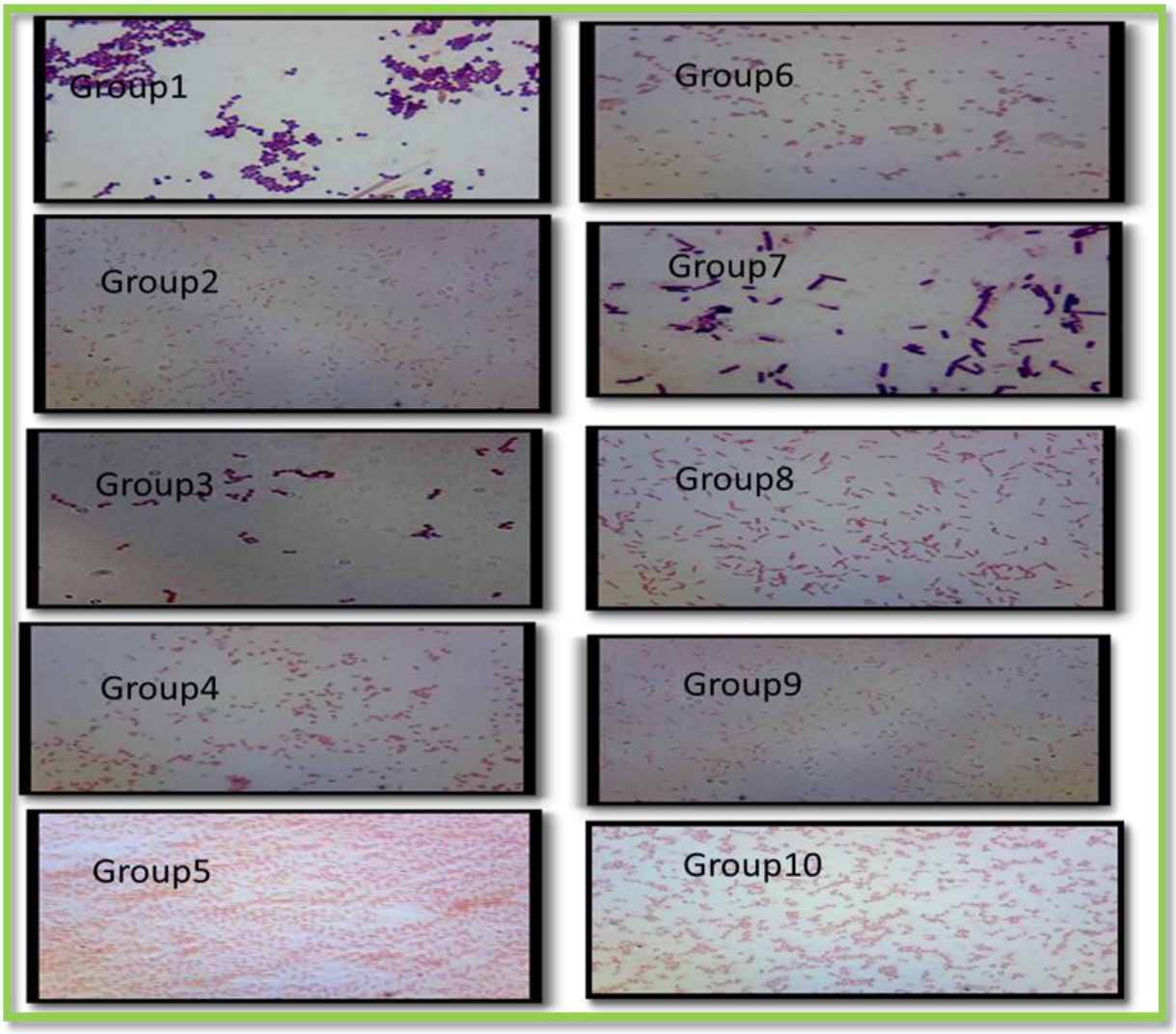


Figure 3.12: Observation of gram staining reaction of representative isolates from 10 groups of colonies from NA plates. Cell smears were observed under 40X (c) and 100X (a, b, d) objective lens.

3.4. Biochemical Test report of Diabetic &Non Diabetic Isolates:

Colony morphology and gram staining reactions for each isolates (Figure 3.12) were determined according to standard guideline (Bergey's Manual of Systematic Bacteriology, Volume 1, 1984) (Table 2). Biochemical identification of presumptive isolates was done using a biochemical clustering software Bio Cluster 2.0 (www. Microbialgen.du.ac.bd/Bio Cluster). Figure 3.13 representing, isolates of group 1 were gram positive cooci, catalase positive, ferments carbohydrate (Lactose, Dextrose, Sucrose), Oxidase negative they are presumptive staphylococcus spp. Group 2 were gram negative rod, oxidase positive, catalase negative, do not have ability to fermentation carbohydrate but utilize citrate. Group 2 isolates were presumptive *Pseudomonous* spp. Isolates of group 3 were also gram positive cocci but their catalase test were found negative and they hydrolyze esculin in presence of bile .They showed carbohydrate non-fermentative properties and were presumptive *Enterococcus* spp. Isolates belonged to group 4 had the ability to ferment sugar glucose (Dextrose & Sucrose), utilize citrate, Methyle red (MR) positive but Voges- proskauer (VP) test negative and showed gram negative rod shape in gram staining , so they were presumptive *Enterobacter* spp .Group 5 isolates had the abilities to ferment glucose and lactose to produce acid and gas and as they had negative oxidase, utilize citrate, gram negative rod, they were categorized as probable *klebsiella* spp. Isolates belonged to group 6 didn't have the ability to ferment glucose and lactose and as they were oxidase negative, not utilize citrate, gram negative short rod; were presumptive *Acinetobacter* spp.

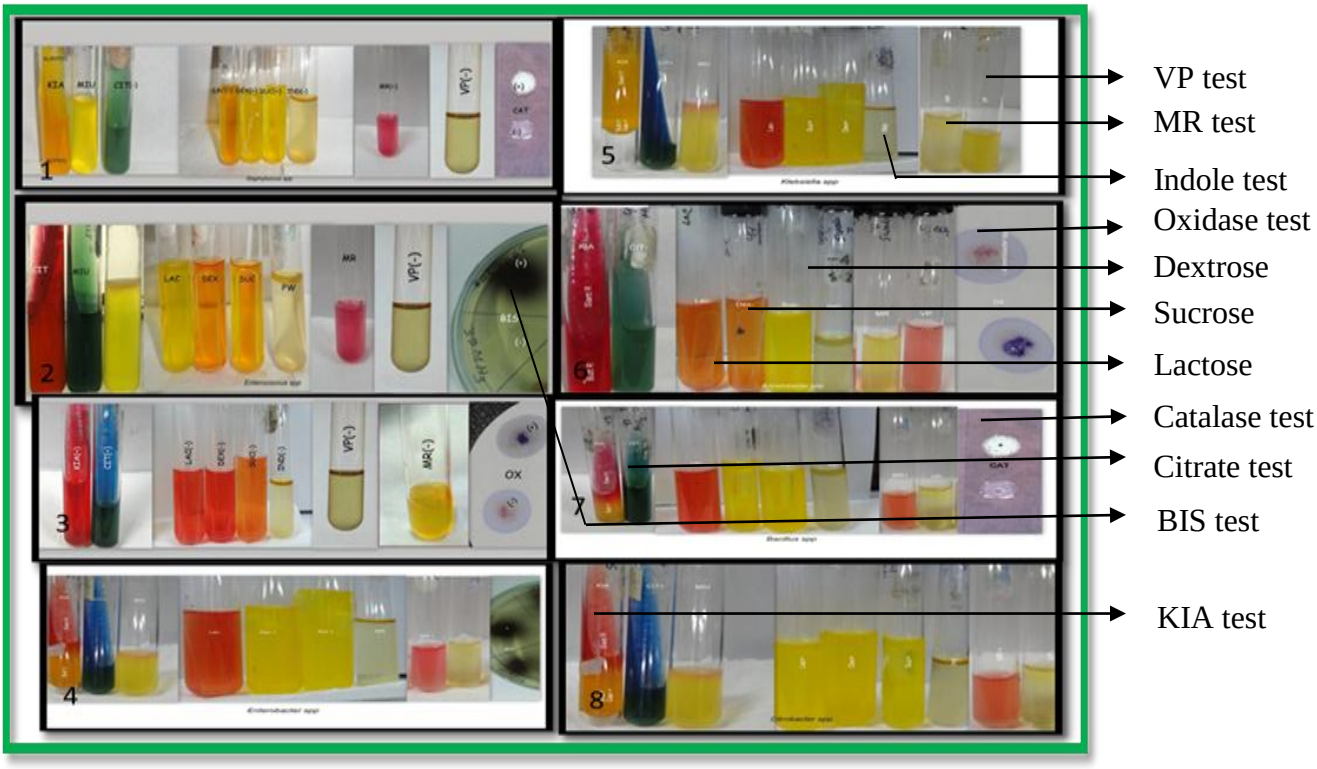


Figure 3.13: Biochemical tests reports of representative isolates from 10 groups.

Isolates of group 7 were gram positive but rod shaped pattern in gram staining; oxidase and catalase positive but non utilize citrate; ferment glucose sugar (Dextrose, Sucrose), positive Methyl red tests; presumptive *Bacillus* spp. Isolates belonged to group 8 have the ability to ferment glucose and lactose and produce acid, gas in KIA medium, ferments carbohydrate (Lactose, Dextrose, Sucrose), oxidase negative but utilize citrate; and belongs to presumptive *Citrobacter* spp. Only 5 *Citrobacter* spp. were unable to utilize citrate. In group 9, only 3 isolates have the ability to produce hydrogen sulfate, ferments glucose but not lactose, oxidase negative, probable *Proteus* spp. But other isolates of group were unable to produce hydrogen sulfate; and grouped in to presumptive *Providentia* spp. Group 10, consists of only one isolate, do not have ability to ferments glucose, lactose but utilize citrate, motile, MR negative, VP positive; and identified as *Alcaligens fecalis.*

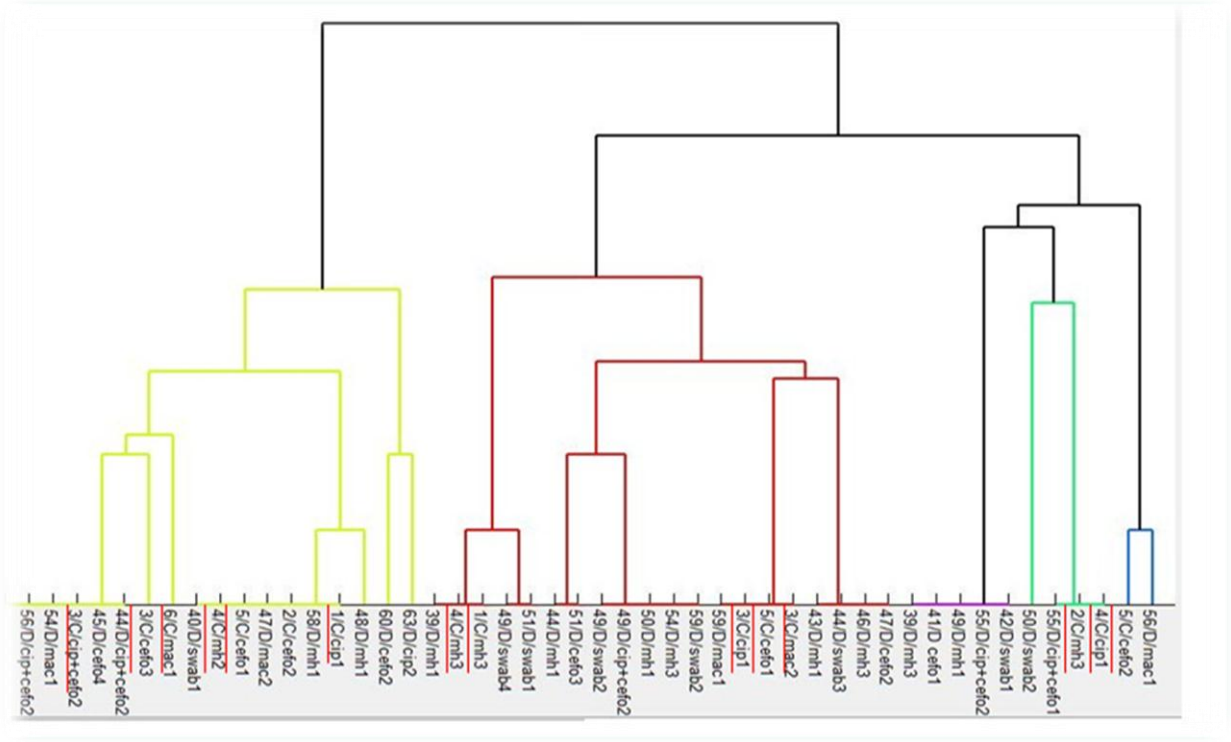
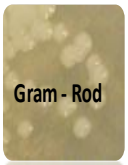


Figure 3.14: Dendogram bacterial cluster according to biochemical tests report of DFI and non-DFI patients isolates.

DFI gram negative isolates had moderate capacity of metabolizing carbohydrates. 49% of the isolates utilized dextrose, 50% of the isolates utilized sucrose, 30% was able to utilize citrate as an alternative carbon source, and 54% were able to oxidize glucose with the production of acid in methyl red test. Non-DFI gram negative isolates had contrasting sugar metabolisms properties than the DFI isolates. Non-DFI 24% isolates utilized dextrose and 40% isolates utilize sucrose and 30% were able to oxidize glucose with the production of acid in methyl red test and utilize citrate as an alternative carbon source. 95% DFI and 83% non-DFI gram positive isolates were catalase positive and 10% were oxidase positive.

Table.3.3. Morphological and biochemical characteristics of isolates from pus samples of DFI and non-DFI patients. DFI isolate were marked in bold. A representative figure of each group of organism and gram staining reactions are mentioned within the column morphology.

Groups	Diabetic "D" Isolates	Non Diabetic "C"Isolates	Colony Character in NA Plate	Bio chemical tests														Probable Organisms
				KIA Butt	KIA Slant	H2S	MIU	Cit	Lac	Dex	Suc	MR	VP	OX	CAT	MT	BIS	
1	**39Dmh1,2,3,39 Dsa1,39Dcip1,2, 39Das2,49Dsa1, 4,49Dmh1,251D sa1,61Dmac1,m h1cip2**	1Cmh3,cip2,cefo2,sa1,4Cm h3,cefo1,cip+cef1,2,6Cmh1, cip1,cip3,cefo2	**Golden Yellow/ whithis Regular, very small,Dry Raised**	**x**	**x**	**x**	**x**	**x**	**x**	**x**	**x**	**x**	**x**	-	+	+	-	 *Staphylococcus* spp.
2	**40Dma1,2,3,40 Dmh1,2,40Dci1, 40Dcip2,3,40Ds a1,40Dsa2,3,4,4 0Dci+cef1,2,41 Dsa4,45Dma1,2 45Dcip+cef1,2,4 7Dma2,48Dmh1 ,mh2,5sa1,54D mh1,2,mac3,56 Dmh2,50Dswa1**	1Cmac2,3,1mh1,2,4,cip1,3D cip+cef1,2,sa2,2mac2,3,mh 1,cip1,cefo2,sa2,3mac2,ma c3,cip+cef3,4Cmh1,mh2,5C mh1,efo1,7Cmh1,cef1	**Pale /Greenish Yellow, Irregular, large, Gummy, Flat**	**R**	R	-	+	+	-	-	-	-	+	+	+	-	-	 *Pseudomonous* spp.
3	**41Dmh1,41Dmh 2,41Dsa2,41Dsa 5,41Dsa6, 41Dcef1,41Dcef 2, 42Dmh1,mh3, 42Dsa2,sa4,55D mh2,cefo2,cip2, cip+cef2**	X	**Whitish, Regular, very small ,Dry, Raised**	**x**	x	x	x	x	x	x	x	x	x	x	x	-	+	 *Enterococcus* spp.
4	**44Dmh1,3,46D mh2,mh4,50Dce f251mac1,2,mh1 ,2,cefo1,2,3,sa2, 3.,52mh1,2,52D sa2,3,56Dmac1, mac2,mh1**	3Ccip1, mh4,mac2,3,5Ccefo1	**Whitish, Regular, Large, Gummy, Raised**	**Y**	R/Y	-	+	+	-	+	+	+	-	-	+			 *Enterobacter* spp.

5	**49Dswab2,3,cip +cef1,2,,50mac1 ,2mh1,2,52awab 1,54mac1,2,mh3 ,59Dmac3,mh2, 3,swa2**	X	**Whitish Creamy, Regular, Very Large, Gummy, Raised**	**Y**	Y	-	+	+	+	+	+	+	-	-	-	-	+	 **Gram - Rod** *Klebsiella* spp.
6	**44Dmac1,2,44D mh2,44DCip+ce fo1,2,44Dcef1,2, 45Dmh1,cef1,2, 3,4,52Dmac1,2, 53mh1,2,3,56Dc ip+cef1,2**	3Ccip+cef1,cip+cef2,4Ccef 1,2,7mac2,3,cip1,cip2,cefo2, cefo3,cip+cef1,2,9Cmac1,2, mh1,2,cip1,2,cefo1,2,cip+ce fo1,2	**Creamy, Regular, Large, Gummy, Raised**	**R**	R	-	-	-	-	+	-	-	-	-	-	-	-	 **Gram - Rod** *Acineobacter* spp.
7	**43Dmh1,2,3,4,4 4Dmh3,44sa1,2, 3,45Dcefo1,2,3, 4,46Dmh1,3,46 Dsa1,sa2,47Dm h1,2,3,47Dsa2,4 7Dsa3,47Dcef2, 47cef3**	X	**Whitish, Irregular, Large, Dry, Flat**	**x**	x	x	x	x	x	x	x	x	x	+	+	-	-	 **Gram + Rod** *Bacillus* spp.
8	**55Dmac1,2,mh1 ,cip1,cefo1,cip+ cef1**	2Cmh3,cip+cef1,2,swab1,s wab3,4Ccip1,1mac1,4,2ma c2,4cp+cef2,cip+cef3,mac1 ,mac2,.10Cmac1,mh1,cip,c efo2,cip+cefo2	**Creamy Whitish, Regular, Medium Size, Gummy , Raised**	**Y**	Y/R	+	+	+	+	+	+	+	-	-	-	-	-	 **Gram - Rod** *Ctrobacter* spp.
9	**56Dmac1,2,mh1**	5Cmac1,2,cefo2,cip1,cip2,c efo2,cip+cef1,2,6mac1,2,m h1,2,3,cip2,cefo1,2,3,cip+ce f1,cip+cef2,	**Pale, Irregular Large,Gu mmy, Flat**	**Y**	Y/R	+	+	+	-	+	+	+	-	-	-	-	-	 **Gram - Rod** *Proteus* spp.
10	**x**	3Ccefo3	**white, very small, Creamy**	**R**	R	-	+	+	-	-	-	-	+	-	-	-	-	 **Gram - Rod** *Alcaligens faecalis.*

X indicates test not done for the isolates

Table 3.4: According to Biochemical tests Organism Growth isolated from DFI patients.

SAMPLE ID Diabetic "D"	Total isolates (213)	Types of isolates	Probable Organism
39/D/FCH	9 isolates	2	*Staphylococcus* spp.
40/D/FCH	16isolates	3	*Pseudomonus* spp. *Bacillus* spp.
41/D/FCH	10 isolates	3	*Enterococcus* spp. *Pseudomonus* spp.
42/D/FCH	8 isolates	2	*Enterococcus* spp. *Pseudomonous* spp.
43/D/FCH	4 isolates	1	*Bacillus* spp.
44/D/FCH	16isolates	3	*Acinetobacter* spp. *Enterobacter* ssp. *Bacillus* ssp.
45/D/FCH	16 isolates	2	*Bacillus* ssp. *Pseudomonus* spp.
46/D/FCH	9 isolates	2	*Pseudomonous* spp. *Enterobacter* ssp.
47/D/FCH	13 isolates	2	*Pseudomonus* spp. *Bacillus* spp.
48/D/FCH	3 isolates	2	*Pseudomonus* spp. *Staphylococcus* spp.
49/D/FCH	8isolates	3	*Klebsiella* spp. *Enterobacter* spp. *Staphylococcus* spp.
50/D/FCH	9isolates	3	*Klebsiella* spp. *Enterobacter* spp. *Pseudomonus* spp.
51/D/FCH	10isolates	2	*Enterobacter* spp. *Staphylococcus* spp.
52/D/FCH	9isolates	3	*Enterobacter* spp. *Acinetobacter* spp. *Klebsiella* spp.
53/D/FCH	3 isolates	1	*Acinetobacter* spp.
54/D/FCH	6isolates	2	*Klebsiella* spp. *Pseudomonus* spp.
55/D/FCH	10 isolates	2	*Citobacrter* spp. *Enterococcus* spp.
56/D/FCH	6 isolates	2	*Pseudomonous* spp. *Proteus* spp.
58/D/FCH	4isolates	2	*Pseudomonous* spp. *Enterobacter* spp.
59/D/FCH	8isolates	2	*Enterobacter* spp. *Klebsiella* spp.
60/D/FCH	8isolates	1	*Pseudomonous* spp.
61/D/FCH	5 isolates	1	*Pseudomonous* spp.
62/D/FCH	8isolates	2	*Pseudomonous* spp. *Staphylococcus* spp.
63/D/FCH	6 isolates	1	*Pseudomonous* spp.
64/D/FCH	4isolates	2	*Pseudomonous* spp.
65/D/FCH	2isolates	2	*Staphyloccus* spp. *Enterococcus* spp.
67/D/FCH	3 isolates	1	*Staphyloccus* spp.

Table: 3.5: According to Biochemical tests Organism Growth isolated from Non-DFI (Control) Patients.

SAMPLE ID Control"C"	Total isolates (131)	No of types of bacteria	Probable Organism
1/C/DMC	17 Isolates	**3**	*Pseudomonous* spp. *Citrobactersp* spp. *Staphylococcus* spp.
2/C/DMC	17 Isolates	**2**	*Pseudomonous* spp. *Citrobacter* spp.
3/C/DMC	17 Isolates	**5**	*Pseudomonous* spp. *Enterobacter* spp. *Citrobacter* spp. *Alcaligens fecalis* spp. *Acinetobacter* spp.
4/C/DMC	12 Isolates	**4**	*Pseudomonous* spp. *Eschereria coli* spp. *Acinetobacter* spp. *Staphylococcus* spp.
5/C/DMC	11 Isolates	**2**	*Pseudomonous* spp. *Providentia* spp.
6/C/DMC	14 Isolates	**2**	*Acinetobacter* spp. *Staphylococcus* spp.
7/C/DMC	12 Isolates	**2**	*Bacillus* spp. *Pseudomonous* spp.
8/C/DMC	11 Isolates	**2**	*Pseudomonous* spp. *Bacillus* spp.
9/C/DMC	10 Isolates	**1**	*Acinetobacter* spp.
10/C/DMC	10Isolates	**2**	*Citrobacter* spp. *Pseudomonous* spp.

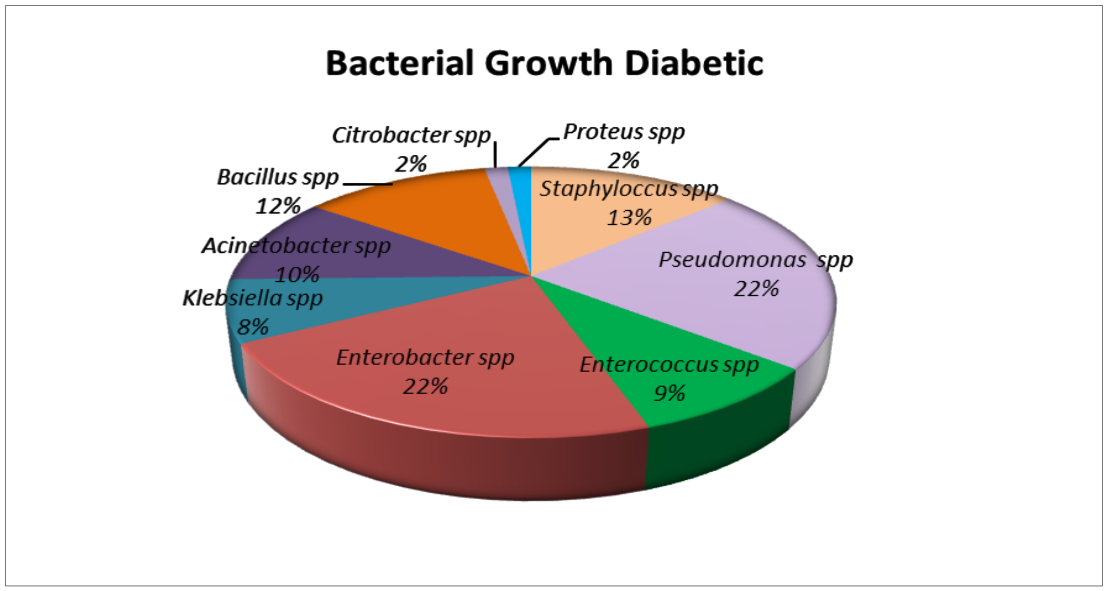
3.5. Composition of Bacterial population with in DFI and non-DFI patients.

Based on morphological and biochemical identification, the most predominant isolated organism in both DFI and non-DFI patient's pus samples was *Pseudomonous* spp. (22% DFI and 29% Non-DFI; Figure 3.15). *Enterobacter* spp. predominated in DFI and was found 22%, whereas, it was 7% in non-DFI patients. *Staphylococcus* spp. was equally detected (13%) in both study subjects. *Klebsiella* spp. and *Enterococcus* spp. accounted 8% and 9% respectively only in DFI patients. *Bacillus* spp. was found 12% in diabetic pus samples but in non-DFI, it was only 3%. The presence *Citrobacter* spp. (29%) was high in pus samples of non-DFI than DFI patients. Presence of *Alcaligens fecalis*, *Providentia* spp. and *Escherichia coli* (each 3%) were only found in non-DFI pus samples respectively. However *Proteus* spp. (2%). was detected only in DFI patient's pus samples.

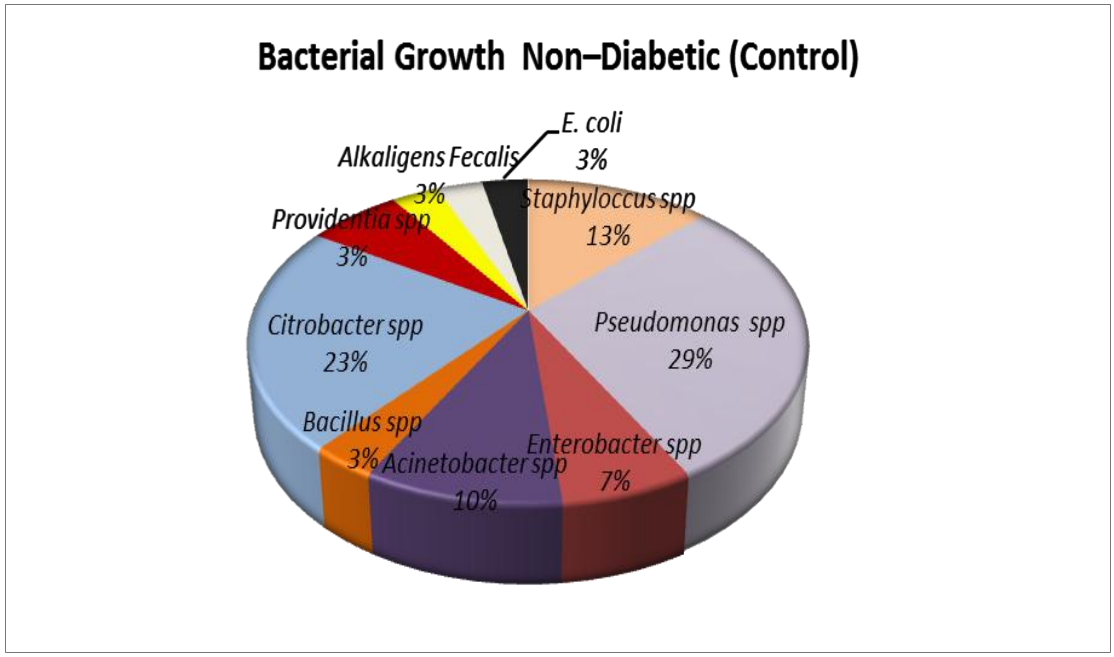
Enterococcus spp. (9%), and *Klebsiella* spp. (8%) occurred only in DFI and the other predominant bacteria in DFI patients over non-DFI were *Enterobacter* spp. (22%) and *Bacillus* spp. (12%). In contrast, non-DFI patient's samples predominated *Pseudomonas* spp. (29%) and *Citrobacter* spp. (29%). In both cases, *Staphylococcus* spp. (13%) and *Acinetobacter* spp. (10%) contained same percentages.

Figure 3.15: Distribution of bacterial genera with in pus samples of (a) DFI and (b) non-DFI (control) Patients.

(a)



(b)



3.6. Isolation of Multidrug Resistant (MDR) organism

Antibiogram of the isolates are showed in (Table 3.6). All isolates either from DFI and non-DFI samples were tested against 21 commonly used antibiotics belonging to 12 different groups. Different groups of isolates showed distinctly resistance to different antibiotics (Table 3.6 and 3.7). Bacteria isolated from DFI samples were more resistance to most of the antibiotics used in this work in contrast to bacteria isolated from non-DFI samples; and same bacteria isolated from two samples were showed different in resistance pattern. Most of the isolates of group 1 were commonly resistant to monobactum (ATM) groups of antibiotics. It was observed that group 2 was mostly resistant to cephalosporin (CAZ, CRO,CXM) groups of antibiotics. Group 3 isolates also showed resistant to monobactum (ATM) groups of antibiotics. Isolates of group 3, group5 and Group7 were found only in diabetic patients. These three groups were commonly resistant to monobactam (ATM) groups of antibiotics. Group 4 was commonly resistant to both monobactam (ATM) & penicillins (AMP) groups of antibiotics. Cephalosporin (CAZ, CRO, CXM) and penicillin (P) groups of antibiotics were found resistant for Group 8 isolates. Group 9 also found resistance to monobactum (ATM) groups of antibiotics (Table 3.6 and 3.7).

(Table 3.8) Representing *Staphylococcus* spp. of DFI patients was found 100% resistant to monobactum (ATM) groups of antibiotics. *Staphyloccous* spp. of DFI patients was 67% resistant to penicillins (AMP) groups of antibiotics but *Staphyloccous* spp. isolated from Non-DFI patients was found 100% sensitive to that penicillins (AMP) groups of antibiotics. However *Staphylococcus* spp isolated from both DFI and non-DFI pus samples were sensitive to carbapenem (IPM) groups of antibiotic. cephalosporins (CAZ,CRO,CXM) groups were found moderately resistant in *staphylococcus* spp. and *Pseudomonous* spp. isolates of DFI patients. P*seudomonous* spp. of DFI patients were 33% resistant to cabapenem (IPM) group of antibiotics. 83% DFI *pseudomonous* spp. were resistant to penicillins (AMP) groups of antibiotics and 78% *pseudomonous* spp. was resistant to monobactum (ATM) groups of antibiotics.

DFI *Enterobacter* spp. was found moderately resistance to penicillin (P), monobactam (ATM) & cephalosporin (CAZ, CRO, CXM) groups of antibiotics (Table 3.8). 50% of non-DFI *Enterobacter* spp. was resistant to penicillins (AMP), monobactum (ATM), tetracycline (DO), lincosamide (lincomycins) (DA), and polypeptides (PB) groups of antibiotics. 86% *Acinetobcter* spp. isolated from DFI patients were resistant to AMP & CXM and 57% *Acinetobacter* spp. were resistance to lincosamide (lincomycins) (DA), cephalosporins (CAZ, CRO, CXM) groups of antibiotics. *Bacillus* spp. isolated from DFI & Non-DFI was respectively 88% & 50% resistance to monobactam (ATM), cephalosporin(CAZ,CRO,CXM), & penicillins (AMP) groups of antibiotics. *Klebsiella* and *Enterococcus* spp. only isolated from DFI patients. *Klebsiella* spp. was showed 100% resistance to penicillin (P) groups of antibiotics & *Enterococcus* spp. was showed 100% resistance to polypeptides (PB), amikacin (AK), rifamycin (RD) groups of antibiotics. *Citrobacter* spp. isolated from both DFI and non-DFI were 100% resistant to cephalosporin (CAZ, CRO, CXM) group of antibiotics (Table 3.8).

Generally we found *Staphylococcus* spp. isolated from DFI was found 100% resistant to monobactam (ATM) groups of antibiotics and 67% resistant to penicillins (AMP)groups of antibiotics however the same was 100% sensitive to that penicillin(P) for non-DFI. *Pseudomonous* spp. isolates of DFI were resistant to cabapenem (IPM), cephalosporins (CAZ,CRO,CXM) and monobactam (ATM) group of antibiotics 33%, 72% and 78% respectively in contrast in Non-DFI it was found resistance to 8%, 62%, and 69% respectively. *Enterobacter* spp. isolated from of DFI was found 56% resistance to penicillins (AMP), monobactam (ATM) & cephalosporin (CAZ, CRO, CXM) groups of antibiotics and in Non-DFI it was found 50% (Table 4). *Acinetobcter* spp. isolated from DFI were 86% to penicillins (AMP) and Cephalosporins (CAZ,CRO,CXM) in contrast in Non-DFI it was found 57%. *Bacillus* spp. isolated from DFI and Non-DFI was found respectively 88% and 50% resistance to monobactam (ATM), cephalosporin (CAZ, CRO, CXM), & penicillin (AMP) groups of antibiotics. *Citrobacter* spp. isolated from both DFI and non-DFI patients were 100% resistant to cephalosporin (CAZ, CRO, CXM) group of antibiotics (Table 3.8). However average 82% DFI isolates and 90% non-DFI isolates were found sensitive to carbapenem (IPM) groups of antibiotics.

Table 3.6: Antibiotic Resistant pattern of 10 groups of isolates from DFI and non-DFI (Control) patients.

	Antibiotics Resistance Patteren			
	Diabetic Id No "D"	**Antibiotics**	Non Diabetic Id No "C"	**Antibiotics**
***Group1 Straphylococcus* spp**	**39D mh1,2, 39Dcip1,2**	AMC,ATM,AMP,AZM,CAZ,CXM,CIP,CL,PB	1Cmh3,cip2,cefo2 Swab1	ATM,CFM,CL,PB
	39Dswab1,39Dmh3	ATM,AMP,AZM,CAZ,CIP,CL	4Cmh3	ATM,CAZ,CRO,CFM
	39 DSwab2	AK,AMC,CRO,CXM,C,CTX,CNF,DA,RD,ATM,AMP,AZM,	4C cefo1	ATM,AMP,AZM,CAZ,CRO,CXM,CFM,CL
	51Dswab1	AMC,ATM,AMP,AZM,CAZ,CXM,CTX,CN,	6Ccip1,3,cefo2	ATM,AMP,CAZ,CXM,CIP,C,CFM
	62Dmac1,mh1,cip2	AMC,ATM,AMP,CAZ,CRO,CXM,CTX,FEP,PB,CL	6Cmh1	AMC,ATM,AMP,CAZ,CRO,CXM,C,CTX,CFMC,CTX,CO,DA
***Group 2 Pseudomonous* spp**	**40Dmac1,2,3,mh1,2,3,cip1,2,3,swab1,2,3,4,cip+cef1,2**	AMC,ATM,AMP,CAZ,CRO,CXM,CIP,CTX,IPM,DO,FEP,F,CO,DA,RD	1Cmac3,mh1,2,4,cip1,3,cefl1, cip+cefo1,2,swab1,2,	AK,AMC,ATM,AMP,AZM,CAZ,CRO,CXM,CFM,CIP,C,CN,DO,,F,CO,DA,RD
	41Dswab2,4,	AK,AMC,AMP,AZM,CAZ,CRO,CXM,CIP,C,CTX,CN,IPM,DO,FEP,F,CO,DA,RD		
	45Dmac1,2,mh1cip+cef1,2	AMC,ATM,AMP,AZM,CRO,CXM,C,CTX,IPM,F,CO,DA,RD	2CMAC2,3,mh1	AK,AMC,ATM,AMP,AZM,CAZ,CRO,CXM,CIP,C,CN,DO,CFM,F,CO,DA,RD
	47Dmac1	AK,AMC,ATM,AMP,AZM,CAZ,CRO,CXM,CIP,C,CTX,IPM,CFM,F,CO,DA,RD,PB	2C cip2,swab2	AK,AMC,ATM,AMP,CAZ,CRO,CXM,CIP,C,CN,DO,CFM,F,CO,DA,RD
	47Dmac2,cefo1,swab1, cip+cef1,2 54DMac4,mh1,2	AK,AMC,ATM,AMP,AZM,CAZ,CRO,CXM,CIP,C,CTX,CN,DO,CFM,F,CO,DA,RD	2Ccefo2	AK,AMC,ATM,AMP,CRO,CXM,CIP,C,CL,DO,CFM,F,CO,DA,RD
	48Dmh1,2	AMP,AZM,CN,CXM,F,CO,DA	4Cmh1,	AMC,ATM,AMP,CXM,DA,RD
	50Dswab1	AMC,ATM,AMP,CRO,CXM,CTX,CN,IPM,	4Cmh2	AMC,ATM,AMP,AZM,CAZ,CRO,CXM,CIM,CTX,DO,CFM,DA,RD
	56D mac1,2,mh1,2	AZM,DO,F,	5Cmh2,3	AMC,AMP,CAZ,CRO,CXM,CIP,C,CTX,CN,DO,CFM
	58Dmh1	AK, DA	5Cmh1,cefo1	AMC,AMP,CAZ,CRO,CXM,IP,CFM,F,DA,RD
	60Dmac1,2,cip2	AK,AMC,ATM,AMP,AZM,CAZ,CRO,CXM,CFM,CN,DO,PB	7 Cmh1,cefo1	AMC,AMP,AZM,CAZ,CXM,C,CTX,CN,DO,CFM,F,RD,DA
	61Dmac1,mh1,2,cefo1,2	AMC,ATM,AMP,AZM,CAZ,CRO,CXM,CTX,CFM,	8Ccip+cefo1,2,cefo1	AMC,ATM,AMP,CAZ,CRO,CXM,CIP,C,IPM,DO,CFM,F,CO,DA,CL,PB,RD
	62Dmac2,mh2	AK,AMC,AMP,CAZ,CRO,CXM,CRX,FEP,C,CIP,CN,DO, PB, CL, CO	10Cmac2,mh2,cip2,cefo2,cip +cefo1	AK,AMC,ATM,AMP,AZM CRO,CXM,CIP,DO,F,DA,RD
	64Dmh1,2	AK,AMC,AZM,CAZ,CRO,CXM,CFM,C,CIP,CN,DO,PB,CL		
***Group3 Enterococcus* spp**	**41Dmh1,2,swab5,6,cefo1,2**	AK,ATM,AMP,AZM,CAZ,CRO,CXM,CTX,CN,DO,CFM,DA,RD,PB	X	X
	42Dmh1,2,3,swab1,2,4	AK,CL,RD		
	49Dmh1,2	ATM,AZM,CO		
	55Dmh2,cip2,cefo,cip+cefo2	AK,AZM,CAZ,CRO,CXM,CIP,C,CTX,DO,CO,DA,CL		

Antibiotics Resistance Pattern				
Diabetic Id No "D"		**Antibiotics**	Non Diabetic Id No "C"	**Antibiotics**
Group4 Enterobacter spp	**44Dmh1,2,4,**	AK,AMC,ATM,AMP,AZM,CAZ,CRO,CXM,C,CTX,CN,FEP,F,DA,RD	3Cmac2,3	AMC,AMP,AZM,CXM,DO,DA,CL,RD
	46Dmh1,2	AMC,ATM,AMP,CAZ,CRO CXM,CTX,CFM,CO,DA,CL,RD,PB		
	50Dswab2,3	ATM		
	51Dmac1,2	AMC,ATM,AMP,CAZ,CRO,CXM,CTX,CN,DA,RD		
	51Dswab2,3	AMP,AZM,DA,RD		
	51Dmh1,2,cfeo1,2,3	AMC,ATM,AMP,CAZ,CRO,CXM,CTX,CN,DA, F , RD	3Ccip1,mh4	AMC,AMP,CXM,DA
	52Dcefo2	ATM,CL,PB		
	52D swab2,3	ATM,AMP,AZM,AMC,AXM,CTX,CN,F,DA	5Ccefo1	AMC,AMP,CAZ,CRO,CXM,IP,CFM,F,DA,RD
	59Dmac1,2,mh1,swab1	DO,CO		
Group5 Klebsiella spp	**49Dswab2,3cip+cefo1,2**	AMC,ATM,AMP,AZM,CAZ,CRO,CXM,C,CTX,CN,DO,FEP,CO,DA,DR	X	**X**
	50Dmac1,2,mh1,2	AMC,AMP,DO,DA,RD		
	51Dswab1	ATM,AMP,AZM,CAZ,CRO,CXM,CTX,CN,DA,RD		
	54Dmh3	AMC,AMP,AZM,CXM,CIP,CN,DO,F,CO,RD,CFM		
	59Dmac3,mh2,3,swab2	AZM,AMP		
Group6 Acinetobacter spp	**44Dmac1,2cip+ceo1,2,cefo1,2**	AK,AMC,ATM,AMP,AZM,CAZ,CRO,CXM,CIP,C,CTX,CN,IPM,CFM,F,CO,DA,RD	3Ccip+cefo1,2,3,swab1,3	AK,AMC,ATM,AMP,AZM,CAZ,CRO,CXM,CIP,CTX,C,CFM,CN,IPM,F,CO,DA,RD
	44Dmh2	AK,AMC,ATM,AMP,AZM,CAZ,CRO,		
	45Dcefo1,2,3,4	CXM,CIP,C,CTX,CN,FEP,F,DA,RD	2Ccfo1	AMC,ATM,AMP,AZM,CXM,DA,RD
	52D mac1,2	ATM,AMP,AZM,CAZ,CRO,CXM,DA		
	53Dmh1,2,3	AMC,ATM,AMP,CAZ,CRO,CXM,CN,F,DA,RD	4Ccfeo1,2	AK,AMC,ATM,AMP,DA,RD,PB,CAZ,CRO,CXM,CIP
	54Dmac1,2	AAMP,AZM,CXM,CIP,CN,DO,F,CO,RD,CFM	7Cmac2,3,cip1,2,cefo2,3,mh2,cip+cefo1,2,9Cmac1,2,mh1,2,cip1,2,cefo1,2,cip+cefo1,2.	AK,AMC,ATM,AMP,AZM,CAZ,CRO,CXM,CIP,C,CTX,CN,CFM,F,DA.FEP
	56Dcip+ceo1,2	AMP,AZM,CAZ,CRO,CXM,CIP,CTX,CN,DO,		

Antibiotics Resistance Pattern				
Diabetic Id No "D"		**Antibiotics**	Non Diabetic Id No "C"	**Antibiotics**
***Group7 Bacillus* spp**	**43Dmh1,2,3,4**	AMC,ATM,AMP,CAZ,CXM,CTX,CFM,CL,PB	X	X
	44Dswab3	AMC,ATM,AMP,CAZ,CRO,CXM,CTX,CFM,DA,CL		
	45Dcefo1,2,3,4	AMC,ATM,AMP,CAZ,CXM,CTX,CFM,F,DA		
	44Dswab1,2,mh3	AK,AMC,ATM,AMP,AZM,CAZ,CRO,CXM,CIP,C,CTX,CN,CFM,F,CO,DA,RD		
	46Dmh3,swab2	AMC,ATM,AMP,CAZ,CRO,CXM,CTX,CFM,RD		
	46Dswab1	AMC,ATM,AMP,AZM,CAZ,CRO,CXM,CTX,CFM,CL,RD		
	47Dcefo2,swab2,3,mh1,2,3,cef3	AMC,ATM,AMP,AZM,CAZ,CRO,CXM,CTX,CFM,CO,CL,PB		
***Group8 Ctrobacter* spp**	**55Dmac1,2mh1cefo1cip1,cip+cefo1**	AMC,ATM,AMP,CAZ,CRO,CXM,CIP,CTX,CN,DO,CO,CL,RD	1Cmac1,4	AMC,AMP,AZM,CRO,CAZ,CXM,CFM,C,CTXCN,CO,DO,DA,F,RD
			2Cmh3,cip+cef1,2,Swab1,3	AK,AMC,ATM,AMP,AZM,CAZ,CRO,CXM,CIP,CTX,C,CFM,CN,IPM,F,CO,DA,RD
			2Cmac1	AK,AMC,ATM,AMP,CAZ,CRO,CXM,CTX,CN,CFM,CO,DO,DA,RD
			10Cmac1,mh1,cip,cefo2,cip+cefo2	AK,AMC,ATM,AMP,AZM,CRO,CXM,CIP,C,CFM,F,CO,DA,RD
			3Cmac1	AMC,ATM,AMP,CRO,CXM,CIP,DO,CFM,CO,DA
			4Ccip1,2,cip+cefo2,3,mac1,2	AMC,ATM,AMP,AZM,CAZ,CRO,CXM,CTX,DO,CO,DA,RD
***Group9 Proteus* spp**	**56Dmac1,2,mh1**	AZM,DO,F,	5Cmac1,2,cefo2,cip+cef1,26Cmac1,2,mh2,3,cip2,3,cefo1,3,cip+cefo1,2,	AMC,AMPAZM,CAZ,CRO,CXM,CIP,C,CTX,CN,DO,CFM,CO,DA,CL,PB
Group 10 Alcaligens Fecalis	X	X	3Ccefo3	DA,RD

AK,Amikacin AMP ampicillin (10 lg); ATM, aztreonam (30 lg); AZM, azithromycin (15 lg); C, chloramphenicol (30 lg); CIP, ciprofloxacin (5 lg); CN,gentamicin (10 lg); CTX, cefotaxime (30 lg); DA, clindamycin (2 lg); DO, doxycycline (30 lg); E, erythromycin (15 lg); F, nitrofurantoin (300 lg);FEP, cefepime (30 lg); IPM, imipenem (10 lg); P, penicillin G (10 lg); PB, polymyxin B (300 lg); RD, rifampicin (05 lg).

Table 3.7: Antibiotics group commonly resistance 10 groups of isolates from DFI and non-DFI (Control) patients.

Groups	Probable Organism	Patients	No of Antibiotic Group Commonly Resistance		Antibiotics
1	*Staphylococcus* spp.	Diabetic	2	Monobactum ,Penicillin	ATM,AZM,AMP,P (4)
		Non Diabetic (Control)	1	Monobactum	ATM,AZM (2)
2	*Pseudomonas* spp.	Diabetic	4	Monobactum , Cephalosporin, Lincosamide ,Nitrofuran	ATM,CRO,CAZ,CTX,CXM,CTX,CFM,F,DA (9)
		Non Diabetic (Control)	2	Penicillin ,Cephalosporin	AMP,CRO,CAZ,CXM (4)
3	*Enterococcus* spp.	Diabetic	3	Monobactum, Cephalosporin ,Plypeptides	ATM,AZM,CRO,CAZ,CTX,CXM,CTX,CFM,PB (8)
		Non Diabetic (Control)	X		
4	*Enterobacter* spp.	Diabetic	5	Monobactum ,Penicillin Cephalosporin ,Lincosamide, Nitrofuran	ATM,AMP,P,CRO,CAZ,CTX,CXM,CTX,CFM,F,DA (10)
		Non Diabetic (Control)	1	Penicillin	AMP(1)
5	*Klebsiella* spp.	Diabetic	3	Monobactum, Cephalosporin ,Amoxyclav	ATM,AMC,CRO,CAZ,CTX,CXM,CTX,CFM (9)
		Non Diabetic (Control)	X		
6	*Acinetobacter* spp.	Diabetic	2	Penicillin ,Cephalosporin	AMP,CRO,CAZ,CXM (4)
		Non Diabetic (Control)	2	Penicillin ,Cephalosporin	AMP,CRO,CAZ,CXM (4)
7	*Bacillus* spp.	Diabetic	6	Monobactum ,Amoxyclav, Penicillin ,Cephalosporin Lincosamide ,Nitrofuran	ATM,AZM,AMP, AMC,P,CRO,CAZ,CTX,CXM,CTX,CFM,F,DA (13)
		Non Diabetic (Control)	X		
8	*Citrobacter* spp.	Diabetic	2	Penicillin ,Cephalosporin	AMP,P,CRO,CAZ,CTX,CXM (6)
		Non Diabetic (Control)	4	Penicillin ,Aminoglycosides, Cephalosporin ,Teracycline	AMP,P,CN,CRO,CAZ,CTX,CXM,DO (8)
9	*Proteus* spp.	Diabetic	1	Penicillin	P (1)
		Non Diabetic (Control)	2	Penicillin ,Cephalosporin	AMP,P,CRO,CAZ,CTX,CXM (6)
10	*Alcaligens* SPP.	Diabetic	x		
		Non Diabetic (Control)	1	Lincosamide	DA (1)

Table 3.7 representing no of antibiotics groups commonly resistance to DFI and non-DFI isolates. DFI *staphylococcus* spp. was commonly found resistance to 2 groups of antibiotics (Penicillin and Monobactam) 4 antibiotics (ATM,AZM,AMP), *Staphylococcus* spp. isolated from non-DFI patients was found commonly resistance to one groups of antibiotics (penicillin) 2 antibiotics (AMP,P). *Pseudomonas* spp. isolated from DFI patients was found commonly resistance to 4 groups of antibiotics (Penicillin, Cephalosporin, Lincosamide & Nitrofuran) 9 antibiotics (ATM, AMP, P, CAZ, CRO, CXM, CTX, DA, F). However, *Pseudomonas* spp. isolated from non-DFI patients was found resistance to only to groups of antibiotics (Penicillin & Cephalosporin), 4 antibiotics (AMP, CRO, CAZ, CXM). *Enterococcus* spp. was only isolated from DFI patients. *Enterococcus* spp. was found commonly resistance to 3 groups of antibiotics (Monobactum, cephalosporin & polypeptides), 9 antibiotics (ATM, AZM, CRO, CAZ, CXM, CTX, PB, CL). *Enterobacter* spp. isolate from DFI patients also showed high resistance pattern, found resistance to 5 groups of antibiotics (Penicillin, Monobactam, Cephalosporin, Lincosamide & Nitrofuran)11antibiotics (ATM, AMP ,P , CAZ, CRO, CXM, CFM, CTX, DA, F).While *Enterobacter* spp. of non-DFI patients was found resistance to only one groups of antibiotics (Penicillin). Found resistance to 2 antibiotics (AMP, P). *Klebsiella* spp. was isolated from only DFI patients pus samples, found commonly resistance to 3 groups of antibiotics (Monobactam, cephalosporin & Amoxyclav) 7 antibiotics (ATM, AMC, CAZ, CRO, CXM, CTX, CFM). *Acinetobacter* spp. isolated from both DFI and non-DFI patients were found commonly resistance to 2 groups of antibiotics (penicillin & cephalosporin) 5 antibiotics (AMP, P, CAZ, CRO, CXM). *Bacillus* spp. isolated from DFI patients showed highest resistance pattern, 6 groups of antibiotics (Monobactam, Amoxyclav, Penicillin, Cephalosporin, Lincosamide & Nitrofuran) was found resistance to 13 antibiotics (ATM, AMP, P, AZM, AMC, CAZ, CRO,CXM,CTX,CFM, FEP, DA, F). *Citrobacter* spp. isolated from non-DFI patients was found highly resistance to 4 groups of antibiotics (Penicillin, Aminoglycosides, cephalosporin & Tetracycline) resistance to 8 antibiotics (AMP, P, CN, CRO, CAZ, CXM, CTX, CFM, DO). DFI *Citrobacter* spp. was found resistance to 2 groups of antibiotics (Penicillin & cephalosporin) found resistance to resistance to 5 antibiotics (AMP, CAZ, CRO, CTX, and CXM)

3.7. Molecular characterization and genotyping:

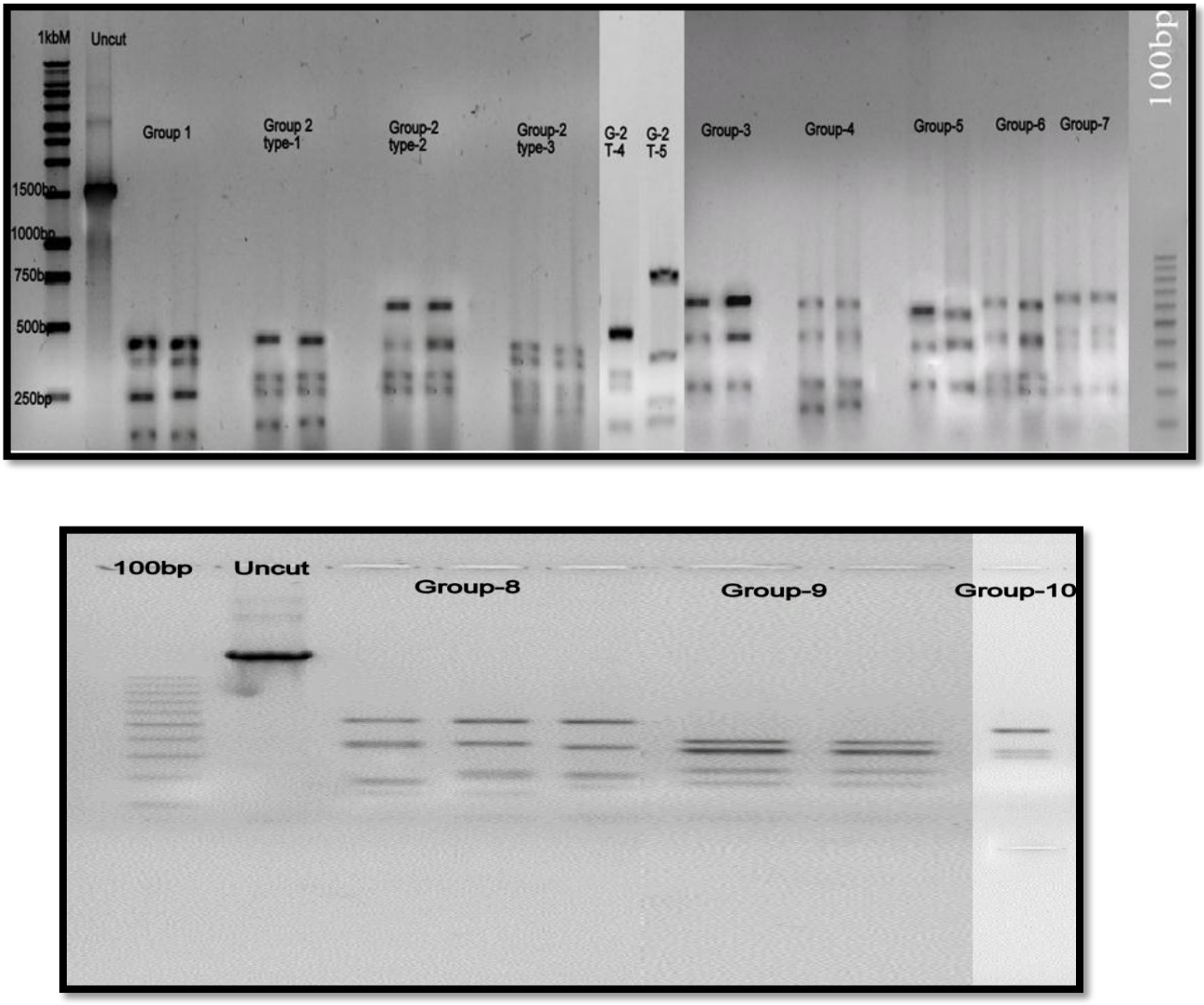


Figure 3.16: Agarose gel electrophoresis (on 1% agarose gel) of PCR specific amplicon of 16S rRNA gene. Each lane indicating isolate code, Marker used was 1 kb DNA ladder (Bioneer, USA) Restriction digestion with Hha1 enzyme of the approximate 1400-1450 bp amplified fragment of 16S rRNA gene showed different restriction pattern for all of the isolates. ARDRA pattern of representative isolates from 10 groups. Isolates Group 1(39/D/MH3,39/D/CIP2)Group2(47/D/MAC2,54/D/MH1,45/D/CIP+CEFO1,3/C/CIP+CEFO2,50/D/SWAB1)Group3(41/D/MH2,55/D/MH2)Group4(44/D/MH1,46/D/MH2)Group5(49/D/AWSAB2,52/D/AWAB1)Group6(44/D/SWAB2,44/D/CIP+CEFO1)Group7(44/D/SWAB2,43/D/MH1)Group8(2/C/MH3,)Group9(56/D/MH1,5/C/MAC1)Group10(3C/CEFO3).

3.7.1. *Amplified ribosomal DNA restriction analysis (ARDRA).*

Template DNA from each of the isolates was subjected to amplification of their 16S rRNA gene using universal primers 27F and 1492 R. PCR product of approximately 1400-1450 bp was successfully amplified from each of the isolates. ARDRA of a PCR product (of approximately 1500 bp) of the different morphogroups of the isolates using restriction enzyme HhaI showed different restriction pattern. ARDRA of 10 morphogroups showed distinct genotypic pattern except for morphogroup 2, isolates of which showed 5 different types of pattern (Figure 3.18).

ARDRA group 1 *Staphylococcus* spp. was showed same restriction pattern. Four fragments were obtained: 450bp, 350bp, 250bp, 100bp. *Pseudomonas* spp. (Group2) showed three types of restriction pattern (Types 1, 2, 3, 4 and 5). From Types 1 four fragments were obtained: 500bp, 300bp, 250bp, 180bp. Type 2 were showed 600bp, 400bp, 300bp, 250bp and Type 3 showed 400bp, 350bp, 300bp, 250bp, 100bp restriction fragments. Type 4 were also showed 4 fragments 500bp, 300bp, 350bp, 150bp. Type 5 were showed 4 fragments 700bp, 400bp, 200bp, 250bp. *Enterococcus* spp. (Group3) was showed four fragments: 500bp, 400bp, and 250bp. *Enterobacter* spp. (Group4) was also showed four fragments 550bp, 400bp, 200bp, 100bp. Three fragments were obtained from *Klebsiella* spp (Group5): 500bp, 400bp, 200bp, 100bp. *Acinetobacter* spp. (Group6) Showed four fragments: 600bp, 400bp, 250bp, and 200bp. Group7 *Bacillus* spp. was showed four fragments were obtained from types 1 *Bacillus* spp. 600bp, 400bp, 350bp, and 200bp. Group 8 *Citrobacter* spp. were showed three fragments; 600bp, 450bp, 300bp. ARDRA group 9 *Proteus* spp. was showed four fragments 500bp, 400bp, 300bp and 200bp. Finally, ARDRA group 10 *Alcaligens* spp. was showed three fragments 450bp, 200bp, 250bp (Table 3.9).

Table: 3.9: Restriction Fragments size and No of fragments of Representative isolates from 10 ARDRA groups after digestion with HhaI enzyme.

ARDRA GROUP		No of Fragments	Fragments Size
Group1		4	450bp,350bp,250bp,100bp
Group2	Type1	4	500bp,300bp,250bp,180bp
	Type2	4	600bp,500bp,300bp,250bp
	Type3	5	450bp,350bp,350bp250bp,100bp
	Type4	4	500bp,350bp,300bp,150bp
	Type5	4	700bp,400bp,250bp,200bp
Group3		3	600bp,400bp,250bp
Group4		4	550bp,400bp,200bp,100bp
Group5		4	500bp,400bp,200bp,100bp
Group6		4	600bp,400bp,250bp,200bp
Group7		4	600bp,400bp,350bp,200bp
Group8		3	600bp,450,300bp
Group9		4	500bp,400bp,300bp,200bp
Group10		3	450bp, 200bp, 250bp

3.7.2 .*Plasmid Profile of representative isolates from 10 ARDRA group:*

Plasmid profile analysis of each morphogroups as well as ARDRA group of the isolates revealed that most of the isolates were plasmid free. (Table 3.10) Among 10 phenotypic as well as genotypic groups, representative DFI isolates *Enterobacter* spp. (Group 4; 44/D/MH1, 46/D/MH2) and *Klebsiella* spp. (Group5; 49/D/SWAB2, 52/D/SWAB1) harbor multiple plasmids. Representative isolates of non-DFI *Pseudomonas* spp. (Group 2; 3C/CIP+CEFO2), *Enterobacter* spp. (Group4; 5/C/CEFO1) and *Citrobacter* spp. (Group 8; 2/C/MH3) harbor plasmids (Figure 3.17). Representatives DFI isoloates *Pseudomonas* spp. (47/D/MAC2, 54/D/MH1, 45/D/CIP+CEFO1, 50/D/SWAB1) were found to be plasmid free showing resistance to penicillins (AMP), monobactam (ATM) and other groups of antibiotics. One of the *Pseudomonas* isolates (40/D/MAC1) showed resistance to more than 12 groups of antibiotics and were plasmid free (Table 3.10, a, b).

.(a) (b)

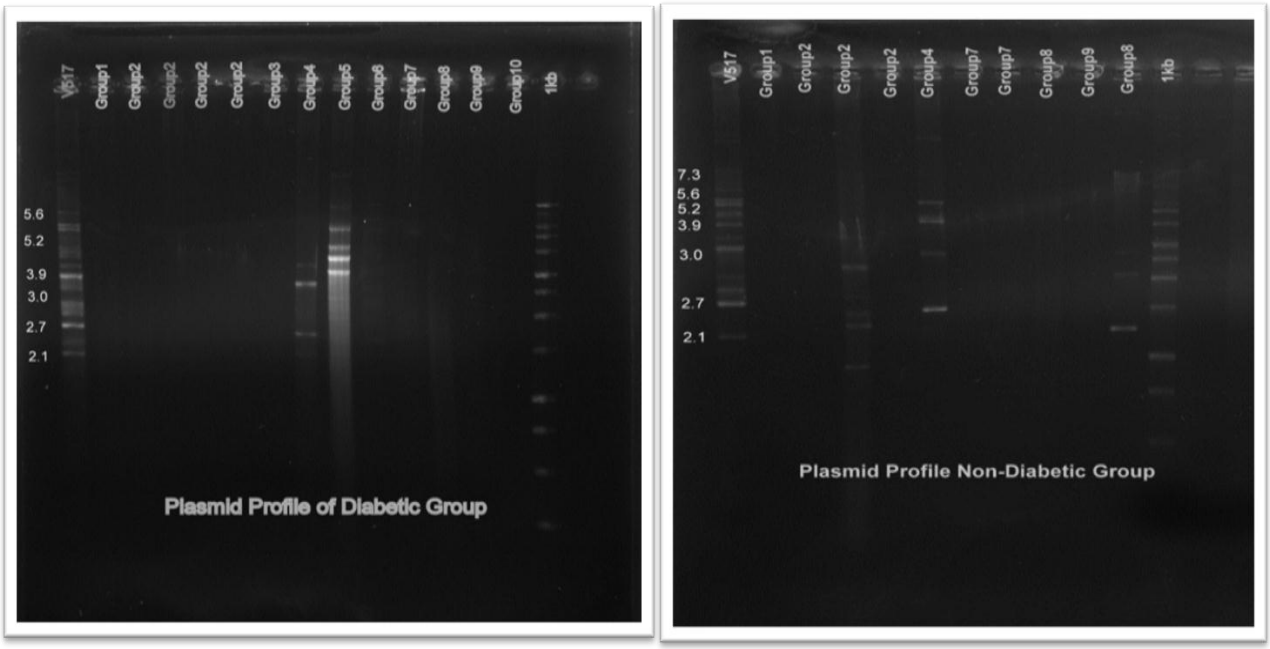


Figure3.17. Agarose gel electrophoresis (0.8% gel) of plasmid DNA showing the plasmid patterns among the representative isolates from 10 ARDRA groups. *E. coli* V517 was used as reference plasmid marker. The gel was run along with molecular marker of 1 Kb DNA ladder (Bioneer, USA).

Table 3.10 Number and size of plasmids of Representative (a) DFI & (b) non-DFI patients isolates from 10 ARDRA groups

(a)

Groups	Plasmid Diabetic Size(Kb)					
	Number of Bands					
	1	2	3	4	5	6
Group1	No plasmid					
Group2	No plasmid					
Group3	No plasmid					
Group4	4.5	4.1	2.4			
Group5	7.6	6.2	5.6	5.1	4.8	4.2
Group6	No plasmid					
Group7	No plasmid					
Group8	No plasmid					
Group9	No plasmid					
Group10	No plasmid					

(b)

Groups	Plasmid Non- Diabetic (Control) Size(Kb)			
	Number of Bands			
	1	2	3	4
Group1	No plasmid			
Group2	4.25	2.8	2.4	1.27
Group3	No plasmid			
Group4	7.9	6.2	5.5	4.8
Group5	No plasmid			
Group6	No plasmid			
Group7	No plasmid			
Group8	4.8	4.1	2.4	
Group9	No plasmid			
Group10	No plasmid			

Table3.10 Representing DFI patients *Enterobacter* spp. (group4) was found harbors 3 plasmid size (2.4kb-4.5kb). Were found moderately resistance to penicillin (AMP), cephalosporin (CAZ, CRO, CXM) & monobactam (ATM) groups of antibiotics. Non-DFI patients *Enterobacter* spp. (groups 4) was found harbors 4 plasmid size (4.8kb-7.9kb) was found 50% resistance to penicillin (AMP), monobactam (ATM), tetracycline (DO) and lincosamide (lincomycins)(DA) groups of antibiotics. DFI *Klebsiella* spp. (Group5) was found harbor 6 plasmids size ranged (4.2-7.6kb) found 100% resistance to penicillins (AMP) groups of antibiotics and found moderately resistance to cephalosporin groups of antibiotics. DFI *Pseudomonas* spp. were found plasmid free.83% DFI patients *pseudomonas* spp. were resistant to penicillins (AMP) Groups of antibiotics and 78% *pseudomonas* spp. were resistant to monobactam (ATM) groups of antibiotics. However, no one of them harbors any plasmid and their resistance to antibiotics may be of chromosomal origin. But Non-DFI patients *Pseudomonas* spp. was found harbor plasmids. They were found highly resistance to penicillin (P) & cephalosporin (CAZ, CRO, CXM) groups of antibiotics. Non-DFI *Citrobacter* spp. was found harbor 3 plasmids (2.4- 4.8 kb) and100% resistance to cephalosporin (CAZ, CRO, CXM) groups of antibiotics. The MDR properties of most of the isolates are originated from plasmid. But most of the groups were found harbor no plasmids and their resistance to antibiotics may be of chromosomal origin.

3.7.3. Sequencing, phylogenetic analysis, and correlation of the species.

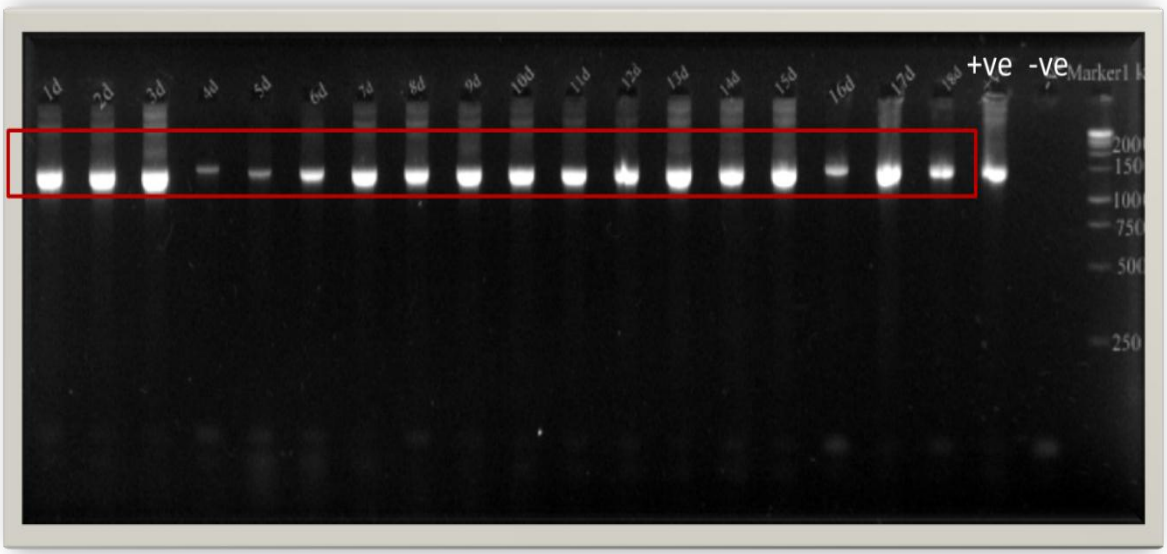


Figure 3.18: Template DNA from each of the isolates was subjected to amplification of their 16S rRNA gene using universal primers 27F and 1492 R. PCR product of approximately 1400-1450 bp was successfully amplified from each of the isolates.

From each of the 10 morphogroups and corresponding ARDRA groups, representative 27 isolates were randomly selected for detailed 16S rRNA gene sequence analysis. From 10 ARDRA groups, 27 isolates were selected. A phylogenetic tree was constructed in MEGA5 software using Neighbour-Joining algorithm and 1000 bootstrap replicates to deduce close phylogenetic relationship. (Exhibit: 3.16.).

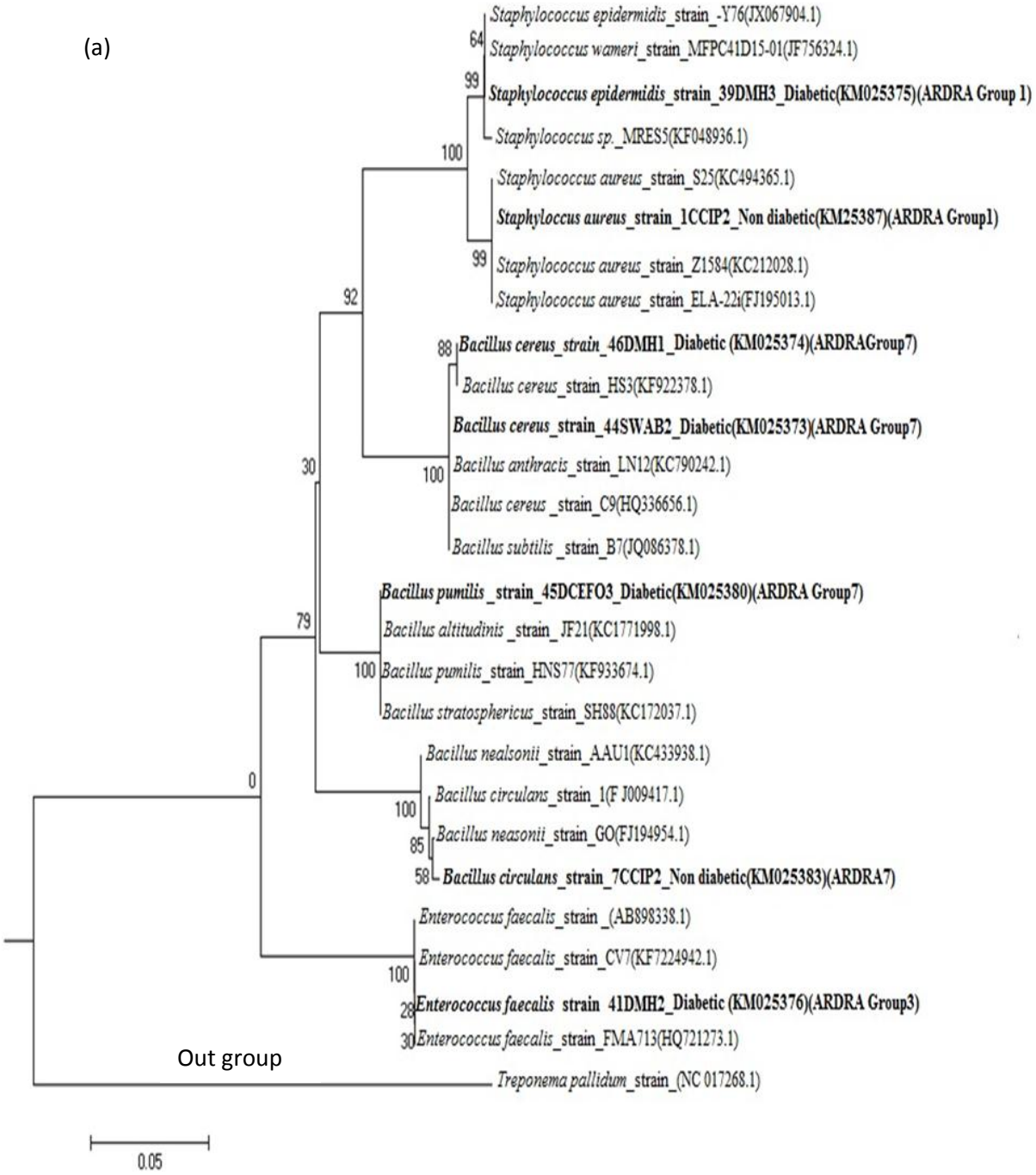
Figure 3.19 and Table 3.10 representing that Among the gram positive organisms, Isolate 39DCIP2 from ARDRA group 1 was phylogenetic ally closely related to *Staphylococcus aureus* strain Z1584 KC212028.1 with a similarity of 100% and isolate 39DMH3 was closely related to *Staphylococcus epdermidis*Y76 (JX067904) strain with a similarity 99%. Isolate 41DMH2 from ADRDA group 3 was phylogenetically closely related to *Enterococcus faecalis*CV7 (KF724942 with a similarity of 100%. Isolate 46DMH1 and 44DSWAB2 from ARDRA group 7 was

phylogenetically closely related to *Bacillus cereus* strain HS3 (KF922378) and *Bacillus cereus* strain C9 (HQ336656) respectively with a similarity 99%. Isolate 7CCIP2 from ARDRA group 7 was closely related to *Bacillus circulans* strain I1 (FJ009417) with a similarity 99%.

Among gram negative organisms, isolates 3CCIP+CEFO2 and 47DCEFO3 from Type 1 of ARDRA group 2 was phylogenetically closely related to *Pseudomonas aeruginosa* strain C1501 (KF976394) and *Pseudomonas aeruginosa* strain LCQ-4 (KF994773) respectively with a similarity 100%. Isolate 54/D/mh1 of genotype 2 of ARDRA group 2 was phylogenetically related to *Pseudomonas stutzeri* strain TH-31 (KF783212) with a similarity of 100%. Isolate 45DCIP+CEFO1 of genotype 3 from ARDRA group 2 were phylogenetically related to *Pseudomonas monteilii* strain EU45 (JF681286) with a similarity 100%. Isolates 48/D/mh1 of genotype 4 from ARDRA group 2 closely related to *Pseudomonas pseudoalcaligenes* strain Z170 (KF835816) with a similarity 99% and isolates 50DSWAB1 from genotype 5 of same ARDRA group phylogeneticaly closely related to *Pseudomonas hibiscicola* strain cp17 (JN082269) with 100% similarity (table 3.10).

Isolate 44DMH1 from ARDRA group 4 was phylogenetically closely related to *Enterobacter hormaechei* strain WW2 (JN993998) with a similarity 100%. Isolates 49DSWAB2 from ARDRA group 5 was phylogenetically related to *Klebsiella pneumoniae* strain zg2010 (JX435602) with a similarity 100%. Isolates 44DMH2 from ARDRA group 6 was phylogenetically related to *Acinetobacter baumannii* OIFC143 (JN668837) strain with a similarity 100 %. Isolates 2CMH3 and 4CCIP1 from ARDRA group 8 was phylogenetically related to *Citrobacter sp.* R5-325 (JQ659727) and *Escherichia coli* c120 (JQ781608) strain respectively with a similarity 99%. Isolates 5CMAC1 and 56DMH1 from ARDRA group 9 was phylogenetically closely related to *Providencia stuartii* S2SA-Sa (JQ828866) and *Proteus mirabilis* FUA1263 (JN102554) strain respectively with a similarity 99%. Finally, isolate 3CCEFO3 from ARDRA group 10 was plylogenetically closely related to *Alcaligenes faecalis* strain SH184 (KC172063) with a similarity of 99% (Figure 3.19 a, b).

(a)


Staphylococcus epidermidis_strain_-Y76(JX067904.1)
Staphylococcus wameri_strain_MFPC41D15-01(JF756324.1)
Staphylococcus epidermidis_strain_39DMH3_Diabetic(KM025375)(ARDRA Group 1)
Staphylococcus sp._MRES5(KF048936.1)
Staphylococcus aureus_strain_S25(KC494365.1)
Staphyloccus aureus_strain_1CCIP2_Non diabetic(KM25387)(ARDRA Group1)
Staphylococcus aureus_strain_Z1584(KC212028.1)
Staphylococcus aureus_strain_ELA-22i(FJ195013.1)
Bacillus cereus_strain_46DMH1_Diabetic (KM025374)(ARDRAGroup7)
Bacillus cereus_strain_HS3(KF922378.1)
Bacillus cereus_strain_44SWAB2_Diabetic(KM025373)(ARDRA Group7)
Bacillus anthracis_strain_LN12(KC790242.1)
Bacillus cereus _strain_C9(HQ336656.1)
Bacillus subtilis _strain_B7(JQ086378.1)
Bacillus pumilis _strain_45DCEFO3_Diabetic(KM025380)(ARDRA Group7)
Bacillus altitudinis _strain_JF21(KC1771998.1)
Bacillus pumilis_strain_HNS77(KF933674.1)
Bacillus stratosphericus_strain_SH88(KC172037.1)
Bacillus nealsonii_strain_AAU1(KC433938.1)
Bacillus circulans_strain_1(F J009417.1)
Bacillus neasonii_strain_GO(FJ194954.1)
Bacillus circulans_strain_7CCIP2_Non diabetic(KM025383)(ARDRA7)
Enterococcus faecalis_strain_(AB898338.1)
Enterococcus faecalis_strain_CV7(KF7224942.1)
Enterococcus faecalis strain 41DMH2_Diabetic (KM025376)(ARDRA Group3)
Enterococcus faecalis_strain_FMA713(HQ721273.1)
Out group
Treponema pallidum_strain_(NC 017268.1)
64
99
100
99
92
88
30
100
79
100
0
100
85
58
100
28
30
0.05

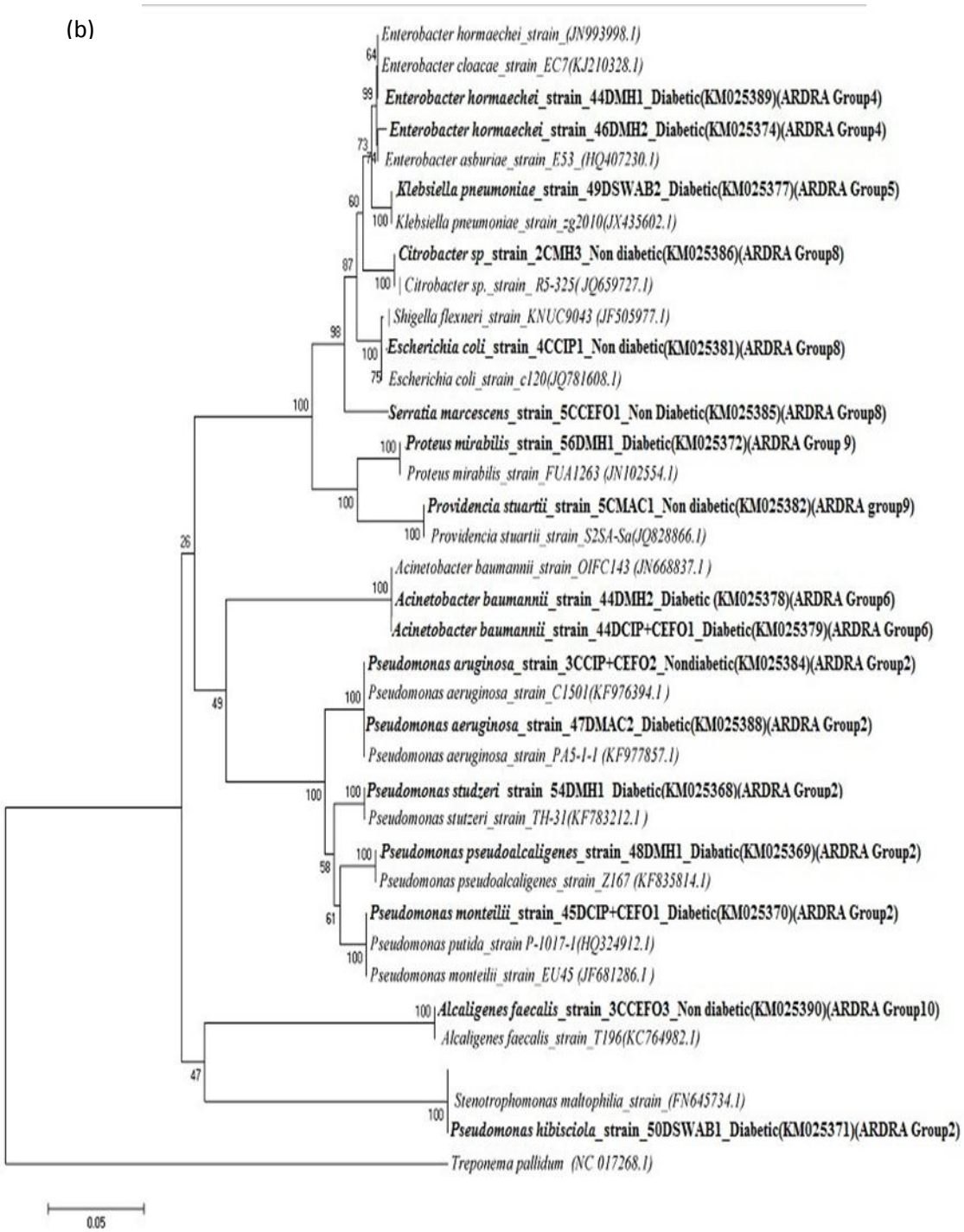


Figure 3.19 Phylogenetic tree of 16S rRNA gene sequences of gram positive (a) and gram negative (b) isolates from DFI and non-DFI patients (Accession numbersKM025368-KM025392shown in bold) and close relative reference isolates retrieved from database with accession numbers. The tree was generated in program MEGA 5 using the Neighbour-Joining algorithm- with the *Treponema pallidum* sequences serving as out group. Bootstrap values (n = 1000 replicates) are shown at branch nodes and the scale bar represents the number of changes per nucleotide position

Table 3.11: Maximum identity profile of 16S rRNA gene sequences of 10 groups of isolates according to BLAST identification.

Morph Groups	Isolates No	Close similarity to(accession numbers)	% maximum identity
Group1	39/C/cip2	*Staphylococcus aureus* Z1584 KC212028.1	100%
	39/D/mh3	*Staphylococcus epdermidis*Y76 (JX067904)	99%
Group2	47D/cefo3	*Pseudomonas aeruginosa*C1501 (KF976394)	100%
	54/D/mh1	*Pseudomonas stutzeri* TH-31 (KF783212)	100%
	45/D/cip+cefo2	*Pseudomonas monteilii* EU45 (JF681286)	100%
	48/D/mh1	*Pseudomonas pseudoalcaligenes*Z170 (KF835816)	99%
	50/D/swab1	*Pseudomonas hibiscicola* cp17 (JN082269)	100%
Group3	41/D/mh2	*Enterococuccus faecalis* CV7 (KF724942	100%
Group4	44/D/mh1	*Enterobacter hormaechei* WW2 (JN993998)	100%
Group5	49/D/swab2	*Klebsiella pneumoniae* zg2010 (JX435602)	100%
Group6	44/D/mh2	*Acinetobacter baumannii* OIFC143 (JN668837)	100%
Group7	46/D/mh1	*Bacillus cereus*HS3 (KF922378)	99%
	44/D/swab2	*Bacillus circulans* I1 (FJ009417)	100%
Group8	2/C/mh3	*Citrobacter sp.* R5-325 (JQ659727)	99%
	4/c/cip1	*Escherichia coli* c120 (JQ781608)	99%
Group9	5/C/mac1	*Providencia stuartii* S2SA-Sa (JQ828866)	99%
	56/D/mh1	*Proteus mirabilis* FUA1263 (JN102554)	99%
Group10	3/C/cefo3	*Alcaligenes faecali* SH184 (KC172063)	99%

Table 3.12: Serum CRP levels of DFI patients.

CRP Level Diabetic	Organism Growth Diabetic Patients	Resistance	Groups Resistance	ID NO
11.1mg/L	*Enterobacter* spp., *Pseudomonas* spp	*DO,DA,RD*	3	58/D/FCH
11.2mg/L	*Enterobacter* spp., *Klebsiella* spp	*AZM,AMP,CO*	2	59/D/FCH
11.5/mg/L	*pseudomonas* spp.	*AK,AMC,ATM,CAZ,CRO,CXM,CN,FEF,CO,DA,CL*	4	48/D/FCH
12/mg/L	*Enterococcus* spp.	*AMC,ATM,AMP,CAZ,CRO,CXM,CTX, CL,RD*	3	41/D/FCH
13mg/L	*Pseudomonas* spp	*AK,AMC,ATM,AMP,CAZ,CRO,CXM,CIP, C,CO*	6	34/D/FCH
15.7mg/L	*Pseudomonas* spp.	*AK,AMC,ATM,AMP,AZM,CAZ,CRO,CXM,CFM,CN,DO,PB*	7	60/D/FCH
15mg/L	*Enterococcus* spp.	*AMP,CAZ,CRO,CXM,CO,CN,*	4	18/D/FCH
16.9mg/l	*Staphylococcus* spp.	*CIP,CO,CN,*	3	27/D/FCH
17.9mg/L	*Staphylococcus* spp.	*AZM,AMP,*	1	30/D/FCH
18.6mg/L	*Pseudomonas* spp., *Staphylococcus* spp.	*AMC,ATM,AMP,CAZ,CRO,CXM,CTX, FEP,PB,CL*	7	62/D/FCH
19.1mg/L	*Klebsiella* spp	*AMC,AMP,CRO,CAZ,CXM,CIP,CN,CEM,DO,C*	7	24/D/FCH
20/mg/L	*Bacillus* spp	*AMC,ATM,AMP,CAZ,CXM,CTX,CFM, CL,PB*	5	43/D/FCH
21.7mg/L	*Enterococcus* spp.	*AMP,AZM*	1	19/D/FCH
22mg/L	*Pseudomonas* spp.	*AMC,ATM,AMP,AZM,CTX,C,CIP,CO, CN,DO,PB,CL*	10	63/D/FCH
27.3mg/L	*pseudomonas* spp.	*AK,CAZ,CRO,CIP,CN*	4	7/D/FCH
28.6mg/L	*Pseudomonas* spp.	*AMC,ATM,AMP,AZM,CAZ,CRO,CXM, CTX,CFM,*	7	61/D/FCH
35mg/L	*Staphylococcus* spp.	*AMC,ATM,AMP,AZM,CAZ,CXM,CIP, CL,PB*	7	39/D/FCH
37.9mg/L	*Klebsiella* spp., *Pseudomonas* spp.	*AK,AMC,ATM,AMP,AZM,CRO,CXM,CIP,C,CTX,CN,IPM,DO,FEP,F,CO,DA, CL,CFM,RD,*	13	54/D/FCH
50mg/L	*Staphylococcus* spp.	*ATM,CRO,CXM,CTX,C,RD,PB,CL,*	6	13/D/FCH
51/mg/L	*pseudomonas spp.*	*AMC,ATM,AMP,CAZ,CRO,CXM,CIP, CTX,IPM,DO,FEP,F,CO,DA,RD*	10	40/D/FCH
53.1mg/L	*Klebsiella* spp., *Staphylococcus* spp.	*AMC,ATM,AMP,CAZ,CRO,CXM,CTX, CL,RD*	9	49/D/FCH
55/mg/L	*Bacillus* spp., *Acinetobacter* spp., *Enterobacter* spp.	*AK,AMC,ATM,AMP,AZM,CAZ,CRO,CXM,CIP,C,CTX,CN,IPM,CFM,F,CO,DA,RD*	12	44/D/FCH
56.2mg/L	*Klebsiella* spp., *Enterobacter* spp., *Pseudomonus* spp.	*AMCATM,AMP,CRO,CXM,CTX,CN,*	6	50/D/FCH
72.1mg/L	*Enterobacter* spp., *Pseudomonas* spp.	*AMP,AZM,CAZ,CRO,CXM,CIP,CTX,CN,DO,*	10	56/D/FCH
93.8mg/L	*Staphylococcus* spp.	*AZM,CO,DA,AMP,CN,DO,CIP,AMP*	7	12/D/FCH
100mg/L	*Pseudomonas* spp., *Bacillus* spp.	*AK,AMC,ATM,AMP,AZM,CAZ,CRO,CXM,CIP,C,CTX,IPM,CFM,F,CO,DA,RD,PB*	13	47/D/FCH
108mg/L	*Enterococcus* spp., *Pseudomonas* spp.	*AK,AMC,ATM,AMP,CAZ,CRO,CXM,CTX,CL,RD*	10	42/D/FCH
112mg/L	*Bacillus* spp.	*AMC,ATM,AMP,CAZ,CRO CXM,CTX,CFM,CO,DA,CL,RD*	11	46/D/FCH
201mg/L	*Acinetobacter* spp., *Pseudomonas* spp.	*AK,AMC,ATM,AMP,CAZ,CRO,CXM,CIP,C,CTX,CN,IPM,DO,CFM,F,DA,RD*	13	45/D/FCH

Table 3.13: Serum CRP levels of Non-DFI patients.

CRP Level Non Diabetic	Organism Growth	Resistance	Group Resistance	ID NO
9.1 mg/L	*Acinetobacter* ssp., *Pseudomonas* ssp.	*AK,AMC,ATM,AMP,AZM,CRO,CXM, CIP,CO*	5	10/C/DMC
13.1 mg/L	*Acinetobacter* spp.	*AMC,AMP,CAZ,CXM,CIP,C,CTX,DO ,DA,CL*	5	9/C/DMC
17.9 mg/L	*Pseudomonus* spp., *Citrobacter* spp., *Acinetobacter* ssp.	*AK,AMC,ATM,AMP,AZM,CAZ,CRO, CIP,C,CN,F,CO,DA,RD*	8	2/C/DMC
34.2 mg/L	*Acinetobacter* ssp., *Staphylococcus* ssp.	*AK,AMC,ATM,AMP,CAZ,CRO,CIP,C, CN,F,DA*	7	6/C/DMC
73.1 mg/L	*Pseudomonus* ssp., *Citrobacter* ssp., *Staphylococcus* ssp.	*AK,AMC,ATM,AMP,AZM,CAZ,CRO, CXM,CFM,CIP,C,CN,DO,,F,CO,DA,*	8	1/C/DMC
90.2 mg/L	*Pseudomonas* ssp., *Citrobacter* ssp., *Acinetobacter* ssp, *Staphylococcus* ssp.	*AMC,AMPAZM,CAZ,CRO,CXM,CIP, C,CTX,CN,DO,CFM,CO,DA,CL,PB*	8	5/C/DMC
96.5mg/L	*Pseudomonas* spp., *Bacillus* ssp.	*AMC,ATM,AMP,CAZ,CRO,CXM,CIP, C,IPM,DO,CFM,F,CO,DA,CL,PB,RD*	8	8/C/DMC

Table 3.14: Serum CRP levels of healthy control subjects.

Healthy Control CRP		
Healthy Control	**CRP Level Healthy Control**	**Organism Growth**
1	6.25	NO growth
2	3.18	NO growth
3	4.96	NO growth
4	5.12	NO growth
5	4	NO growth

3.8. Serum C reactive protein tests of DFI, Non-DFI and healthy control patients:

Normal range of CRP level in human serum is ≤10mg/l. Table 3.12 representing serum CRP level of DFI patients. In DFI patients serum CRP levels was found ranged from 10 mg/L- 201 mg/L. Among the DFI patients 40% patients showed CRP level >50mg/L. DFI patients 42/D/FCH, 47/D/FCH and 46/D/FCH patients CRP level was found >100mg/L. they were found commonly infected with *pseudomonas* spp. and *Bacillus* spp. 16% of DFI patients was found CRP level <20mg/L. In non-DFI patients serum CPP levels was found ranged from 9.1mg/L-90.5mg/L. (Table 3.13) representing that, Among the non-DFI patients 25% patients showed CRP level >50mg/L and they were found commonly infected with *pseudomonas* spp. *Citrobacter* spp. and *staphylococcus* spp. DFI patients *pseudomonas* spp. and *Bacillus* spp. was found resistance to more than 12 groups of antibiotics. Non-DFI *Pseudomonas* spp., *Citrobacter* spp. and *Staphylococcus* spp. was found resistance to more than 7 groups of antibiotics. Healthy control patients have no diabetic and foot infection. In healthy control patients CRP levels was found with in normal range (Table 3.14).

Figure 3.20: Average serum CRP level of DFI, Non-DFI and Healthy control patients.

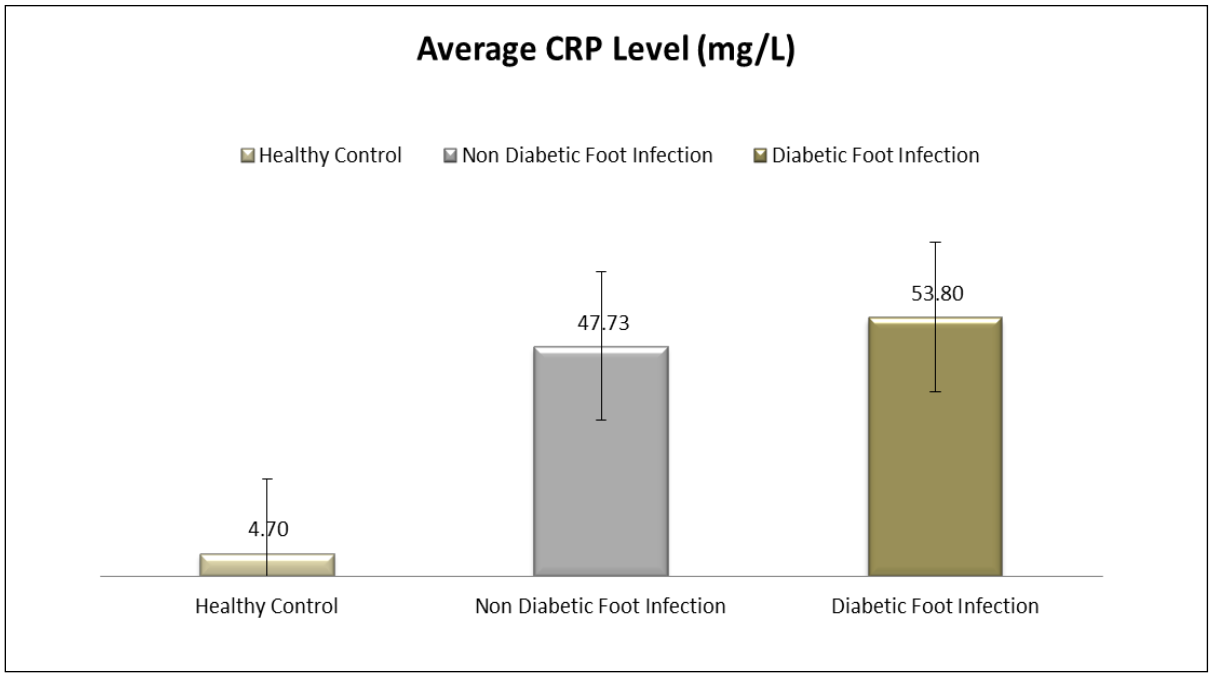


Figure 3.20 and table 3.15 demonstrates mean and ± SD (standard deviation) values of CRP of three (DFI, Non-DFI and healthy control) study groups. Normal range of CRP level in human serum is ≤10mg/l. In DFI patients serum CRP was found ranged from 11.1 – 201 mg/l (mean value, 53.80 ± 49.93mg/l), whereas, in non-DFI patients, it was from 9.1-96.5mg/L (mean value, 47.73 ± 37.84mg/l) and in healthy control it was 4-6.25 mg/l. The mean CRP values for the Diabetic patients with foot infection were (53.80 ± 49.93mg/l) and in non-DFI patients the mean CRP levels (47.73 ± 37.84mg/l) in both foot infected patients the value were found significantly higher compared to healthy control (4.70 ± 1.17mg/l, p<0.01) (Table 3.15).

Table 3.15: Mean and ± SD (standard deviation) values of serum CRP of three study groups

Serum sample	Range value CRP mg/l	Mean ± SD CRP(mg/l)
Diabetic Foot infection	11.1 – 201	53.80 ± 49.93
Non Diabetic foot infection	9.1-96.5	47.73 ± 37.84
Healthy control	4-6.25	4.70±1.17

It was observed CRP value increased with chronic DFI and non-DFI patients' history and concomitantly change the microbes compositions in our samples. A correlation was deduced between the no of antibiotic group resistance by the organisms and the respective levels of CRP in DFI and non-DFI patients (Figure3.22 a, b). The results shows that- (i) DFI patients whose serum CRP level 10 to 50 mg/L, were commonly infected with *Pseudomonas* spp. *Enterobacter* spp. and *Klebsiella* spp. Polymicrobial infection was observed in 33.3% cases. These organisms were found commonly resistance to 7 groups of antibiotics; All Non-DFI patients with CRP values between 10 to 50mg/L was infected with *Acinetobacter* spp. However, Polymicrobial infection (Combination of *Acinetobacter* spp. and one or two of others) was observed in 75% cases. These organism were resistance to 5 groups of antibiotics (ii) DFI Patients with CRP values between 50 to 100mg/L were commonly infected with *Enterobacter* spp. and *Pseudomonas* spp. Polymicrobial infection was observed in 62.5% cases. These organisms were

found commonly resistance to 9 groups of antibiotics; Non DFI patients with CRP values between 50 to 100mg/L commonly infected with *Pseudomonas* spp. *Citrobacter* spp. *Staphylococcus* spp. Polymicrobial infection was observed in 100% cases. These organism were resistance to 8 groups of antibiotics (iii) whose serum CRP level >100mg/L was found only in DFI patients and commonly infected with *Bacillus* spp. and *Pseudomonas* spp. Polymicrobial infection was observed in 100% cases. These organisms were found commonly resistance to more than 12 groups of antibiotics (Table3.16 a, b).

(a)

Correlation with DFI (n=30) Isolates reistant pattern and CRP values					
CRP value Range mg/L	**Common Organism Growth**	**No of Antibiotic commonly Resitant**	**Groups**	**No of patients**	**Polymicrobial n (%)**
10 to 50	*Pseudomonas* spp *Enterobacter* spp *Klebsiella* spp	AMC,ATM,AMP,CAZ,CRO,CXM,CFM,CN,DO (9)	7	18	33.30%
50 to 100	*Enterobacter* spp *Pseudomonas* spp	AMC,ATM,AMP,CRO,CXM,CTX,CN,IPM,PB,DO (10)	8	8	62.50%
> 100	*Bacillus* spp *Pseudomonas* spp	AK,AMC,ATM,AMP,AZM,CAZ,CRO,CXM,CIP,C,CTX,IPM,CFM,F,CO,DA,RD,PB (18)	12	4	100%

(b)

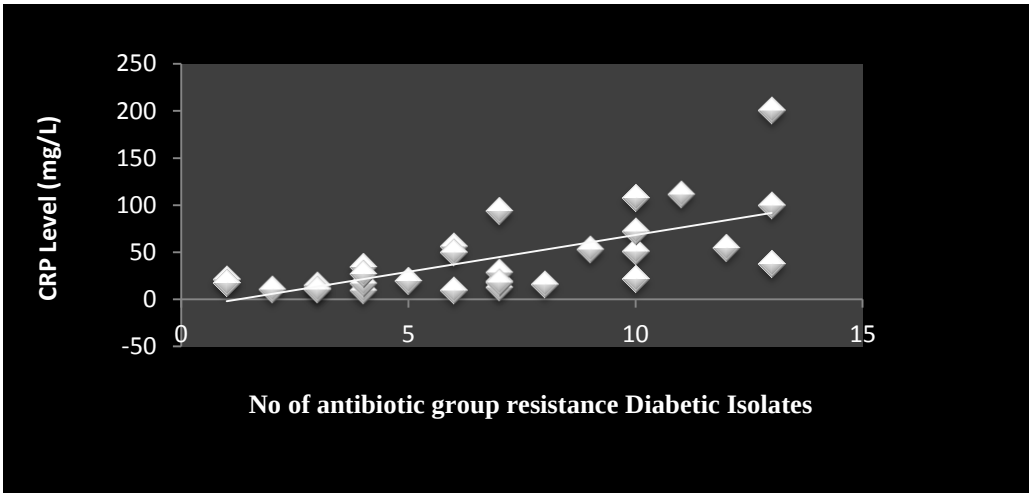
Correlation with Non-DFI Isolates resistant pattern and CRP values					
CRP value Range mg/L	**Common Organism Growth**	**No of Antibiotic commonly Resitant**	**Groups**	**No of patients**	**Polymicrobial n (%)**
10 to 50	*Acinetobacter* spp.	AMC,ATM,AMP,AZM,CAZ,CRO,CXM,CTX,CFM (9)	6	4	75%
50 to 100	*Pseudomonas* spp. *Citrobacter* spp. *Staphylococcus* spp.	AMC,AMP,AZM,CAZ,CRO,CXM,CIP,C,CTX,DO,CO,DA (11)	8	6	100%
> 100	X				

Table 3.16: The correlation between serum CRP level, Organism growth and no of antibiotics resistance (a) Diabetic patients (b) Non-diabetic patients

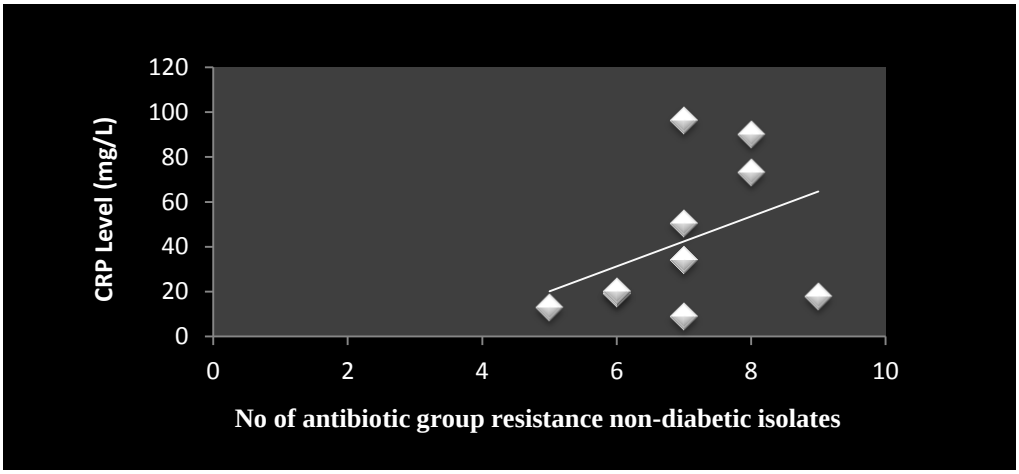
Therefore, a highly significant positive correlation was detected in DFI patients between no of antibiotic groups' resistance by the organisms and levels of CRP in DFI patients. The regression analysis of the data showed a positive correlation in DFI sample with r = 0.656, $p<0.01$ Fig.3.22 (a) and (b) for non-DFI r =0.0387, $p<0.26$ (FIG 3.21 a, b)

Figure 3.21: The correlation between the serum CRP levels and no of antibiotic resistance by (a) DFI and (b) Non-DFI isolates.

(a) Diabetic



(b) Non-diabetic (control)



Chapter 4

Discussion

4.0 Discussion:

The pathophysiological condition of DFI patients may favor growth of different bacterial pollution compared to non-DFI patients, because limit of blood flow and antimicrobial agent's delivery slowed down in the infected zone (Shameem *et al*., 2012). DFI is a common phenomenon in the progress of DM and about 25% DM patients experience DFI which impaired micro vascular circulation, limits entrance of phagocytic cells (Neutrophils) to the infected tissue and reduced the concentration of antibiotic in infected zone (Ozer *et al*., 2012). There is very limited number of literature published on the microbial association in case of DFI, particularly in Bangladesh. The present investigation demonstrated- **i)** Demography played a role in the occurrence of DFI in diabetics patients; **ii)** Polymicrobial and increased bacterial population association were observed in DFI and non-DFI patients, but with different composition; **iii)** Bacterial populations were MDR and had an association with CRP; and **iv)** MDR properties were either chromosomal or plasmid mediated.

4.1. (i) *Impact of demography in the DFI and non-DFI patients*:

Our findings demonstrates that demographic factors influence the incident of the foot infections in both DFI and Non-DFI patients; in our study we found that DFI patients with more than 40 years of age are more susceptible to foot infection whereas non-DFI less than 40 years of age were predominant. This finding is comparable with findings of (Shler *et al*., 2012). Average ages of DFI was 58 years and among them male were the predominant subjects in both DFI and non-DFI cases. This finding also correlated with findings (Gadepalli *et al.*, 2006; Raja *et al*., 2007 and Mayfield *et al.*, 1996). Most of the patients were from rural area (lower middle income family) and majority of them were using tube well water. Majority of the DFI patients were small scale business men (33%) and housewives (21%). These findings correlated to the report of Hena *et al*. (2010). Among the non-DFI patients majority of patients were daily worker (80%).

4.2. **(ii)** ***Polymicrobial association observed in DFI and non-DFI patients*****:**

Our study showed that most of the DFI (85%) and non-DFI (100%) patient's infections were polymicrobial in nature. These Polymicrobial infections were responsible for chronic wound and more complex infection. Similar with our finding in the study of (Citron *et al.*, 2007 and Anandi *et al*., 2003;) found most of the DFI patients had polymicrobial infection (80%) and (69%) respectively. However, this contrast with finding of Dhanasekaran *et al*., (2003) where (60%) of DFI patients infection were mono microbial.

In this study most of the specimen from DFI (93.3% n=67) and non-DFI (71.4%, n=10) were culture positive. In both of DFI and non-DFI cases the isolates were predominantly gram negative (66% and 70% respectively). Results of DFI cases were compatible with the findings of other studies (Abdulrazak *et al.*, 2005;Anandi *et al.*, 2004 and Ozkara *et al*., 2008), where the incidence gram negative organisms were more than 60%; however, they did not include specimens from Non-DFI patients. These contradicted with findings of Mantey *et al.*, (2000) and Dang *et al*.(2003) where of gram positive organisms were predominant (70%).

The most prevalent species observed in Bangladeshi foot infected populations is *Pseudomonous* spp. (Shameem *et al*., 2012). In our DFI and non-DFI Patients, the incidence of the organism was 22% and 29% respectively. This finding is supported by Murugan *et al* (2010) who worked with DFI patients only and found the frequency of the organism was more than (50%).

Through phenotypic and genotypic characterization, five species of *Pseudomonous* were isolated from DFI patients, namely, *P. aeruginosa, P. stutzeri, P. psudoalcaligenes, P. monteilii,* and *P. hibiscola*. However, only one species, namely, *P. aruginosa* was isolated from non-DFI patients. Only *Pseudomonas aeruginosa* was found in the study of (Pappu *et al*., 2011, and Hena *et al*., 2010) but others did not identify the species of Pseudomonas.

Pseudomonas spp. is invasive and release different toxins & enzyme (pyocyanin, lecithinase, collagenase, lipase, hemolysin, and fluorescein) which play role for tissue destruction. Among DFI isolates *P. stutzeri* –a superbug (isolate 40/D/MAC1, 40/D/MAC2, 40/D/CIP+CEFO1, CIP+CEFO2 isolates) were found 100% resistant to 12 groups of antibiotics (AMP, ATM,CRO, CXM, CTX, IPM, DO,CIP, F, DA, RD, PB etc.) that are commonly prescribed by the practioners

in Bangladesh. It is specially an opportunistic pathogen and different from other members in relations to colony morphology (unusual shape and consistency), nutritional characteristics, biological factors and reported to be multidrug resistant (MDR) (Lalucat *et al.*, 2006).

P. aruginosa is also frequently causes severe tissue damage and an important cause of nosocomial infection. Seventy percent of *P. aruginosa* isolated in this study was resistant to penicillins (AMP), cephalosporins (CRO, CAZ, CXM), carbapenem (IPM) and tetracycline (DO) groups of antibiotics. This result was comparable with that Murugon *et al*., (2010) who found 100% of their isolates resistant to above mentioned antibiotics. In our study *P. montelli isolated* from DFI patients were found highly resistant to penicillins (AMP), cephalosporins (CAZ, CRO, CXM), carbapenem (IPM), nitrofurantoin (F), refamycin (RD), lincosamide (DA) groups of antibiotics. *P.hibiscola.* also resistance to penicillin (AMP) & cephalosporin (CAZ, CRO, CXM) groups of antibiotics (Table3.7).

Enterobacter spp. is emerging in Bangladeshi DFI populations. In this study, *Enterobacter* spp. was one of the predominant organisms which were isolated from 23.3% DFI and 10% non-DFI patients. The species isolated from both of DFI and non-DFI was *Ent. hormaechai.* Shameem *et al*., (2012) found 4.4% *Enterobacter* spp. in Bangladeshi DFI patients. *Enterobacter* spp. isolated from DFI patients was commonly resistance to penicillins, cephalosporins, nitrofurantoin groups of antibiotics. This antibiotic resistant pattern of *Enterobacter* spp. is comparable with poultry source of isolates reported from our laboratory (Nandi *et al*., 2013). The involvement of *Enterobacter* spp. in DFI and Non-DFI combined with its association with other sources and antibiotics resistant pattern, consider it as an emerging pathogen particularly in Bangladesh. Therefore, more attention must be recommended.

B. cereus infection can occur in neuropathic patients and has been reported in a patient with severe combined immunodeficiency disease (Boulton *et al.*, 2005). In our study, prevalence of *B cereus* was 17% in DFI patients and 3% in non-DFI patients. In DFI, *B cereus* strains were resistant to monobactam, amoxyclav, penicillins, cephalosporin, nitrofurantoin groups of antibiotics. In this study, isolate 7CCIP2 was cultured from a non-DFI patient and found phylogenetically closely related to *B. circulans* strain NBRC13634 (AB680471.1) with a similarity 99%.

B. circulans was reported as opportunistic pathogen for immune-compromised patients. These bacteria are facultative anaerobe, which can multiply in blood, human host tissues in the absence of effective immune responses. This is a nosocomial bacterium responsible for sepsis (inflammatory response) that causes damages of vital body organ heart, lungs, kidney, and liver (Alebouyeh *et al.*, 2011 and Richard *et al.*, 1988). Other *Bacillus* spp. (*B. subtilis*) are reported as causes of septicemia in immune-compromised patients (Oggioni *et al.*, 1998).

B. circulans was found 100% resistant to 10 groups of antibiotics such, as cephalosporins, monobactams, penicillins, quinolones, lincosamide lincomycins and rifamycin etc. *B. circulans* 7CCIP2 produce exotoxin (hemolysin) and is able to destroy Red blood cell (RBC) and hemoglobin (Mukry *et al*., 2010).

Bacillus circulans can cause potential complications of chronic inflammation of the internal coats of the eye, fatal sepsis, disseminated intravascular coagulation and wound infection. *Bacillus circulans* has also been reported in malignant patients, Non Hodgekin lymphoma patients, infection in teeth and cerebrospinal infected patients (Logan *et al*., 1985, Roncoroni *etal*., 1985, Goudswaard *et al*., 1995 and Gurol *et al*., 2007). This statement of emerging of MDR *B. circulans* is a threat for Bangladesh and as far we know this is the first case found in our population. Therefore, more studies are required to understand how this bacterium has acquired this resistant phenotype and its association for polymicrobial infection.

Staphylococcus species comprised 13% of all organisms isolated from both DFI and non-DFI in this study. Only *Staphylococcus epidermidis* were isolated from DFI and only *Staphylococcus aureus* were isolates from Non-DFI patients. Ozer *et al*. (2010) reported that 10.8% of the bacterial infection in case of DFI was occurred by *Staphylococcu*s spp. *Staphylococcus aureus* are the most frequent pathogens contributing to progressive and extensive tissue damage. *Staphylococcus aureus* and *Staphylococcus epidermidis* are the most common pathogen cultured from bone samples in the feet of diabetic patients (Lipsky *et al*., 2010).

Colonization of *Staphylococcus* spp. were less frequently occurred on intact human skin, but it can cause severe infections when penetrates anatomic barriers (Yao *et al.*, 2005). Methicillin-resistant *Staphylococcus aureus* (MRSA) is commonly isolated from 10–40% of diabetic wounds (Hefni *et al.*, 2013). In our study we found that 30% of the *Staphylococcus aureus* are MRSA. Many studies have previously reported that *Staphylococcus* spp. was the main causative pathogen for DFI (Dang *et al*., 2003 and Lipsky *et al*., 2004). However, our study and other recent reports(Samir *et al*., 2009; Ozer *et al*., 2010; Citron *et al.*, 2007 and Ozer *et al*., 2012) supported the predominance of gram negative organisms in case DFI.

Acinetobacter boumannai (A. boumannai) is an important nosocomial pathogen (Manikal *et al.*, 2011). Its incidence is increasing day by day. In present investigation, frequency of *A. boumannai* was 10% irrespective of DFI and Non-DFI. This frequency was much higher (33%) in the study of Hefni *et al*. (2013). *A. boumannai in* this study were found highly resistant to penicillin (86%), cephalosporins (60%) groups of antibiotics. However, resistance to carbapenem was only 14%. But in other studies showed that *A. boumannai* were 76% resistance to carbapenem groups of antibiotics (Rasha *et al*., 2014) . Studies of Abbo *et al*., (2005) revealed that 88% MDR *A. boumannai* were hospital acquired. All strains of *A. boumannai* in this study were isolated hospital admitted patients. .

Enterococci are opportunistic pathogen and cause infection in immuno-compromised patients, such as DM patients (Rubin *et al*., 1998). In our study we found *Enterococcus faecalis* strains (9%) in DFI patients; in non-DFI patients *Enterococcus* spp. was not isolated. This compares with finding of Ozer *et al*.(2012) where the isolation rate was 14.9% in DFI patients.

Frequency of *Klebsiella pneumoniae* (*K. pneumoniae*) was 7% in DFI patients in the present investigation. This correlates with finding of Shameem *et al*.(2012) where the frequency was 8.8%; but contrast with findings of Bansal *et al*.(2008) and Srivaraman *et al*., (2011) where the frequency were 17% and 20.5% respectively. However, *K. pneumoniae* was not isolated in non-DFI patients. *K. pneumoniae* isolated from DFI patients was MDR. Strains of these bacteria were 100% resistant to penicillin groups of antibiotics followed by aminoglycosides (60%), tetracycline (60%), rifamycin (60%), cephalosporins (40%) and lincosamide (40%) groups.

Citrobacter spp. a component of normal intestinal flora (Holmes *et al*., 1998), is an opportunistic nosocomial pathogen causing infection in immune- compromised patients (Ryan *et al*., 2004). In our study, prevalence of this organism in non-DFI patients was 33% and only 2% in DFI patients. Prevalence of this organism in DFI patients correlates with that in the study of Esma *et al*., (2012) and Pittet *et al*., (1999) where the prevalence were 5.3% and 3.6% respectively.

Alcaligens faecalis(*Al.faecalis*) is a bacterium having antagonistic properties against the growth of other bacteria (Bernan *et al.,* 1997 and Zahir *et al.,* 2013). This microorganism, was also able to inhibit the growth of many bacteria such as *B subtilis* (Li *et al*., 2007; Li *et al*., 2008). In our study, we found *Al faecalis* (3%) in only Non-DFI patients. However, polymicrobial growth *(Pseudomonas* spp., *Acinetobacter* spp., and *Enterobacter* spp.) was revealed along with *Al faecalis* in present study.

Proteus spp. was responsible for incessant and extensive tissue destruction to the infected site (Armstrong *et al*.,1998). In our studies we found 2% *Proteus* spp. in DFI patients. However ,Samir *et al*., (2009) and Shameem *et al*., (2012) found 33.3% and 16.4% of *Proteus* spp. respectively in their studies.

This study revealed 3% *E. coli* in non-DFI patients. *E. coli* was not isolated from DFI patients. Our findings are compatible with the findings of Ozer *et al*.,(2010) and Citron *et al*., (2007) but contrast with that of Shameem *et al*., (2012) and Pappu *et al*., (2012) where the frequency of isolation of *E.Coli* from DFI patients were 11.9% and 12 % respectively. Comparable with our study, they found *E. coli* 70% resistance to cephalosporin groups of antibiotics.

The statistical calculation (Comparative bacterial significance analysis: at 90% confidence level) revealed that the rate of occurrence of three organisms e.g. *Enterococcus* spp. |Z|=2.2125, *Klebsiella* spp. |Z|= 1.732, *Bacillus* spp. |Z| =1.9034 had significant association in DFI patients compared to non-DFI patients. However in non-DFI patients rate of occurrence of *Citrobacter* spp. |Z|=14.1901 were significant. Common genera found in both DFI and non-DFI was *Pseudomonas,* spp., *Staphylococcus* spp. and *Acinetobacter* spp. and their frequency in both cases were comparable.

4.3. (iii) *Multidrug resistant bacterial association with Diabetics Foot Infection correlates with C-reactive protein value*

DFI patients have chronic non-healing ulcers due to several underlying factor such as poor glycemic control, peripheral neuropathy, poor blood supply to the extremities and Polymicrobial infection in foot. This leads to chronic subclinical inflammation (CSI) and delays the healing process (Weigelt *et al.*, 2009; Boulton *et al*., 2008; Jeffcoate *et al*., 2003). DFI patients with CSI is always treated with combination of antibiotics, because the microbes are highly resistant to different groups of antibiotics; therefore, DFI patients has history of long treatment (Heurtier *et al.*, 2004; Kandemir *et al.*, 2007; Sivaraman *et al*., 2011). In our study also, bacterial populations associated with DFI were comparatively more resistant to antibiotics than non-DFI.

Inflammatory reaction is one of the major complications of DFI (Sallam *et al*., 2012). CRP, an acute phase protein, is an important marker of CSI and play important role in acute injury, systemic inflammatory response, and infection. CRP binds with specific molecules (receptor for CRP) of dead cell surface or surface of pathogens, activate complement and help clearing them through phagocytosis by macrophages (opsonin-mediated phagocytosis) It plays a role in innate immunity as an early defense system against infection in both mammal and insect. Its antimicrobial activity in insect model can clear pathogen within 6-48 hours (Ng *et al*., 2004 and Tan *et al*., 2005).

Serum CRP levels increase up to 1000-fold in response to acute and chronic inflammatory response. Therefore, CRP is an important marker of inflammation. Sallam *et al*., (2012) revealed a strong correlation between the levels of CRP in the patients with DFI they also found a correlation between increase of CRP level with insulin resistance syndrome (T2DM). Serum CRP level is also used as a diagnostic tool for management of DFI patients (Jeandrot *et al.*, 2008 and Lipsky *et al.*, 2004).

Different types of Pathogen are associated with different inflammatory response and patients who were infected with Gram negative organism have high CRP levels. CRP is a predominant gram negative endotoxin recognition protein and produce strong immune inflammatory response (Shler *et al*., 2012). In our study, we found predominance of gram negative organism in both DFI and non-DFI patients. (Pseudomonas was isolated from most of both DFI and non-DFI patients. followed by *Enterobacter* spp. *Klebsiella* spp. and *Bacillus* spp. from DFI and *Acinetobacter* spp. *Staphylococcus* spp. and *Citrobacter* spp. from non-DFI patients).The correlation of *Pseudomonas* spp. with higher CRP level was also found by Ng *et al.*, (2004) and Tan *et al.*, (2005).

We measured serum CRP of 30 DFI (randomly), 10 non- DFI patients and 5 healthy controls. Serum CRP level of DFI and non-DFI patients were found significantly higher ($p<0.01$) compared to healthy control serum CRP. These findings are comparable with other studies (Weigelt *et al*., 2009). We found predominance of gram negative organism in pus samples of patients having DFI and high CRP levels. DFI patients particularly having infected with *Pseudomonas* spp. and *Bacillus* spp. have high CRP levels (>100mg/L). These results are supported by reports of Ng *et al*., (2004). *Bacillus* spp. and *Pseudomonas* spp. isolated from DFI patients were found resistance to more than 12 groups (AK, AMC, ATM, AMP, CAZ, CIP, C, IPM, F, CO, RD, PB etc.) of antibiotics. In non-DFI patients who were infected with *Pseudomonas* spp. *Citrobacte*r spp. and *Staphylococcus* spp. having high CRP level (50-100mg/L), these organisms were resistance to 8 groups (AMC, AMP, AZM, CAZ, CIP, C, DO, CO etc.) of antibiotics (Table 3.16 a, b). So it is evident that there is a correlation among high CRP level, Polymicrobial infection and multiple drug resistance in DFI and non-DFI patients. The higher the CRP levels, the more incidences of Polymicrobial infection and multidrug resistance involving increasing number of antibiotics. The level of CRP is higher and number of antibiotics being resistant is more in DFI patients than non-DFI patients. The above discussion is also supported by correlation analysis: co-relation coefficient, r = (0.656, $p<0.01$) in DFI patients and in non-DFI (r =0.0387, $p<0.26$) (FIG 3.21; a, b).

4.4. **(iv)** ***MDR DFI & non-DFI isolates are Plasmid free:***

MDR properties within the bacteria may be due to the genetic elements of antibiotic resistance may be positioned on chromosome or on mobile genetic elements including plasmids, transposons or integrons and these mobile elements serve as vectors promoting distribution of antibiotic resistant gene (Davies *et al*., 1995). Among these mobile elements, plasmids play a very important role in the transmission of these resistance markers between the organisms both horizontally and vertically. In the present study, DFI (24.4%) & non-DFI (61.0%) isolates were found to harbor numbers of plasmids and the rest percentages were plasmid free. These results implicated that MDR properties occurred in the isolated bacteria may be chromosomal or plasmid or both borne. In DFI patients, 2 groups of isolates (*Enterobacter* spp. & *Klebsiella* spp.) harbored plasmid ranged from 2.4 Kb to 7.6Kb. In non-DFI patients 3 groups of isolates (*Pseudomonas* spp., *Enterobacter* spp. & *Citrobacter* spp.) harbor plasmid ranged from (1.27 Kb to 7.9Kb.). Other Isolates of both DFI (76.6%) & non-DFI (39%) having MDR properties but they are plasmid free.

Chapter 5

Concluding Remarks

5.0 Concluding Remarks:

The present study demonstrates the microbial diversity in DFI and non-DFI patients of Bangladeshi populations. Altered microbial compositions were found in case of DFI and non-DFI patients and also we originate a correlation with microbial resistance pattern and CSI (evident by elevated CRP level). The finding of the present investigation concludes:

- Diabetic male patients having ages more than > 40 years are more susceptible to foot infection compare to < 40 years of old. DFI showed in rural area at alarming levels and prevalent more in lower income groups implicating a poor maintained healthcare.

- In Bangladeshi (DFI and non-DFI) patients Gram negative organisms are most prevalent. Phylogenetic analysis of 16S rRNA gene of the isolates revealed the prevalence of pathogenic gram negative organisms, mostly *Pseudomonas* spp. e.g., *P. aeruginosa, P. monteilii , P. hibisocola* and *P. stutzeri* with clinical significance. *Enterobacter* spp. e.g. *Ent. hormaechai* –a emerging MDR pathogen is prevailing in DFI populations. *A. boumannii and K pneumoniae* are also prevalent.

- Phylogenetic analysis of 16S rRNA gene of the isolates also showed commonness of gram positive organisms frequently *Bacillus* spp. e.g. *B. cereus. Staphylococcus* spp. e.g. *S. aureus, S.epidermidis. Enterococcus faecalis are also* predominant.

- *Enterococcus* spp. (9%), and *Klebsiella* spp. (8%) occurred only in DFI patients and the other predominant bacteria in DFI over non-DFI patients were *Enterobacter* spp. (22%) and *Bacillus* spp. (12%). In contrast, non-DFI patient's samples predominated *Pseudomonas* spp. (29%) and *Citrobacter* spp. (29%). In both cases, *Staphylococcus* spp. (13%) and *Acinetobacter* spp. (10%) contained same percentages. The statistical calculation (Comparative bacterial significance analysis: at 90% confidence level) revealed that the rate of occurrence of three organisms; *Enterococcus* spp. $|Z|=2.2125$, *Klebsiella* spp. $|Z|= 1.732$, *Bacillus* spp. $|Z| =1.9034$ is significant in DFI patients compare to non-DFI patients. However in non-DFI patients rate of occurrence of *Citrobacter* spp. $|Z|=14.1901$ were significant.

- All of the isolates showed MDR properties at alarming levels. Most of the isolates from DFI patients were commonly resistant to cephalosporins (CAZ,CRO,CXM) and monobactam(ATM) groups of antibiotics.

- The MDR properties of the isolates from both patients sources may either plasmid or chromosome / or both plasmid and chromosome borne.

- Serum CRP level was found higher in DFI patients than non-DFI patients. In DFI patients who had higher serum CRP value were commonly infected with *Pseudomonas* spp. and *Bacillus* spp. and these species were showed super bug MDR properties. There is significant correlation between MDR properties of the isolates and elevation of serum CRP levels in DFI patients.

Recommendations:

Therefore, the altered microbial association with their antimicrobial properties and CSI described in this thesis recommends clinical practioners for an alternate treatment and medication of DFI patients. The thesis further recommends that i. proper public health maintenance and early diagnosis of DFI and Non-FDI are extremely important; and ii. For appropriate choice of antibiotics to treat the patients, diagnosis of the bacteria involved with wound and its' antibiogram must be done.

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Chapter 6

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Chapter 7

Appendix

Appendix-I

Unless otherwise mentioned, all media were sterilized by autoclaving at 121° C for 15 minutes at 15 lbs pressure. Distilled water was used for preparation of all media. The media used in this thesis have been given below:

1. **Mac Conkey Agar Media (OXOID)**

Ingredients	Amount (g/L)
Peptone	20.0
Lactose	10.0
Bile salts	1.5
Sodium chloride	5.0
Neutral red	0.03
Crystal violet	0.001
Agar	15.0
p^H	7.1±0.2

2. **Muller Hinton Agar (OXOID)**

Ingredients	Amount (g/L)
Beef Extract	2.0
Casein hydrolysate	17.5
Starch	1.5
Agar	15.0
pH	7.3

3. **Nutrient Agar (OXOID)**

Ingredients	Amount (g/L)
Peptone	**5.0**
Sodium Chloride	5.0
Beef extract	3.0
Agar	15.0
pH	7.0

4. Nutrient Broth (OXOID)

Ingredients	Amount (g/L)
"Lab lemco" powder	1.0
Yeast extract	2.0
Peptone	5.0
NaCl	5.0
pH	7.4±0.2

5. Peptone water

Ingredients	Amount (g/L)
Peptone	10.0
NaCl	5.0

6. Kligler Iron Agar (Oxoid)

Ingredients	Amount (g/L)
Peptone mixture	20.00
Sodium Chloride	5.00
Ferric Ammonium Citrate	0.50
Phenol Red	0.025
Lactose	10.00
Dextrose	1.00
Sodium Thiosulfate	0.50
Bacteriological Agar	15.00

7. Voges-Proskaur (VP), Methyl Red (MR) broth

Ingredients	Amount (g/L)
Buffered peptone	7.0
Dextrose	5.0
Dipotassium phosphate	5.0
pH	6.9±0.2

8. Simmon's Citrate Agar (Simmons', 1926, Modified)

Ingredients	Amount (g/L)
NaCl	5.0
$MgSO_4$	0.2
$(NH4)_3PO_4$	1.0
K_2HPO_4	1.0
Sodium citrate	2.0
Agar	15.0
pH	6.8±0.2

9. Carbohydrate broth

Ingredients	Amount (g/L)
Trypticase	20.0
NaCl	5.0
Sugar (Lactose/Sucrose/Dextrose)	5.0

10. Starch agar

Ingredients	Amount (g/L)
Peptone	5.0
Meat extract	3.0
Soluble starch	2.0
Agar	15.0

11. Lauria broth

Ingredients	Amount (g/L)
Tryptone	10.0
Yeast extract	5.0
NaCl	10.0

Questionnaire

TITLE: CHARACTERIZATION OF MICROBIAL AGENTS IN DIABETIC FOOT INFECTION AND ITS CORRELATION WITH CHRONIC SUBCLINICAL INFLAMMATION

ID: DATE:

1. Name……..
2. Address…….Mobile:

   ……..
3. Age…….....Sex……
4. History of patient illness

   (a) Pain

   (b) Fever

   (c) Discharge from the lesion: O/E (i)Tenderness

   (ii) Fowl smelling

   (iii) Others
5. Family history of Diabetes……..
6. Occupation……
7. Socioeconomic status……
8. Urban/Rural……
9. Shoes wear/not……
10. Type of water used….. Tap/River/pond / Tube well.
11. Treatment Taking…….
12. Fasting:
13. ABF:
14. CRP:
15. HbA1c:

Appendix-II

Solutions and Reagents used

Preparations of the stock solutions used in this work are given below: (all the working solutions used in this work were prepared from the stock solutions).

Normal saline

Normal saline was prepared by dissolving 0.85 g NaCl in 100ml of distilled water and sterilized by autoclaving, pH was adjusted to 7.8.

1 M Tris-Cl

121.1 g tris-base was dissolved in 800 ml of distilled water. The pH was adjusted to the desired value by adding concentrated HCl and the final volume was made up to 1 L by distilled water. The solution was sterilized by autoclaving and stored at RT.

3M NaCl

175.3 g of NaCl was dissolved in distilled water to a final volume of 1L. The solution was autoclaved and stored at RT.

10 M NaOH

40 g of NaOH pellet was dissolved in 100 ml distilled water. The solution was stored in an airtight bottle at RT.

0.5 M EDTA

186.1 g of $Na_2EDTA.2H_2O$ and 20.0 g of NaOH pellets were added and dissolved by stirring to 800 ml distilled water on a magnetic stirrer. The pH was adjusted to 8.0 with a few drops of 10 M NaOH and final volume was made up to 1L with distilled water. The solution was sterilized by autoclavingand stored at RT.

3 M sodium acetate

40.81 g of Na_2 (CH_3COOH).H_2O was dissolved in 80 ml of distilled water. The pH was adjusted to 5.2 with glacial acetic acid. The final volume was adjusted to 100 ml with distilled water and the solution was sterilized by autoclaving. It was stored at 4°C.

TAE buffer

242 g of tris-base, 57.1 ml of glacial acetic acid, 100 ml of 0.5 M EDTA (pH 8.0) was talen and distilled water was added to the mixture to make 1L. 1X concentrated TAE buffer was made by adding 10 ml 50X TAE buffer with 490 ml idtilled water and stored at RT.

Ethidium bromide solution

10 µl of ethidium bromide was dissolved in 100 ml TAE buffer to make a final concentration of 20 mg/ml and stored at 4°C in the dark.

0.5 McFarland standard

A 0.5 McFarland turbidit standard was prepared by adding 0.5 ml of 1.175% (w/v) barium chloride ($BaCl_2.2H_2O$) solution to 99.5 ml of 1% sulphuric acid.

Boronic acid solution

1.5 g of boronic acid was dissolved in 5 ml of DMSO. The solution was stored at 4°C.

Phosphate buffer solution:

359 µl of 1M K_2HPO_4 solution and 142µl of 1M KH_2PO_4 solution was dissolved in distilled water to make the final volume of 50 ml and final concentration of 0.01 M. The p^H was adjusted to 7.2 and stored at 4°C.

Kovac's reagent

Ingredients	Amount
p-dimethaylamino benzaldehyde	0.5 g/L
Amyl alcohol	75 ml/L
Hydrochloric acid, concentrated	25 ml/L
Distilled water	Up to 1 L

Methyl Red indicator

Ingredients	Amount (g/L)
Methyl Red	0.1 g
Ethyl alcohol	300 ml
Distilled water	500 g

Gel loading buffer*

Ingredients	Amount (g/L)
Sucrose	6.7
Bromophenol blue	0.04
Distilled water	Up to 1 L

*Stored at 4°C

ATP™ Genomic DNA Mini Kit (Blood/Culture Cell/Bacteria) Catalog No. AGB100/AGB300

Reagents	Purpose
GT Buffer	For the resuspension of pelleted cells
GB Buffer	For lysis of cells
GD Column	For Binding of the DNA molecules
Collection Tube	For collection of flothrough
W1 Buffer	For washing purposes
Wash Buffer (ethanol added)	For washing purposes
Elution Buffer	For elution of DNA from the GD column

Jena Bioscience Fast-n-Easy mini prep plasmid DNA Purification kit .Catalog No. PP-2041

Reagents	Purpose
Lysis Buffer	For lysis of cells
Neutralization Buffer (containing RNase A)	For Nutralizaton
Spin Column	For Binding of the plasmid DNA molecules
Collection Tube	For collection of flothrough
Activation Buffer	Foe activation of Spin Column
Washing Buffer	For washing purposes
Elution Buffer	For elution of the plasmid DNA from the Spin Column

PureLink™ PCR Purification Kit. Catalog No. K3100-02

Reagents	Purpose
Binding Buffer (Isopropanol added)	Help in binding of PCR product
Spin Column	For Binding of PCR product
Collection Tube	For collection of flow through
Wash Buffer	For washing purposes
Elution Buffer	For elution of the purified DNA from the Spin column

Appendix III

Instruments & Apparatus

The important instrument and apparatus used through the study are listed below:

ABI Prism 3130 Genetic Analyzer	USA
AlphaImager HP System Versatile Gel Imaging	USA
Autoclave, Model no: HL-42AE	Hirayama corp, Japan
Centrifuge, Mode1:5804	Eppendorf, Germany
Class II Microbiological safety cabinet	Labcaire, USA
Electric balance, Scout, SC4010	USA
Freezer (-30°C)	Liebherr, Germany
Horizontal gel elctrophoresis apparatus Hl-SET	UK
Incubator	Japan
Microcentrifuge, Mikro20	Germany
Microcentrifuge tube	Eppendorf, Germany
Micropipettes	Eppendorf, Germany
Microwave oven, Model: D90N30 ATP	Butterfly, China
NanoDrop 2000	Thermo Scientific, USA
PCR Thermocycler	Biometra , Germany Applied Biosystems, USA
pH meter, Model no: MP220	Eppendorf, Germany
Power pack	Toledo, Germany
Refrigerator (4°C)	Vest frost
Room temperature horizontal shaker	Gerhardt, Germany
Sterilizer, Model no: NDS-600D	Japan
Water bath, Model:SUM	England

Isolate No	Sequences
39/D/mh3	TGCAGTCGAGCGAACAGACGAGGAGCTTGCTCCTCTGACGTTAGCGGCGGACGGGTGAGT AACACGTGGATAACCTACCTATAAGACTGGGATAACTTCGGGAAACCGGAGCTAATACCG GATAATATATTGAACCGCATGGTTCAATAGTGAAAGACGGTTTTGCTGTCACTTATAGAT GGATCCGCGCCGCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATGCGTAG CCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGA GGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGT GATGAAGGTCTTCGGATCGTAAAACTCTGTTATTAGGGAAGAACAAATGTGTAAGTAACT ATGCACGTCTTGACGGTACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCG GTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGT TTTTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGAAA ACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATAT GGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGATGTGCGAAAG CGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAA GTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGG AGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGC ATGTGGTTTAATTCGAAGCAACGCGAAGA
54/D/mh1	TGCAGTCGAGCGGATGAGTGGAGCTTGCTCCATGATTCAGCGGCGGACGGGTGAGTAATG CCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGCATAC GTCCTACGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATGAGCCTAGGTCGG ATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGG ATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGG AATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTC GGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGA CGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGG TGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCGTTAAGTTGGA TGTGAAAGCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCGAGCTAGAGTATGGC AGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAG TGGCGAAGGCGACCACCTGGGCTAATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAA CAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGGATCCT TGAGATCTTAGTGGCGCAGCTAACGCATTAAGTCGACCGCCTGGGGAGTACGGCCGCAAG GTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTC GAAGCAACGCGAAGAACCT
48/D/mh1	TGCAAGTCGAGCGGATGAAGGGAGCTTGCTCCCTGATTTAGCGGCGGACGGGTGAGTAAT GCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTTCCGAAAGGAACGCTAATACCGCATA CGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCG GATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAG GATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGG GAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTT CGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCGTTTGGCTAATATCCAAGCGTTTTG ACGTTACCGACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGG GTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCGTTAAGTTGG ATGTGAAAGCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCGAGCTAGAGTACGG TAGAGGGTAGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCA GTGGCGAAGGCGACTACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAA ACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGGTTCC TTGAGAACTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAA GGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATT CGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGCTGAGAACTTTC

45/D/cip+cef1	CATGCAGTCGAGCGGATGACGGGAGCTTGCTCCTTGATTCAGCGGCGGACGGGTGAGTAA TGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGCAT ACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTC GGATTAGCTAGTTGGTGGGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGA GGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGG GGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCT TCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTT GACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAG GGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCGTTAAGTTG GATGTGAAAGCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCGAGCTAGAGTACG GTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACC AGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCA AACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGAATC CTTGAGATTTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCA AGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAAT TCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATGCAGAGAACTTT
50/D/swab1	CAGTCGAACGGCAGCACAGAGGAGCTTGCTCCTTGGGTGGCGAGTGGCGGACGGGTGAGG AATACATCGGAATCTACTCTGTCGTGGGGGATAACGTAGGGAAACTTACGCTAATACCGC ATACGACCTACGGGTGAAAGCAGGGGATCTTCGGACCTTGCGCGATTGAATGAGCCGATG TCGGATTAGCTAGTTGGCGGGGTAAAGGCCCACCAAGGCGACGATCCGTAGCTGGTCTGA GAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGT GGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATACCGCGTGGGTGAAGAAGGC CTTCGGGTTGTAAAGCCCTTTTGTTGGGAAAGAAATCCATCTGGTTAATACCCGGGTGGG ATGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGA AGGGTGCAAGCGTTACTCGGAATTACTGGGCGTAAAGCGTGCGTAGGTGGTCGTTTAAGT CCGTTGTGAAAGCCCTGGGCTCAACCTGGGAACTGCAGTGGATACTGGGCGACTAGAATG TGGTAGAGGGTAGCGGAATTCCTGGTGTAGCAGTGAAATGCGTAGAGATCAGGAGGAACA TCCATGGCGAAGGCAGCTACCTGGACCAACATTGACACTGAGGCACGAAAGCGTGGGGAG CAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGCGAACTGGATGTTGGGT GCAATTTGGCACGCAGTATCGAAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTC GCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTT AATTCGATGCAACGCGAAGAACCTTACCTGGCCTTGACAT
3/C/cip+cef2	TGCAGTCGAGCGGATGAAGGGAGCTTGCTCCTGGATTCAGCGGCGGACGGGTGAGTAATG CCTAGGAATCTGCCTGGTAGTGGGGGATAACGTCCGGAAACGGGCGCTAATACCGCATAC GTCCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATGAGCCTAGGTCGG ATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGGTCTGAGAGG ATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGG AATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTC GGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGA CGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGG TGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCAGCAAGTTGGA TGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTACTGAGCTAGAGTACGGT AGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAG TGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAA CAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGGATCCT TGAGATCTTAGTGGCGCAGCTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCCGCAAG GTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTC GAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGCTG
5/c/cefo1	TGCAGTCGAGCGGTAGCACAGGGGAGCTTGCTCCCTGGGTGACGAGCGGCGGACGGGTGA GTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACC GCATAGCGTCGACAAGACCAAAGGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCC CAGATGGGATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGT CTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAG CAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGA ACGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAGGTGGTGAGCTTAATACGCTCA TCAATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAAT ACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTT AAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTTGAAACTGGCAAGCTAG AGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGG AATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGG GGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTGGAGGT TGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACG GCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGG TTTAATTCATGCAACGAAATAACCTTAC

41/D/mh2	GCAGTCGACGCTTCTTTCCATCCCGAGTGCTTGCACTCATTGGAAAGAGGAGTGGCGGAC GGGTGAGTAACACGTGGGTAACCTACCCATCAGAGGGGGATAACACTTGGAAACAGGTGC TAATACCGCATAACAGTTTATGCCGCATGGCATAAGAGTGAAAGGCGCTTTCGGGTGTCG CTGATGGATGGACCCGGGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCCAC GATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACT CCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGC CGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACAAGGACG TTAGTAACTGAACGTCCCCTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCA GCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGC GCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGA AACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCG TAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAG GCTCGAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATG AGTGCTAAGTGTTGGAGGATTCCGCCCTTCAGTGCTGCAGCAACGCATTAAGCACTCCGC CTGGGAGTACGACCGCAAGGTTGAACTCAAGGATTGAGGGGCCCGCACAAGCGGTGGAGC ATGTGGTTTAATTCAGAGCACGCGAAGACCTTACCAGGT
46/D/mh2	TGCAAGTCGAACGGTAAGCAGGAAGCAGCTTGCTGCTTTGCTGACGAGTGGCGGACGGGT GAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATA CCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCGGATGTGCC CAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGT CTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAG CAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGA AGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGCGATAAGGTTAATAACCTTG TCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAAT ACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTC AAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAG AGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTATCGGTGAAATGCGTAGAGATCTGGAAG GATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGG GGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGT TGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACG GCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAACATGTGG TTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACAT
49/D/swab2	ATGCAAGTCGAGCGGTAGCACAGAGAGCTTGCTCTCGGGTGACGAGCGGCGGACGGGTGA GTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACC GCATAACGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCATGCCATCAGATGTGCCCA GATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCT GAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCA GTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAG GCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGCGATAAGGTTAATAACCTTGTC GATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC GGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAA GTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAG TCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAA TACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGG AGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGTTG TGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGC CGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTT TAATTCGATGCAACGCGAAGAACCTTACC
44/D/mh2	TGCAAGTCGAGCGGGGGAAGGTAGCTTGCTACCGGACCTAGCGGCGGACGGGTGAGTAAT GCTTAGGAATCTGCCTATTAGTGGGGGACAACATCTCGAAAGGGATGCTAATACCGCATA CGTCCTACGGGAGAAAGCAGGGGATCTTCGGACCTTGCGCTAATAGATGAGCCTAAGTCG GATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCGGGTCTGAGAG GATGATCCGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGG GAATATTGGACAATGGGGGGAACCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTT ATGGTTGTAAAGCACTTTAAGCGAGGAGGAGGCTACTTTAGTTAATACCTAGAGATAGTG GACGTTACTCGCAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAG GGTGCGAGCGTTAATCGGATTTACTGGGCGTAAAGCGTGCGTAGGCGGCTTATTAAGTCG GATGTGAAATCCCCGAGCTTAACTTGGGAATTGCATTCGATACTGGTGAGCTAGAGTATG GGAGAGGATGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACC GATGGCGAAGGCAGCCATCTGGCCTAATACTGACGCTGAGGTACGAAAGCATGGGGAGCA AACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGTCTACTAGCCGTTGGGGCC TTTGAGGCTTTAGTGGCGCAGCTAACGCGATAAGTAGACCGCCTGGGGAGTACGGTCGCA AGACTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAAT TCGATGCAACGCGAAGAACCTTACCTGGCCTTGACATAC

44/Dcip+cef1	CATGCAAGTCGAGCGGGGGAAGGTAGCTTGCTACCGGACCTAGCGGCGGACGGGTGAGTA ATGCTTAGGAATCTGCCTATTAGTGGGGGACAACATCTCGAAAGGGATGCTAATACCGCA TACGTCCTACGGGAGAAAGCAGGGGATCTTCGGACCTTGCGCTAATAGATGAGCCTAAGT CGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCGGGTCTGAG AGGATGATCCGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTG GGGAATATTGGACAATGGGGGGAACCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCC TTATGGTTGTAAAGCACTTTAAGCGAGGAGGAGGCTACTTTAGTTAATACCTAGAGATAG TGGACGTTACTCGCAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAG AGGGTGCGAGCGTTAATCGGATTTACTGGGCGTAAAGCGTGCGTAGGCGGCTTATTAAGT CGGATGTGAAATCCCCGAGCTTAACTTGGGAATTGCATTCGATACTGGTGAGCTAGAGTA TGGGAGAGGATGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATA CCGATGGCGAAGGCAGCCATCTGGCCTAATACTGACGCTGAGGTACGAAAGCATGGGGAG CAAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGTCTACTAGCCGTTGGGG CCTTTGAGGCTTTAGTGGCGCAGCTAACGCGATAAGTAGACCGCCTGGGGAGTACGGTCG CAAGACTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTA ATTCGATGCAACGCGAAGAACCTTACCTGGC
45/Dcefo3	ATGCAGTCGAGCGGACAGAAGGGAGCTTGCTCCCGGATGTTAGCGGCGGACGGGTGAGTA ACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGAGCTAATACCGG ATAGTTCCTTGAACCGCATGGTTCAAGGATGAAAGACGGTTTCGGCTGTCACTTACAGAT GGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAG CCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGA GGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGT GATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCAAGAGTAACT GCTTGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCG GTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGT TTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAA ACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGT GGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAG CGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAA GTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGG AGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGC ATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACAT
7C/cip2	TAATGCAAGTCGAGCGGACTTTAAAAGCTTGCTTTTAAAGTTAGCGGCGGACGGGTGAGT AACACGTGGGCAACCTGCCTGTAAGACTGGGATAACTTCGGGAAACCGGAGCTAATACCG GATAATCCTTTTCCTCTCATGAGGAAAAGCTGAAAGACGGCATCTCGCTGTCACTTACAG ATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGT AGCCAACCTGAGAGGGTGATCGGCCACACTGGGACTGAAACACGGCCCAAACTCCTACGG GAGGCAGCAGTAGGGAATCTTCCGCAATGGGACAAAAGTCTGACGGAGCAACGCCGCGTG AGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACAAGAGTA ACTGCTTGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCC GCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGC GGTCCTTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAAGGGTCATTGGAAACTG GGGGACTTGAGTGCAGAAGAGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGA TGTGGAGGAACACCAGTGGCGAAGGCGACTCTTTGGTCTGTAACTGACGCTGAGCCGAAG CTGGACAACAGATAATACTGTAGTCAGCGTAACGATGATGC
44/Dswab2	TATACTGCAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGG TGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAA TACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTT ATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGAT GCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCT ACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGC GTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGT TGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGC AGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGC AGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAA CTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTA GAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGC GCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGA GTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCG CCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCG GTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCT

4/C/cip1	GCAGTCGAACGGTAACAGGAAGCAGCTTGCTGCTTCGCTGACGAGTGGCGGACGGGTGAG TAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCG CATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAG ATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTG AGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAG TGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGG CCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGGAGTAAAGTTAATACCTTTGCTC ATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACG GAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAG TCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGT CTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAAT ACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGA GCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGT GCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCC GCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTT AATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCACAGAACTT
2/C/mh3	ATGCAAGTCGAACGGTAACAGGAAGCAGCTTGCTGCTTTGCTGACGAGTGGCGGACGGGT GAGTAATGTCTGGGAAACTGCCCGGTGGAGGGGGATAACTACTGGAAACGGTAGCTAATA CCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCACCGGATGTGCC CAGATGGGATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATCCCTAGCTGGT CTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAG CAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGA AGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGGGATGTGGTTAATAACCATA TTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAAT ACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTC AAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTTG AGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGG AATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGG GGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTATTTGGAGGT TGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAATAGACCGCCTGGGGAGTACG GCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGG TTTAATTCGATGCAACGCGAA
56/D/mh1	AAAGCTTGCTTTCTTGCTGACGAGCGGCGGACGGGTGAGTAATGTATGGGGATCTGCCCG ATAGAGGGGGATAACTACTGGAAACGGTGGCTAATACCGCATAATGTCTACGGACCAAAG CAGGGGCTCTTCGGACCTTGCACTATCGGATGAACCCATATGGGATTAGCTAGTAGGTGG GGTAAAGGCTCACCTAGGCGACGATCTCTAGCTGGTCTGAGAGGATGATCAGCCACACTG GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGG CGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTAGGGTTGTAAAGTACTT TCAGCGGGGAGGAAGGTGATAAGGTTAATACCCTTGTCAATTGACGTTACCCGCAGAAGA AGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGG AATTACTGGGCGTAAAGCGCACGCAGGCGGTCAATTAAGTCAGATGTGAAAGCCCCGAGC TTAACTTGGGAATTGCATCTGAAACTGGTTGGCTAGAGTCTTGTAGAGGGGGGTAGAATT CCATGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAATACCGGTGGCGAAGGCGGCCCC CTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCT GGTAGTCCACGCTGTAAACGATGTCGATTTAGAGGTTGTGGTCTTGAACCGTGGCTTCTG GAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGA ATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAA CCTTACCTACTCT
5/C/mac1	TGCAGTCGAGCGGTAACAGGGGAAGCTTGCTTCTCGCTGACGAGCGGCGGACGGGTGAGT AATGTATGGGGATCTGCCCGATAGAGGGGGATAACTACTGGAAACGGTGGCTAATACCGC ATAATCTCTTAGGAGCAAAGCAGGGGACCTTCGGGCCTTGCGCTGTCGGATGAACCCATA TGGGATTAGCTAGTAGGTAAGGTAATGGCTTACCTAGGCGACGATCCCTAGCTGGTCTGA GAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGT GGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGC CCTAGGGTTGTAAAGTACTTTCAGTCGGGAGGAAGGCGTTGATGTTAATACCATCAACGA TTGACGTTACCGACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGG AGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTAATTAAGT TAGATGTGAAATCCCCGGGCTTAACCTGGGAATGGCATCTAAGACTGGTTAGCTAGAGTC TTGTAGAGGGGGGTAGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAATA CCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAG CAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTGGAGGTTGTT CCCTAGAGGAGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCG CAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTA ATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATC

বাংলাদেশ ডায়াবেটিক সমিতি
DIABETIC ASSOCIATION OF BANGLADESH

Memo No. BADAS-ERC/EC/14/00137 Date: May 6, 2014

To

Mousumi Karmaker
M.Phil (Thesis part)
Department of Microbiology
University of Dhaka

Subject: Ethical Clearance

The Ethical Review Committee (ERC) of the Diabetic Association of Bangladesh (BADAS) has approved your protocol on "Characterization of microbial agents in diabetic foot infection and its correlation with chronic subclinical inflammation".

(Dr. KMS Aziz)
Chairman
Ethical Review Committee

122 KAZI NAZRUL ISLAM AVENUE SHAHBAG DHAKA 1000 BANGLADESH PHONE : 8616641-50, 880-2-9661551 FAX : 9677772, Web : www.dab-bd.org