BACTERIAL DIVERSITY WITH EMERGING ANTIMICROBIAL RESISTANCE OF DIABETIC FOOT INFECTION AND THEIR CURRENT DETECTION TECHNIQUES: A REVIEW

By

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of Bachelor of Science in Biotechnology

Department of Mathematics and Natural Sciences
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Declaration

It is hereby declared that

1. The thesis submitted is my/our own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I/We have acknowledged all main sources of help.

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Approval

The thesis/project titled "Bacterial Diversity with emerging antimicrobial resistance of diabetic foot infection and their current detection techniques: A review" submitted by Sayeed Akhtar Alvi (Id-16136028) of Spring Semester, 2016 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Science in Biotechnology on February, 2021.

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Ethics statement:

This thesis was done with integrity, honesty and complete fairness.

Abstract:

Diabetic foot ulcer is becoming one of the major complications around the world with associated consequences such as lower-extremity amputation, high morbidity, mortality and hospitalization. It is on its way to become the next global epidemic. Major part of the concern comes from the diverse bacterial and fungal population that is found at the infection site and their growing antimicrobial resistance. If the threat of antimicrobial resistance is not dealt with than it will rise to become the main cause of mortality and below knee amputation in case DFI. Also, most of the time main focus is given on detecting bacterial population which causes the fungal population to go unnoticed and act as the silent enemy. Bacterial and fungal prevalence scenario from different countries have been discussed in this study along with the alarming antibiotic resistance scenario around the globe. Furthermore, choosing the correct technique to identify them also plays a vital role. With proven lacking's of the culture-based methods maybe it is time to move on to the faster and more specific molecular methods. As, many of the molecular techniques have already proven to be more efficient. This review discussed the bacterial and fungal prevalence along with their growing antimicrobial resistance and evaluated different biochemical and molecular techniques in identification process.

Keywords: DFI, causative agents, antibiotic resistance, wound infection, molecular methods, biochemical tests

Dedication

I dedicate this thesis to my parents, my grandfather and grandmother.

Acknowledgement

I express my deepest gratitude to the almighty to have enabled and aided me in successfully completing my thesis. Throughout my venture I have worked with many people and I express my deepest gratitude and warmest thanks to all of them.

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It will be my honor to cherish the knowledge and skills gained from this experience and to nurture them and apply them in the best possible way for the betterment of society.

Sayeed Akhtar Alvi

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Table of Contents

List of tables

List of Acronyms

DFI	Diabetic foot infection
PCR	Polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
MRSA	Methicillin-resistant *Staphylococcus aureus*

Chapter 1

Introduction

1.1 What is Diabetes?

Diabetes mellitus or commonly known as diabetes is a chronic, metabolic disease. It causes blood glucose or blood sugar levels in our body to rise. Which in time leads to harmful effects on our heart, blood vessels, eyes, kidneys and nerves.(World Health Organization) Diabetes occurs when the pancreas cannot produce enough insulin or when our body cannot efficiently use the insulin that has been produced by the pancreas. Insulin is a hormone which controls blood sugar levels in a human body. The condition when the blood sugar level is in increasing state than usual is called Hyperglycemia. It is a common consequence of uncontrolled diabetes mellitus which results in significant damage to nerves and blood vessels.(World health organization, 2020) It has slowly become one of the top 10 reasons of death globally.(Roth et al., 2018) It was evident that an increase premature mortality by 5% due to diabetes in the period of 2000 to 2016 and was responsible for 1.6 million deaths globally.(World health organization, 2020) A person suffering from diabetes has 10-30% less life expectancy than a non-diabetic individual.("Intensive Diabetes Treatment and Cardiovascular Disease in Patients with Type 1 Diabetes," 2005) According to available data so far, there was a global increase occurrence of diabetes from 11.3 million in 1990 to 22.9 million in 2017.(Lin et al., 2020) The global prevalence increased from 211.2 million in 1990 to 476 million in 2017.(Lin et al., 2020) In Bangladesh, the rate increased from 4% in 1995-200 to 9% in 2006-2010.(Saquib et al., 2012) It is estimated that 13% of the Bangladeshi population will be suffering from diabetes by 2030.(Beckman, 2012)

1.2 Different types of Diabetes

There are different types of diabetes: Diabetes mellitus (Type 1 and 2), gestational diabetes etc. Type 1 diabetes occurs when there is a shortage in insulin production. I this case daily administration of insulin is required. Some of the symptoms of Type 1 insulin are, hunger, weight loss, vision changes and fatigue.(World health organization, 2020) Type 2 diabetes occurs when the human body cannot use the insulin that has been produced. Majority of the world population is suffering from this type of diabetes.(World health organization, 2020) When blood glucose values are higher than normal but also below the diagnostic value, then it is called Gestational diabetes. It is similar to hyperglycemia. This type of diabetes occurs in pregnant women and causes them complications during pregnancy and delivery. The women and the newborn both have higher chances of developing type 2 diabetes in future.(World health organization, 2020)

1.3 Causes of Diabetes:

Diabetes can be caused by a variety of reasons. For instance, type 1 diabetes occurs when an individual's own immune system attacks and destroys insulin-producing beta cells of the pancreas. On the other hand, lifestyle factors and genes play a major role in causing type 2 diabetes. Individuals who are physically inactive, overweight or obese are more likely to develop type 2 diabetes. Because, weight gain leads to insulin resistance and insulin resistance is largely responsible for type 2 diabetes. In this condition, muscle, liver and fat cells do not use insulin well. As a result, body needs more insulin to help glucose enter into cells and ultimately pancreas cannot produce enough insulin to keep up with the demand. Having family history of diabetes make the future generation more likely to suffer from the disease. Sometimes, individuals with no previous family history can also develop diabetes. This is caused by Monogenic mutation, where a single gene suddenly gets mutated on its own.(Symptoms & Causes of Diabetes, 2016) Cystic fibrosis also responsible for diabetes by causing scarring in the pancreas which prevents the pancreas from making enough insulin.(Symptoms & Causes of Diabetes, 2016) Hemochromatosis can cause diabetes by enabling the body to store too much iron and resulting in damaging the pancreas. Sometimes hormonal imbalance can cause insulin resistance leading to diabetes. This happens in the form of Cushing's syndrome, Acromegaly, Hyperthyroidism etc. Medicines can damage beta cells as well. For example, anti-seizure drugs, psychiatric drugs, drugs against HIV, pentamidine (drug used for the treatment of pneumonia), glucocorticoids etc.(Symptoms & Causes of Diabetes, 2016)

1.4 What is DFI (Diabetic foot infection)?

One of the major problems related to diabetes is diabetic foot infection or DFI. It is an infection of the soft tissue or bone below the malleoli. Usually the infection happens in the site of skin trauma or ulceration.(G E Reiber et al., 1999) The characteristic comparison between diabetic patients who developed diabetic foot ulcer and patients that did not are as follows, Older age (61.7 vs 56.1), longer diabetic duration (11.3 vs 7.4), lower body mass index (23.8 vs 24.4), high percentage of smokers (29.1% vs 17.4%), hypertension (63.4% vs 53.1%), diabetic retinopathy (63.6% vs 33.3%).(Zhang et al., 2017) Comparing the before mentioned data, patients who developed DFI exhibited higher percentage of numbers.

Globally 6.3% people are affected by DFI.(Zhang et al., 2017) North America tops the chart with

the highest ratio of DFI. An estimated 13% people suffer from DFI in North America. Followed by Africa (7.2%), Asia (5.5%), Europe (5.1%) and Oceania (3%).(Zhang et al., 2017) The scenario in Bangladesh is alarming as well. In a study conducted on individuals visiting 16 Bangladeshi hospitals and medical centers revealed that 44.5% of the study population was at risk of DFI. Individuals living in rural locations were more at risk (45.6%).(Banik et al., 2020)

1.5 Risk factors of DFI

There are several risk factors related to DFI. The main being neuropathy and peripheral vascular disease. Neuropathy produces anatomic abnormalities in the foot by affecting the sensory and motor components of the nervous system, which leads to unusual extension of the infected foot. Autonomic dysfunction causes reduced sweating leading to development of dry skin which results in fissures or cracks ultimately resulting in infection. Amputation and gangrene are the next two major risks associated with DFI.(Al-Rubeaan et al., 2015) Poor glycemic control, cigarette smoking, diabetic nephropathy are also some of the common risk factors. According to a large cohort study, 46.64% and 29.36% DFI patients suffered from Retinopathy and nephropathy. 46.64% patients suffered from vasculopathy. 56.78% were affected by hypertension.(Al-Rubeaan et al., 2015) The overall major amputation rate was found to be 26.2% in patients with diabetic foot wound.(Kim et al., 2018)

1.6 Objective:

The objective of this study was to discuss about the bacterial and fungal diversity and the role of antimicrobial resistance leading to DFI, along with their current detection techniques.

Chapter 2

Causative agents and prevalence

2.1 Causative agents:

Diabetic foot infection is one of the main causes of hospitalization of diabetic patients.(Mohammuddunnobi et al., 2018) It is a complex heterogeneous disorder that affects 15% of the patients during their lifetime.(Anand et al., 2016) Diabetic foot infection depends on several factors such as, high blood sugar level, suppressed immunity, inadequate blood supply and neuropathy.(Aherrao, Shahi, Dwivedi, Kumar, et al., 2012) However, the primary cause of Diabetic foot infection are different microbial agents.(Benjamin A. Lipsky, 1999) Study revealed that 85% of lower limb amputation is caused by polymicrobial infection which is one of the most extreme outcomes of DFI.(Menon et al., 2006; Pecoraro et al., 1991; Viswanathan, 2007) Polymicrobial infections involve a variety of aerobic and anaerobic infections such as, *Staphylococcus aureus*, *Streptococcus spp*., *Enterobacteriaceae spp*., *Bacteroides fragilis*, *Peptococcus spp*. and *Peptostreptococcus spp*.(Benjamin A. Lipsky et al., 2012) The aerobes and anaerobes are divided into gram negative and gram positive bacteria as well, which will be discussed later in the article. Apart from bacteria mycotic agents such as fungi play a major role as well. Filamentous fungi and yeasts have been detected to cause DFI by various studies. *Candida spp*., is determined to be the main agent involved in DFI.(Fata et al., 2011)

2.2 Prevalence:

Individuals who are affected by diabetes are more likely to develop infection than normal individuals and the rate of developing foot infection can be as high as 25%.(Singh et al., 2005) The reason behind this are the various microbial agents. At the same time, microbial flora of the lower limb, various metabolic factors, use of antibiotic and food hygiene is responsible for the variety of microorganisms found in DFI.(Chincholikar & Pal, 2002)

Among the diabetic patients' correlation was found between age and infection occurrence rate. The age standardized rate for prevalence increased from 161.7 (1990) to 164.8 (2017) for Type 1 Diabetes and 4576.7 (1990) to 5722.1 (2017) for type 2.(Lin et al., 2020) According to a study conducted on patients from Diabetic Foot Care Hospital and Dhaka Medical College Hospital, Bangladesh, the average age was found to be 56.25 years.(Karmaker et al., 2016) In another study conducted by BIRDEM General Hospital, Bangladesh, the average age was 51.57±12.13 years.(Mohammuddunnobi et al., 2018) In Malaysia a study conducted by Hospital Universiti Sains detected that age of the patients who were most frequently affected by DFI was in the range

of 41-60 years.(Sharifah Aisyah et al., 2019) 57.6 years was the average age found by investigation conducted on another Malaysian hospital.(NS Raja, 2007) From a survey on diabetic patients admitted to S.L. Raheja Hospital and Diabetic Research Centre, Mumbai, India, the average age was found to be 65 years.(Kareliya et al., 2019) According to another research the average was detected to be 60.8±10.2.(Chellan et al., 2010) Therefore, according to these it can be said that the prevalence of DFI is most among patients of advance age ranging from 50-70 years.

DFI was found to be more prevalent among males than females. In accordance with a study carried out in BIRDEM General Hospital, Bangladesh, the male to female ratio was 2.3:1.(Mohammuddunnobi et al., 2018) Similarly, in a study conducted on patients from a hospital in Malaysia the male to female ratio was 1.58:1.(NS Raja, 2007) In relation to another report, it was found that 73.7% males were suffering from DFI.(Chellan et al., 2010) Furthermore, 74% males were found to be suffering from DFI among the total patients by another study.(Kareliya et al., 2019)

It has already been mentioned that, different types of bacteria are the most prevalent organism found in DFI. They can be gram positive, gram negative, aerobic, anaerobic. According to an investigation conducted on Diabetic Foot Care Hospital and Dhaka Medical College Hospital, Bangladesh, *Enterococcus spp.* (9%), *Klebsiella spp.* (7%), *Bacillus cereus* (17%). were found to be the most dominant. They also detected five strains of pseudomonas such as, *P.aeruginosa*, *P. stutzeri*, *P. pseudoalcaligens*, *P. monteilii*, and *P. hibiscicola*. According to their findings, *Ent. hormaechei* (22%) was the organism that was found in highest number among patients. Although *Citrobacter spp*. was only found in 2% patients, it was responsible for 5% infections in immune compromised patients. Furthermore, staphylococcus species amounted for 13% of all isolates. But, the most important finding was the presence of a nosocomial pathogen called *A. baumannii* (10%).(Karmaker et al., 2016) In accordance with a study conducted by BIRDEM General Hospital, Bangladesh, Polymicrobial infection was found in 75.3% cases.(Paul et al., 1970) Polymicrobial infection was also found in high numbers in patients with high grade foot ulcers.(Paul et al., 1970) In this study gram negative organism was found in high numbers (80%), such as Pseudomonas (48%), *Proteus sp*. (33%). While *Staphylococcus aureus* (21.3%) was the most frequent among the 19.3% gram positive pathogen found. Fungus was detected as well, although in very low percentage (0.7%).(Paul et al., 1970) Some of the other organisms detected

were, Coagulase negative *Staphylococcus, Pseudomonas sp., Klebsiella sp., Escherichia coli., Acinetobacter sp., Citrobacter sp., Serratia sp.* and *Providencia sp.*(Paul et al., 1970)

A study conducted by Hospital Universiti Sains Malaysia reported that gram negative organisms were the principal agent in causing DFI, as 62.4% such organism was found by them. *Pseudomonas spp*. (27.8%), *Proteus spp.* (10.5%), *Klebsiella spp*. (8.3%), *Enterobacter spp*. (6%), *Acinetobacter spp*. (4.5%) and *Escherichia. Coli* (3.8%) were the gram-negative organisms detected. Gram positive (38%) organisms included, *Staphylococcus aureus* (20.3%), *Streptococcus agalactiae* (9.8%) and *Enterococcus spp*. (3%). They also reported that 28.8% individuals were infected by polymicrobial infection.(Sharifah Aisyah et al., 2019) Another study from Malaysia also indicates that gram negative bacteria (52%) is the most frequently detected organism in DFI. Some of the most frequently detected organisms were, *Proteus spp*. (28%), *P. aeruginosa* (25%), *Klebsiella pneumoniae* (15%), *E. coli* (14.9%) and *Enterobacter cloacae* (13.9%). On the other hand, *S. aureus* (44%), Group B Streptococci (25%) and *Enterococcus spp* (9%) were the most frequently detected gram-positive organism. Five anaerobic bacteria were detected in this study. They were, *Peptostreptococcus spp*., 3 *Bacteroides spp* and *Clostridium spp*.. They also reported 43% polymicrobial infection.(NS Raja, 2007)

United States-based multicenter clinical trial conducted a study from 2001-2004. According to their research, 83.8% patients were suffering from polymicrobial infection. But, more importantly 43.7% patients were infected by four or more organisms. In this study gram positive (57.2%) bacteria were more prevalent. 48% patients were infected by only aerobes and 43.7% were infected by both aerobic and anaerobic bacteria. On the other hand, only 1.3% patients were infected by only anaerobic bacteria. Frequently detected aerobic bacteria included, Nonfermenting gram-negative rods (7.7%), *Pseudomonas spp*. (9.3%), Enterobacteriaceae group (32.4%), *Corynebacterium spp*. (25.6%), Miscellaneous gram-positive rods (11.7%), *Enterococcus spp*. (33.9%), *Staphylococcus spp.* (85.5%), oxacillin resistant *S. aureus* (11.7%), oxacillin sensitive *S. aureus* (36.1%), *S. epidermidis* (15.9%), oxacillin sensitive *S. epidermidis* (3.3%), *S. haemolyticus* (4.8%), *S. lugdunensis* (4.8%). Coagulase-negative staphylococci (7.9%) and *Streptococcus spp*. (41.9%) were also reported to be found. Anaerobic bacteria included, Bacteroides fragilis group (12.1%), *Fusobacterium spp*. (2.4%), *Porphyromonas spp*. (11.7%), *Prevotella spp*. (14.1%), Anaerobic cocci (48.2%), *Clostridium spp*. (4.4%), Non spore forming gram-positive rods (9.5%).

Gram positive bacteria consisted of 80% aerobic organism. Among them *S. aureus* (76.6%) was the most prominent. Other organisms included, Coagulase negative staphylococci, *S. epidermidis, Staphylococcus lugdunensis, Staphylococcus haemolyticus, Staphylococcus simulans, Staphylococcus hominis, Streptococcus agalactiae, Streptococcus mitis, Streptococcus milleri, Enterococci, Helcococcus, Aerococcus, Gemella, Corynebacterium tuberculostearicum, Corynebacterium amycolatum, Corynebacterium xerosis* and *Corynebacterium urealyticum*. Gram negative organisms included, *Pseudomonas aeruginosa, Proteus mirabilis*, Klebsiella species, while Enterobacteriaceae (63.3%) was identified as the largest group of gram negative rod.(Citron et al., 2007)

A research from Kenyatta National Hospital, Nairobi detected 64.71% gram negative and 29.41% gram positive bacteria in DFI patients. Frequently detected organisms were, *S. aureus* (16.47%), *E. coli* (15.29%), *Proteus mirabilis* (10.59%), *Klebsiella pneumoniae* (7.06%) and *P. aeruginosa* (7.06%). Other detected organisms were, *Staphylococcus lentus, Staphylococcus simulans, Staphylococcus xylosus, Acinetobacter baumannii, Burkholderia cepacia, Kocuria kristinae, Leuconostoc mesenteroides, Pantoea agglomerans, Providencia stuartii and Raoultella ornithinolytica.*(Mutonga et al., 2019)

A study conducted on patients admitted to endocrinology ward at All India Institute of Medical Sciences reports that, most of the patients were infected from aerobic bacteria only (65%). On the other hand, 1.2% patients were infected with anaerobic bacteria only and the rest of the 33.8% were infected by both. A staggering 70% patients were suffering from a polymicrobial infection while 12.5% patients were infected by more than three species. Frequently detected aerobic gram-negative bacteria (51.4%) included, Proteus species (12.6%), *E. coli* (12.0%), *Pseudomonas aeruginosa* (9.8%), Acinetobacter species (9.3%), Klebsiella species (6.6%) and 0.5% Citrobacter and Enterobacter species each. Aerobic gram positive (33.3%) organisms included, *S. aureus* (13.7%), Enterococcus species (11.5%), Coagulase negative Staphylococci (6.6%), Micrococcus species (1.6%). Anaerobic gram negative (7.1%) included, Veillonella species (1.6%), Bacteroides species (1.6%), *Bacteroides fragilis* (1.6%), *Bacteroides eggerthii* (1.1%), *Bacteroides vulgaris* (0.5%), *Bacteroides ovatus* (0.5%). Anaerobic gram positive bacteria (8.2%) comprised off, *Peptostreptococcus assachrolyticus* (4.4%), Peptrostreptococcus species (1.6%), *Peptrostreptococcus anaerobius* (0.5%), *Clostridium perfringens* (0.5%), *Clostridium septicum*

(0.5%), *Eubacterium lentum* (0.5%).(Gadepalli et al., 2006) Another study detected 73.75% aerobic and 26.25% anaerobic organisms among the study population. Frequently detected anaerobes were *Peptrostreptococcus spp* (42.85%), *Bacteroides spp*. (28.57%), *Veillonella spp.* (14.28%), *Porphyromonas spp* (9.52%) and *Clostridium perfringens*. In this study 49.32% gram positive and 27.27% gram negative organism were also found. The most frequent isolates were, *Proteus spp*. (32.20%), *Staphylococcus aureus* (20.33%), *Klebsiella spp*. (18.64%), *Enterobacter spp*. (5.08%), *Pseudomonas spp*. (3.38%), *Escherichia coli* (3.38%), *Enterococcus spp*. (10.20%), Diptheroids (8.16%) and Citrobacter.(Haldar et al., 2017)

A study from Turkish Society of Clinical Microbiology and Infectious Diseases reports detecting 52% monomicrobial infection. However, the fatality rate was higher among individuals with polymicrobial infection (13% vs 2.3%). The prevalence of gram-negative bacteria (56.1%) was higher than gram positive bacteria according to this study. Some of the most frequent isolates were, *S. aureus* (20%), *P aeruginosa* (19%), *E. Coli* (12%). They also detected 79% coagulase negative Staphylococcus and 21% multidrug resistant P. aeruginosa.(Saltoglu et al., 2018)

Apart from bacteria, fungus also play a very important role in causing DFI. It is even regarded as the hidden enemy of DFI because of the difficulty in detection and low availability of data. Patients who had to be amputated within 15 days of admission had higher amount of fungal infection.(Sanniyasi et al., 2015)

According to an investigation, researchers detected fungal infection in 16.2% patients. The frequent fungal isolates were, *Candida albicans* (2.9%), *Candida krusei* (2%), Aspergillus (2%), Penicillium (1%) while *Candida tropicalis* (10.5%) was the most frequent one.(Sanniyasi et al., 2015)

A study which focused on isolating fungi from deep tissue of diabetic foot wounds detected 27.2% fungal species which included, *Candida parapsilosis* (25.5%), *C. tropicalis* (22.7%), *T. asahii* (12.8%), *C. albicans* (10.6%), *Aspergillus sp*. (5%), *C. guilliermondii* (2.8%), Non-albicans *Candida sp*. (2.8%), *C. glabrata* (2.8%), *Fusarium sp*. (2.8%), *Candida sake* (2.8%), *Zygosaccharomyces sp*. (2.1%), *Kodamaea ohmeri* (2.1%), *Candida globose* (1.4%), *C. krusei* (0.7%), *Penicillium sp*. (0.7%), *C. lusitaniae* (0.7%), *Candida famata* (0.7%), *Candida melibiosica* (0.7%).(Chellan et al., 2010) In this study they found that 5.8% individuals were infected by fungus only and 21.4% individuals had both fungal and bacterial infection.(Chellan et al., 2010)

Investigation conducted by S.L.Raheja Hospital and Diabetic Research Centre, Mumbai found that, among 41 patients who had to undergo below knee amputation, 70.73 had fungal infection. In this research they found that, 40% of the study population were infected by fungal species. The most frequent isolates were, *Candida albicans* (40%), *Candida krusei* (10%), Cladosporium (10%), *Aspergillus Niger* (10%), *Penicillium Marneffei* (15%), *C. Glabrata* (7.5%) and Fusarium (7.5%).(Kareliya et al., 2019)

Investigation on patients admitted to JSS Hospital, Mysore, India was able to detect fungal species along with bacterial species. The fungal species found included, Candida, *Aspergillus Niger, Aspergillus fumigates, Aspergillus flavus,* Fusarium, Trichophytons (Dermatophyte), Penicillium, Acremonium.(Raza & Anurshetru, 2017)

Fungal species detected from a study on patients admitted to Emam Reza Hospital, Iran was comprised of, *C. albicans* (9.1%), *C. tropicalis* (4.1%), *C. parapsilosis* (0.83%), *C. galbrata* (0.83%), *C. krusei* (0.83%), *Candida spp*. (3.3%), *T. mentagrophytes* (2.5%), *Rhodotorula spp*. (0.83%), *Acremonium spp*. (0.83%), *Scopulariopsis spp*. (0.83%), *A. fumigatus* (0.83%).(Fata et al., 2011)

All of the bacterial and fungal prevalence in DFI can be summarized in the following tables.

Table 1: List of the bacteria responsible for DFI

Organism		Prevalence (%)	
Enterococcus spp.		3%-33.9%	(Citron et al., 2007; Gadepalli et al., 2006; Haldar et al., 2017; Karmaker et al., 2016; NS Raja, 2007; Sharifah Aisyah et al., 2019)
Klebsiella	*Klebsiella spp.*	6.6%-18.64%	(Gadepalli et al., 2006; Haldar et al., 2017; Karmaker et al., 2016; Sharifah

			Aisyah et al., 2019)
	Klebsiella pneumoniae	7.06%-15%	(Mutonga et al., 2019; NS Raja, 2007)
Bacillus cereus		17%	(Karmaker et al., 2016)
Enterobacteria	*Enterobacter hormaechei*	22%	(Karmaker et al., 2016)
	Enterobacter spp.	5.08%-6%	(Gadepalli et al., 2006; Haldar et al., 2017; Sharifah Aisyah et al., 2019)
	Enterobacter cloacae	13.9%	(NS Raja, 2007)
	Enterobacteriaceae group	32.4%	(Citron et al., 2007)
Staphylococcus	Staphylococcus species	13%	(Karmaker et al., 2016)
	Staphylococcus aureus	13.7%-76.6%	(Citron et al., 2007; Gadepalli et al., 2006; Haldar et al., 2017; Mutonga et al., 2019; NS Raja, 2007; Paul et al., 1970; Saltoglu et al., 2018; Sharifah Aisyah et al., 2019)
	Oxacillin resistant *Staphylococcus aureus*	11.7%	(Citron et al., 2007)
	Oxacillin sensitive	36.1%	(Citron et al., 2007)

	Staphylococcus aureus		
	Staphylococcus epidermidis	15.9%	(Citron et al., 2007)
	Oxacillin sensitive *Staphylococcus epidermidis*	3.3%	(Citron et al., 2007)
	Staphylococcus haemolyticus	4.8%	(Citron et al., 2007)
	Staphylococcus lugdunensis	4.8%	(Citron et al., 2007)
	Staphylococcus spp.	85.5%	(Citron et al., 2007)
	Coagulase negative Staphylococci	6.6%-79%	(Citron et al., 2007; Gadepalli et al., 2006; Saltoglu et al., 2018)
Acinetobacter	*Acinetobacter baumannii*	10%	(Karmaker et al., 2016)
	Acinetobacter spp	4.5%	(Sharifah Aisyah et al., 2019)
	Acinetobacter species	9.3%	(Gadepalli et al., 2006)
	Pseudomonas aeruginosa	7.06%-25%	(Gadepalli et al., 2006; Mutonga et al., 2019; NS Raja, 2007; Saltoglu et al., 2018)
	Pseudomonas spp.	3.38%-48%	(Citron et al., 2007; Haldar et al., 2017; Paul et al., 1970;

			Sharifah Aisyah et al., 2019)
Proteus	*Proteus spp.*	10.5%-33%	(Haldar et al., 2017; NS Raja, 2007; Paul et al., 1970; Sharifah Aisyah et al., 2019)
	Proteus mirabilis	10.59%	(Mutonga et al., 2019)
	Proteus species	12.6%	(Gadepalli et al., 2006)
E. coli		3.38%-14.9%	(Gadepalli et al., 2006; Haldar et al., 2017; Mutonga et al., 2019; NS Raja, 2007; Saltoglu et al., 2018; Sharifah Aisyah et al., 2019)
Streptococcus	*Streptococcus agalactiae*	0.8%-25%	(NS Raja, 2007; Sharifah Aisyah et al., 2019)
	Streptococcus spp.	41.9%	(Citron et al., 2007)
Non-fermenting gram-negative rods		7.7%	(Citron et al., 2007)
Corynebacterium spp.		25.6%	(Citron et al., 2007)
Miscellaneous gram-positive rods		11.7%	(Citron et al., 2007)
Fusobacterium spp.		2.4%	(Citron et al., 2007)
Porphyromonas spp.		11.7%	(Citron et al., 2007)
Prevotella spp		14.1%	(Citron et al., 2007)
Anaerobic cocci		48.2%	(Citron et al., 2007)
Clostridium	*Clostridium spp.*	4.4%	(Citron et al., 2007)

	Clostridium perfringens	0.5%	(Gadepalli et al., 2006)
	Clostridium septicum	0.5%	(Gadepalli et al., 2006)
Non spore forming gram-positive rods		9.5%	(Citron et al., 2007)
Citrobacter species		0.5%	(Gadepalli et al., 2006)
Micrococcus species		1.6%	(Gadepalli et al., 2006)
Veilonella	Veilonella species	1.6%	(Gadepalli et al., 2006)
	Veilonella spp.	14.28%	(Haldar et al., 2017)
Peptostreptococcus	*Peptostreptococcus spp.*	42.85%	(Haldar et al., 2017)
	Peptostreptococcus asaccharolyticus	4.4%	(Gadepalli et al., 2006)
	Peptostreptococcus anaerobius	0.5%	(Gadepalli et al., 2006)
Bacteroides	Bacteroides fragilis group	12.1%	(Citron et al., 2007)
	Bacteroides spp.	1.6%-28.57%	(Gadepalli et al., 2006; Haldar et al., 2017)
	Bacteroides fragilis	1.6%	(Gadepalli et al., 2006)
	Bacteroides eggerthii	1.1%	(Gadepalli et al., 2006)
	Bacteroides vulgaris	0.5%	(Gadepalli et al., 2006)
	Bacteroides ovatus	0.5%	(Gadepalli et al.,

			2006)
Diptheroids		8.16%	(Haldar et al., 2017)
Eubacterium lentum		0.5%	(Gadepalli et al., 2006)

Table 2: List of Fungi responsible for DFI

Organism		Prevalence (%)	
Aspergillus	Aspergillus species	2%-5%	(Chellan et al., 2010; Sanniyasi et al., 2015)
	Aspergillus niger	10%	(Kareliya et al., 2019)
	Aspergillus fumigatus	0.83%	(Fata et al., 2011)
Penicillium	Penicillium	1%	(Sanniyasi et al., 2015)
	Penicillium marneffei	15%	(Kareliya et al., 2019)
Candida	*Candida tropicalis*	4.1%-22.7%	(Chellan et al., 2010; Fata et al., 2011; Kareliya et al., 2019; Sanniyasi et al., 2015)
	Candida parapsilosis	0.83%-25.5%	(Chellan et al., 2010; Fata et al., 2011)
	Candida glabrata	0.83%-7.5%	(Chellan et al.,

			2010; Fata et al., 2011; Kareliya et al., 2019)
	Candida spp.	3.3%	(Fata et al., 2011)
	Candida guilliermondii	2.8%	(Chellan et al., 2010)
	Candida sake	2.8%	(Chellan et al., 2010)
	Candida globosa	1.4%	(Chellan et al., 2010)
	Candida famata	0.7%	(Chellan et al., 2010)
	Candida melibiosica	0.7%	(Chellan et al., 2010)
	Candida lusitaniae	0.7%	(Chellan et al., 2010)
	Candida albicans	2.9%-40%	(Chellan et al., 2010; Fata et al., 2011; Kareliya et al., 2019; Sanniyasi et al., 2015)
	Candida krusei	0.7%-2%	(Chellan et al., 2010; Fata et al., 2011; Kareliya et al., 2019; Sanniyasi et al., 2015)
Kodmaea ohmeri		2.1%	(Chellan et al., 2010)

Rhodotorula spp.	0.83%	(Fata et al., 2011)
Scopulariopsis spp	0.83%	(Fata et al., 2011)
Acremonium spp.	0.83%	(Fata et al., 2011)
T. mentagrophytes	2.5%	(Fata et al., 2011)
Fusarium	7.5%	(Kareliya et al., 2019)
Cladosporium	10%	(Kareliya et al., 2019)
Trichosporon asahii	12.8%	(Chellan et al., 2010)

Chapter 3

Detection techniques

Determination of various microorganism that play a major role in the foot infection of diabetic patients is very important. Because it is the usual reason behind high morbidity of diabetic patients, which results in serious complications such as gangrene and amputations.(Grayson, 1995) Different types of detection tests are done worldwide. They are either biochemical methods or molecular techniques.

In a study by Birdem General Hospital, Bangladesh, they used variety of biochemical tests, which included, fermentation, indole, nitrate disk reduction, special-potency disk test, catalase andurease test, sodium polyanethol sulphonate disk test, bile esculin hydrolysis test, lipase and lecithinase test, colony observation of fluorescence study and pigment production test.(Mohammuddunnobi et al., 2018) However, anerobic bacteria is very difficult to detect. To detect anaerobes a specialized detection test called the, simple two step combustion technique in candle jar was used.(Mohammuddunnobi et al., 2018) The same techniques were used by a study in India as well.(Haldar et al., 2017) This modified candle jar technique was cheaper and simpler option than the traditional gas pak system which is used to detect anaerobes.(Haldar et al., 2017; Mohammuddunnobi et al., 2018)

PCR, DGGE, 16S rRNA gene sequencing analysis, metagenomics and metatranscriptomics have emerged as an option for researchers and scientists to get a deep understanding of the bacterial population.(Gayle E. Reiber et al., 1999) *Delftia acidovorans, Serratia nematodiphila, Streptococcus salivarius, Fusobacterium nucleatum, Flavobacterium succinicans, Staphylococcus pettenkoferi* are among the species that have been detected by 16 rRNA method. However, these organisms could not be detected by other detection techniques.(Gayle E. Reiber et al., 1999)

Shotgun metagenomic sequencing detects bacterial population at the infected site. Many uncommon organisms including, *Corrnebacterium striatum, Propionibacterium spp., Pophyromonas somerae, Brevibacterium massiliense, Klebsiella oxytoca* and Coagulase-negative species such as, *Staphylococcus pettenkoferi, Staphylococcus simulans and Staphylococcus lugdunensis* were detected using this technique.(Kalan et al., 2018)

A study on patients from Riyadh Medical Complex used API tests 20E, API-20Strep to determine the pathogens involved. In that study 98.5% aerobic and only 1.5% anaerobic bacteria was detected.(Alodaini, H. A. et al., 2018) Vitek 2 and API 20A was used for the identification of species and the most frequent isolates were, *Pseudomonas aeruginosa* (21.4%), *S. aureus* (17.9%),

Klebsiella pneumoniae (17.9%), *Staphylococcus epidermidis* (13.6%).(Demetriou et al., 2013) In another study where API system was used for identification, 97% aerobic bacteria was detected. Among which, 67.3% was gram negative and 29.6% was gram-positive.(Abdul Kadir et al., 2012)

A study compared the effectiveness of conventional culture methods and 16s rDNA PCR to detect anaerobic organisms. 52% patients was determined to have anaerobic infection by PCR, whereas only 8% patients could be determined by conventional culture methods.(Aherrao, Shahi, Dwivedi, Kumar, et al., 2012) Similarly another study also reports that, they were able to detect 65 pathogens by bacteria specific PCR.(Noor et al., 2018) Another study used PCR to specifically detect *S. aureus.* They were able to detect 44% *S. aureus* from the study population. They also targeted the mecA gene to detect MRSA and found 25% of the samples to be positive for MRSA.(Sandhu et al., 2014)

In a research among patients admitted to Kenyatta National Hospital, Nairobi, comparison was done between biochemical tests and molecular techniques. the samples were divided and both traditional culture-based detection methods and RT-PCR based detection was carried out. Sensitivity of culture-based method could not be determined due to no result. However, RT-PCR showed 58.8% sensitivity to detect *S. aureu*s,(Mutonga et al., 2019) showing that molecular tests were more sensitive than biochemical tests.(Mutonga et al., 2019) Molecular tests helped detecting organisms that were not detected by biochemical tests, although it was less specific than biochemical tests.(Mutonga et al., 2019)

An in-depth study was carried out to determine the benefit and drawbacks of molecular and biochemical tests to detect pathogens in DFI. The sample population was divided in half and biochemical tests were carried out in one half while molecular tests were carried out in another. After comparing the results, it was found that, 88% of total sample was positive for *S. aureus* by RT-PCR while culture-based method was only positive for 57%. In case of *S. pyogenes* 15% were positive for RT-PCR and only 1% for culture-based method. Among *S. agalactiae*, 30% were positive for PCR and 22% for culture-based method. In case of *S. dysgalactiae*, 22% and 13% samples were found to be positive by PCR and culture-based method, respectively. Based on this result it can be said that, molecular detection is more efficient than biochemical method.(Stappers et al., 2015)

Chapter 4

Antibiotic resistance

The primary concern while tackling any infection is to try to slow down the infection rate or to eradicate it completely. Usually, antibiotic treatment is the main method of treatment in such cases. However, over the year's bacteria have been evolving and developing resistant towards various drugs and medicines.

An investigation conducted on patients admitted to two Bangladeshi hospitals detected that, Staphylococcus spp. was 100% resistant towards monobactam and 67% resistant to penicillin-G group. *Acinetobacter spp*. was 86% resistant to penicillin and cephalosporin antibiotic group. *Bacillus spp.* was 88% resistant to monobactam, cephalosporin and penicillin group. Citrobacter was 100% resistant to cephalosporin group. Also, 82% of the study population were resistant to carbapenem antibiotic group.(Karmaker et al., 2016)

United States multicenter clinical trial conducted an investigation from 2001-2004. They found that, Enterococci and MRSA strains were resistant to ertapenem. They also detected that, cephalexin, clindamycin and ciprofloxacin were not that much effective with ciprofloxacin being the most ineffective one. A gram-negative organism called *Stenotrophomonas maltophillia* was resistant towards almost all antibiotics. However, they found that vancomycin, daptomycin and linezolid were effective against all gram positive organisms.(Citron et al., 2007)

According to a study, aerobic gram-negative organisms showed higher resistance to a number of drugs. Amoxycillin (92%), amoxycillin-clavulanic acid (60%) and cephalosporins (72%) showed maximum resistance towards the aerobic gram negatives. In case of anaerobes, high resistance was observed towards clindamycin (38.09%), penicillin (23.81%), cefoxitin (19.05%), imipenem (4.76%) and metronidazole.(Haldar et al., 2017)

Research conducted by Department of Medical Microbiology, UMMC, Malaysia discovered that, *S. aureus was* resistant towards methicillin (16%), vancomycin (100%), rifampin (100%), fusidic acid (7%), erythromycin (16%) and clindamycin (7%). Enterococci was resistant against, imipenem (8%), ampicillin (17%) and co-trimoxazole (25%). All isolates of group B streptococci were effective against penicillin, ampicillin, vancomycin, imioenem, cefuroxime and clindamycin.(NS Raja, 2007)

According to an investigation conducted on patients admitted to a hospital in Kenya, *Staphylococcus aureus* was detected to be resistant towards benzylpenicillin and trimethoprim.

Furthermore, *E. coli* was highly resistant to ampicillin, aztrenam, cefuroxime and TMPSMX. While, *P. mirabilis* was resistant to ampicillin and *S. fonticola* species were resistant to ampicillin, amoxicillin, cefazolin, cefepime, ceftazidime, piperacillin-tazobactam and TMPSMX. 30.77% *S. aureus* and 40.38% gram-negative bacilli were multi drug resistant organism in this study.(Mutonga et al., 2019)

According to another study, Enterobacteriaceae family exhibited high resistant to ß-lactam. 90% *Acinetobacter spp*. strains were also resistant to ß-lactam. Most importantly they detected that almost all of the strains of *Acinetobacter spp*. had developed resistance against the mainstream antibiotics. *Pseudomonas aeruginosa* exhibited highest resistance to cefepime. 67% staphylococci strains exhibited resistance to cefoxitin. Enterobacteriaceae and staphylococci exhibited almost 90% resistance to ampicillin. 87% strains of enterococci was resistant to tetracycline and erythromycin.(Murali et al., 2014)

BIRDEM General Hospital, Bangladesh conducted a research which focused on antimicrobial resistance pattern of the gram-negative organisms. From their report we come to know that, 43.8% *S. aureus* were methicillin resistant. It was also resistance to cotrimoxazole (62.5%), ciprofloxacin (75%) and tetracycline (56.3%). *Pseudomonas sp.* showed high resistant to, augmantin (75%), ceftazidime (66.7%), ceftriaxone (75%), cotrimoxazole (97.2%), tetracycline (80.6%) etc. *Proteus sp*. showed high resistance to, ceftazidime (84%), cotrimoxazole (88%), ciprofloxacin (88%), tetracycline (84%). *Klebsiella sp*. was highly resistant to, cefotaxime (85.7%), cefuroxime (90.8%). *E. coli* exhibited high resistance to, cefuroxime (81.8%), ceftazidime (72.7%), ceftriaxone (72.7%), tetracycline (72.7%).(Paul et al., 1970)

MRSA is a major threat in DFI patients. According to a study, they found 36% MRSA from the study population and they were highly resistant towards ciprofloxacin and erythromycin.(Mottola et al., 2016) Another study reported that, MRSA was 100% resistant towards penicillin, 94.22% towards co-amoxiclav and 81.22% towards gentamicin.(Jafari-Sales et al., 2018)

Fungal species are developing resistance towards antibiotics as well. For instance, they were found to be resistant towards, flucytosine (1.5%), fluconazole (3.9%), amphotericin B (6.9%), voriconazole (6.9%) and itraconazole (17.7%).(Chellan et al., 2010)

In another study, fugal species were found to be 100% resistant to clindamycin + amikacin and

cloxacillin + pipracillin + tazobactum and cephalosporins.(Kareliya et al., 2019)

Chapter 5

Discussion

A high percentage of patients suffering from DFI were male and their age was in the region of 50-70 years old.

Among various studies, *Staphylococcus aureus, E. coli, Pseudomonas aeruginosa, Proteus spp., Klebsiella spp., Enterobacter spp.* and *Enterococcus spp.* were found to be most frequent organisms detected. On the other hand, *Candida albicans, Candida krusei, Candida tropicalis, Candida glabrata* and *Candida parapsilosis* were the most commonly detected fungi across various studies. *Staphylococcus aureus* causes soft tissue and bone infections and a major part remains present at the lower part of the feet.(Ambrosch et al., 2011) It can even invade and enter into osteoblasts(Shi & Xianlong, 2012), fibroblasts and endothelial cell.(Proctor et al., 2006) It is also highly resistant to antibiotic and antibiotic therapy.(Baumert et al., 2002; Garcia et al., 2013) Thus, making *S. aureus* the most commonly detected organism worldwide.

Among biochemical tests vs molecular method. Molecular method was found to be more efficient and reliable. Because, biochemical tests are more time consuming, it needs suitable culture conditions for growth, it also requires viable pathogens. Furthermore, they have lower detection sensitivity and might underestimate the bacterial prevalence.(Stappers et al., 2015) However, molecular method is more fast and sensitive to detecting pathogens.(Stappers et al., 2015) Molecular methods were able to detect that 52% patients were infected with anaerobic infection while biochemical method could only detect 8% from the same sample.(Aherrao, Shahi, Dwivedi, & Kumar, 2012) So, based on this and from the data discussed earlier in the article it can be said that Molecular tests are the better of the two.

The DFI scenario in Bangladesh is not promising compared to the global scenario. Lack of technical facilities and education is most probably the main reason behind it. Here, Fungal data was found from only one study.(Mohammuddunnobi et al., 2018) Although, detecting the fungi present in the infection site is very important for the treatment process. Even neighboring countries such as India is doing a lot of research with fungal infection. The reason behind the unavailability of data is due to lack of research and infrastructure needed to detect fungi. It was found that, gram negative organisms were more prevalent in Bangladesh.(Karmaker et al., 2016)·(Paul et al., 1970) This can be explained with our climate. As gram negative organisms thrive in hot and warm weathers.(Schwab et al., 2014) To back up this statement, Studies from Malaysia(Sharifah Aisyah et al., 2019) and India(Chellan et al., 2010) having similar weather also reported the same. On the

other hand a study from USA reported gram positive to be the more prevalent.(Citron et al., 2007) In terms of detection techniques used, Bangladesh is still reliant on biochemical methods,(Mohammuddunnobi et al., 2018) while globally molecular methods have shown to be more advantageous.(Aherrao, Shahi, Dwivedi, & Kumar, 2012; Mutonga et al., 2019; Stappers et al., 2015)

Detection of DFI has some limitations. The wounds sites are full with various colonies of organisms. Because of which culture-based methods can return no definitive result.(Lavery et al., 2006; Prompers et al., 2007) Besides, in the biochemical methods only the organisms which we know how to find is grown. As a result, there is a chance that these organisms are actually laboratory weeds, which means we are not being able to detect the real organisms responsible. Also, it takes 2-3 days to cultivate and determine the sensitivity pattern of the organisms. During this 2-3 day period patients have to be given empiric antibiotic treatment which is not appropriate in 1/4th of cases.(B. A. Lipsky et al., 2014) Moreover, in cases of polymicrobial infection, pathogenic and harmless colonizers cannot be differentiated. Furthermore, patients who are already under antibiotic treatment sometimes gives false negative results in biochemical tests.(Percival et al., 2015) Also, molecular methods were found to be less specific than culture based methods.(Mutonga et al., 2019)

It will be recommended to use molecular techniques (RT-PCR) for the detection process. Through this process most species of organism can be detected in a matter of hours instead of days. It also has the ability to identify smaller concentrations of organism than the biochemical method (standard cultures). Culture based methods often gave false negative results if the patient had a history of previous antibiotic use, this problem is not present in case of RT-PCR detection. Besides, through PCR multiple types of organism can be detected together whereas only one organism could be detected per culture.(Aherrao, Shahi, Dwivedi, & Kumar, 2012) Overall, RT-PCR is globally available technique. So, it would be financially affordable for mid/low income countries as well.

Chapter 6

Conclusion

Diabetic foot infection is becoming a global threat day by day. From the discussion we see that, there are various types of pathogens involved. Even fungi pose a serious threat to the patients. Not only that. The pathogens are evolving. They are becoming more and more resistant towards the antibiotics that were used to treat the patients till present time. As we see in the discussion, some have already become 100% resistant towards specific antibiotics. Which will only increase in future.

In order to prevent this. We need to focus on early detection and applying easy and quick detection techniques. Currently we are still unable to completely detect all the pathogens involved, one of the reasons being false positive results related complications of the biochemical tests which are used worldwide and specially in developing/underdeveloped countries. Thus, molecular techniques need to be implemented all over the globe to ensure accurate detection.

It is recommended that we try to tackle antibiotic resistance by ensuring proper usage of antibiotics. We also need to launch mass awareness campaigns to educate the population about the importance of healthy lifestyle and physical exercise to control obesity. Proper sanitation needs to be ensured along with implementation of easy and quick detection techniques for timely detection.

Chapter 7

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