UNIVERSITY OF ZULULAND

Isolation and Characterization of Bacteria Recovered from Wounds of Hospitalized Diabetic Patients in Northern KwaZulu-Natal

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DECLARATION

The experimental work presented in this dissertation was conducted in the Department of Biochemistry and Microbiology and the Department of Botany at the University of Zululand from February 2015 - November 2016 under the supervision of Dr D. Penduka, Prof A.M. Zobolo and Prof A.R. Opoku.

This study presents original work by the author, where outside sources were used, proper attributions have been made in the text.

I declare the above statement to be true

……

Wendy Mthembu

……

A.R. Opoku

DEDICATION

I wish to dedicate this piece of work to the Madiba and Mthembu family for their endless love and support. My family has been the pillar of my strength.

ACKNOWLEDGEMENTS

I am grateful for the opportunity to further my studies up to this level. It has not been easy but God has been merciful.

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- Dr Shoba for kindly assisting in the collection of samples.
- The hospitals for allowing us to collect samples

"…..With God Nothing Is Impossible……."

GENERAL ABSTRACT

Diabetes mellitus is a world health concern with about 2.28 million cases recorded in South Africa in 2015. One of the complications of diabetes is the development of chronic wounds that contribute to longer hospitalization and bacterial infection. If not treated properly, the wounds may lead to the emergence of antibiotic resistant bacteria that require viable alternative treatment.

The aim of the study was to isolate and identify bacteria present in wounds of diabetic patients, characterize their antibiotic susceptibility patterns and determine the potential of medicinal plant extracts to inhibit bacterial growth.

The wound specimen were collected and plated on selective and differential media. Identification was done through biochemical characterization, API and 16S rDNA sequencing. The antibiotic susceptibility patterns were determined through the Kirby-Bauer disk diffusion assay while the antibacterial activities of the plant extracts were evaluated through the agar-well diffusion method. The plants were first screened for the presence of phytochemicals and extracts of dichloromethane, acetone and water were prepared separately. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of the active extracts were determined.

A total of 42 isolates were recovered from 83% of the patients sampled from three hospitals (X, Y, and Z). Gram-negative bacilli from *Enterobacteriaceae* were predominant followed by *Staphylococci spp* and *Enterococcus faecalis*. The bacteria exhibited resistance to penicillin (100%), ampicillin (91%), cefepime (60%), ceftazidime (55%) and gentamicin (52%). Hospital X's bacteria were found to be most resistant to erythromycin (80%) and ciprofloxacin (70%), while hospital Z's bacteria were most resistant to vancomycin (50%) and penicillin (50%), with Hospital Y's bacteria showing the most resistance to imipenem (45%). Multidrug resistance patterns were exhibited by Enterococci (83%), Enterobacteriaceae (55%), non-Enterobacteriaceae bacilli (50%), Staphylococci (50%) and Gram-positive bacilli (33%). Most bacteria tested on plant extracts were resistant. However, zones of inhibition ranging from 11±0.1-22±0.6 mm, were observed against the acetone extract of *P. glomerata* and the aqueous and acetone extracts of *C. fendleri*. The MIC values ranged from 10- 20 mg/ml and MBC values at 5-

> 20 mg/ml. The observed antibacterial activities could be attributed to the presence of phytochemicals in the plants.

The resistance associated with bacteria recovered in diabetic wounds is a serious health concern that limits antibiotic treatment options as it has been observed the study, therefore necessitating the need to explore lead active compounds in medicinal plants that may aid wound healing.

LIST OF ABBREVIATIONS USED

AGE-	Advanced Glycation End-product
API-	Analytical Profile Index
ATCC –	American Type Culture Collection
CLSI -	Clinical and Laboratory Standard guidelines
DAG-	Diacylglycerol
DCM -	Dichloromethane
DFU-	Diabetic foot ulcer
DMSO -	DimethylSulphoxide
DNA -	Deoxyribonucleic acid
ECM-	Extracellular matrix
EPS-	Extracellular polymeric substance
GAPDH-	Glyceraldehyde 3-phosphate dehydrogenase
INT -	Iodo-nitro-tetrazolium chloride
MBC -	Minimum bactericidal concentration
MDR-	Multidrug resistant
MIC -	Minimum inhibitory concentration
MMPs-	Metalloproteinases
mm -	millimeters
MRSA-	Methicillin resistant *Staphylococcus aureus*
NADPH-	Nicotinamide adenine dinucleotide phosphate
NF-κB -	Nuclear factor kappa B

NOS-	Nitric oxide synthase
PAI-1-	Plasminogen activator inhibitor-1
PAMP -	Pathogen associated molecular patterns
PCR-	Polymerase chain reaction
PKC-	Protein kinase C
rDNA-	Ribosomal Deoxyribonucleic acid
rRNA-	Ribosomal Ribonucleic acid
RNA-	Ribonucleic acid
ROS-	Reactive oxygen species
TGF α -	Transforming growth factor α
TGF β-1 -	Transforming growth factor β-1
TLR2 -	Toll-like receptor 2
WHO -	World Health Organization

RESEARCH OUTPUT

1. Conference Presentation

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CHAPTER ONE

Introduction

Diabetes mellitus is one of the major causes of lower limb amputations worldwide (Sibidla, 2014; Dreifke *et al.,* 2015). During diabetes, the failure of insulin-stimulated glucose uptake by fat and muscles cells cause glucose concentrations in the blood to remain high (King and Loeken, 2004; Blake and Trounce, 2014). This defect accelerates the lower extremity arterial disease in combination with neuropathy. The blood vessel formation is impaired leading to a decrease in collateral vessel formation and non- healing ulcers (Giacco and Brownlee, 2010; Akhi *et al.,* 2015). Wound healing in diabetics is also impaired due to factors such as the compromised function of white blood cells that enable bacteria to become intense and cause infection (Bertestanu *et al.,* 2013; Moura *et al.,* 2013).

Diabetic wound infections have become a worldwide health concern associated with both aerobic and anaerobic Gram positive or Gram negative bacteria (Grice *et al.,* 2010; Kamel *et al.,* 2014), which have become less sensitive to antibiotic treatment (Bessa *et al.,* 2013; Akhi *et al.,* 2015). There are some diabetic wounds that have been infected by multidrug-resistant microorganisms such as Methicillin-resistant *Staphylococcus aureus* (Gadepalli *et al.,* 2006) and the *Enterobacteriaceae* spp which are the most commonly recovered Gram negative bacteria (Crouzet *et al.,* 2011; Kamel *et al.,* 2014). In the past, *Enterobacteriaceae* spp have been implicated in the prevalence of extended spectrum beta-lactamases (Priyadarshini *et al.,* 2013; Wellington *et al.,* 2013; Adesoji *et al.,* 2016) which poses a threat to the β-lactam produced antibiotic treatment (Holmes *et al.,* 2016). A large proportion of funds is spent on expensive antimicrobial agents to try and combat the infection without carefully studying the infecting bacteria (Han *et al*., 2011) that contribute to the development of antibiotic resistance (Kamel *et al.,* 2014; Akhi *et al.,* 2015). In this regard it essential to assess the microbiological profiles of the wounds to develop a correct antibiotic therapy guide (Akhi *et al.,* 2015).

The need for alternative treatment also becomes essential so as to curb the increasing incidences of antibiotic resistance (Igbinosa *et al.,* 2009; Laxminarayan *et al.,* 2013;

Bloom *et al.,* 2015). According to various traditional medicinal practices throughout the world, wound treatment has been based on medicinal plants (Ayyanar and Ignacimuthu, 2009; de Wet *et al.*, 2013). Medicinal herbs or extracts (alone or in combination), have been used traditionally to treat wounds, making them a viable source of antibacterial compounds

Scope of the Study

This study focuses on isolating and characterizing bacteria recovered from diabetic wounds and assessing the distribution of bacteria across the different patients in three hospitals in Northern KwaZulu-Natal, SA. As part of characterization, the anti-bacterial activities of some antibiotics and extracts of *Platycarpha glomerata*, *Chamaesyce fendleri, Carpobrotus dimidiatus* and *Jatropha zeyheri* were evaluated against the isolates from diabetic patients' wounds.

Aim

To isolate and characterize bacteria present in wounds of diabetic patients hospitalized in the Northern KZN.

Objectives

- To aseptically collect specimen from diabetic wounds using sterile swabs.
- To isolate the bacteria present using primary culture media.
- To presumptively identify the isolated bacteria using morphological characterization and biochemical tests.
- To confirm the isolated bacteria identities by sequencing the 16S rDNA through PCR.
- To collect, identify, screen for phytochemicals and extract plant crude material
- To determine the susceptibility patterns of the bacteria to selected conventional antibiotics and selected plants' crude extracts *in-vitro*.

Lay-out of the dissertation

Chapter 1: Introduces the project, highlights on the scope of the study and gives the aim of the study.

Chapter 2: Reviews the literature on diabetes, diabetic complications, wounds, bacterial infection, antibiotic resistance, medicinal plants that may have antibacterial potentials

Chapter 3: Describes the isolation and identification of bacteria recovered from diabetic wounds of hospitalized patients

Chapter 4 Evaluates the antibiotic susceptibility patterns of bacteria isolated from diabetic wounds

Chapter 5 Assesses the antibacterial potentials of medicinal plants on antibiotic-resistant bacteria isolated from diabetic wounds.

Chapter 6 General discussion and conclusion.

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CHAPTER TWO

LITERATURE REVIEW

2.1. Diabetes Mellitus

Diabetes mellitus is a metabolic disorder that is caused by the insufficient production of pancreatic insulin in the body or failure of the body to respond to the insulin produced (Sibidla, 2014). There are two principal forms of diabetes (Figure 2.1): Type 1 (insulin-dependent) diabetes and Type 2 (non-insulin dependent) diabetes. Type 1 is caused by the immune system's destruction of beta cells of the pancreas leading to total insulin deficiency. Type 2 diabetes is caused by a combination of resistance to the actions of insulin and progressive loss of insulin (ADA, 2014; Blake and Trounce, 2014; ADA, 2016), especially in target organs (Blake and Trounce, 2014).

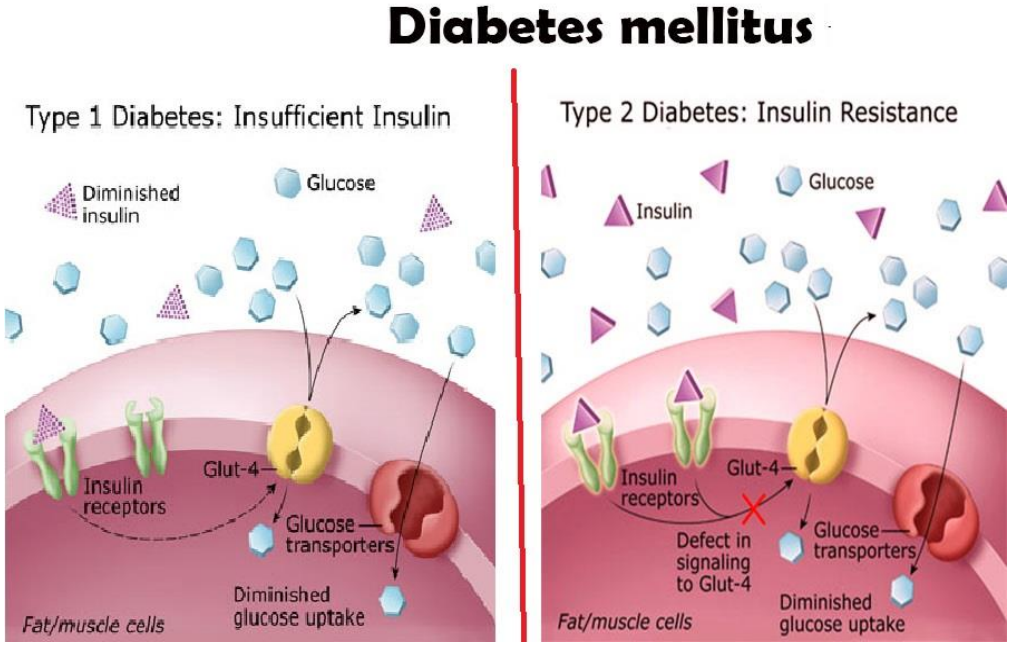

Figure 2.1: Type1 and Type 2 diabetes mellitus. Type 1 diabetes is caused by diminished insulin production which leads to a buildup of glucose in the blood stream. Type 2 diabetes results from defects of glucose transporters that lead to hyperglycaemia and insulin resistance in the cells (Mondal, 2013).

Approximately 383 million people are living with diabetes worldwide and the projections are expected to increase to 592 million in 2035, making diabetes a very costly disease for the healthcare system (Domingueti *et al.,* 2016). In South Africa 2.28 million cases of diabetes were reported in 2015 (International Diabetes Federation (IDF), 2015). The rapid cultural and social change, increasing urbanization, unhealthy eating, and reduced physical activity are closely linked to the increased incidences of diabetes (Society for Endocrinology, Metabolism and Diabetes of South Africa, SEMDSA, 2012).

2.1.1. Chronic Complications of Diabetes

Diabetes mellitus is a common endocrine disorder characterized by hyperglycaemia and predisposes to chronic complications affecting the eyes, blood vessels, nerves and kidneys (Ahmed, 2005; ADA, 2012, 2013, 2014).The injurious effects of hyperglycaemia are categorized into two namely: macrovascular (coronary artery disease, peripheral arterial disease, and stroke), and microvascular (diabetic nephropathy, neuropathy, and retinopathy) (Cade, 2008; Fowler, 2008).The vascular complications are the leading cause of morbidity and mortality among patients living with type1 and type 2 diabetes (Domingueti *et al.,* 2016). The microvascular complications affect organs with cells that do not require insulin for glucose uptake. These cells experience high concentrations of intracellular glucose (Ahmed, 2005; Giacco and Brownlee, 2010). High blood glucose could directly contribute to ulcer formation which may be characterized by poor healing in diabetics (Brownlee, 2001; Domingueti *et al.,* 2016).

Hyperglycaemia plays a significant role in the pathogenesis of diabetic complications through unified metabolic mechanisms (Brownlee, 2001; Ahmed, 2005; Brownlee, 2005; Giacco and Brownlee, 2010). The four main hypothetical mechanisms include increased polyol pathway flux, increased advanced glycation end-product (AGE) formation, activation of protein kinase C (PKC) isoforms, and increased hexosamine pathway flux (Brownlee, 2001; Brownlee, 2005; Cade, 2008; Giugliano *et al.,* 2008; Giacco and Brownlee, 2010; Blake and Trounce, 2014). All the four mechanisms are activated by the mitochondrial overproduction of the reactive oxygen species (ROS) (Giacco and Brownlee, 2010; Domingueti *et al.,* 2016).

2.1.1.1. Increased polyol pathway flux

Aldose reductase, an enzyme present in tissues such as nerves, retina lenses, glomerulus and vascular cells (Giacco and Brownlee, 2010; Brownlee, 2001; Blake and Trounce, 2014) is activated during a hyperglycaemic state resulting in increased enzymatic conversion of glucose to the polyalcohol sorbitol, with a decrease in NADPH (Fig 2.2). A decrease in NADPH results in the inhibition of the activity of glyceraldehyde-3-phosphatase (GAPDH) and an increased concentration of triose phosphate, which could lead to an increase in the formation of methlyglyoxal, a precursor of AGEs and diaclyglycerol (DAG) which may also activate protein kinase C (PKC). (Brownlee, 2001; Giacco and Brownlee, 2010; Morris *et al.,* 2013; Blake and Trounce, 2014). The decrease in NADPH could induce intracellular oxidative stress due to the mitochondrial imbalance of the reactive oxygen species and the body's ability to detoxify the reactive intermediates hence directly leading to poor wound healing (Blakytny and Jude, 2006; Gan, 2013; Blake and Trounce, 2014, Domingueti *et al.,* 2016).

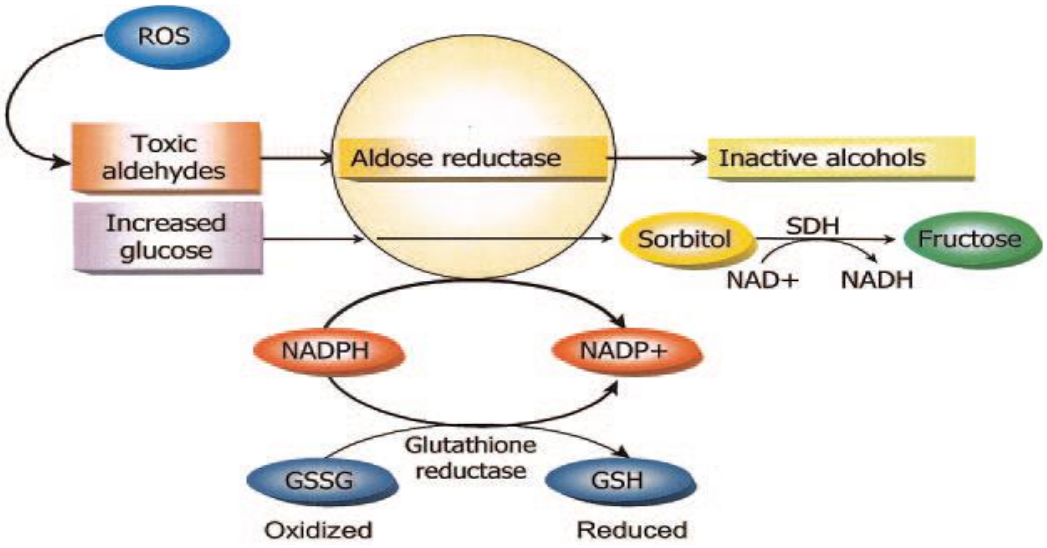

Figure 2.2.: The polyol pathway flux (Brownlee, 2005). Toxic aldehydes are reduced by the enzyme aldose reductase into inactive alcohols. The other function of the enzyme is to reduce intracellular glucose to sorbitol and later sorbitol is oxidised to fructose. The process of reducing glucose to sorbitol consumes NADPH and glutathione is reduced in the process. (Brownlee, 2005) Decreased levels of reduced glutathione (GSH) have been found in diabetic ulcers and where aldose reductase is over expressed (Brownlee, 2001; Blakytny and Jude, 2006) and this can contribute to induction of superoxide and hydrogen peroxide (Gan, 2013) causing oxidative stress (Blakytny and Jude, 2006).

2.1.1.2. Increased intracellular formation of advanced glycation end-products (AGEs)

AGE precursors damage cells using three mechanisms (Figure 2.3): the modification of intracellular proteins involved in the regulation of gene transcription, the modification of extracellular matrix that results in cell dysfunction and the modification of circulating proteins that have the ability to activate AGE receptors responsible for the production of inflammatory cytokines and growth factors that can induce vascular pathology (Brownlee, 2001; Brownlee, 2005; Giacco and Brownlee, 2010; Morris *et al.,* 2013; Manigrasso *et al.,* 2014).

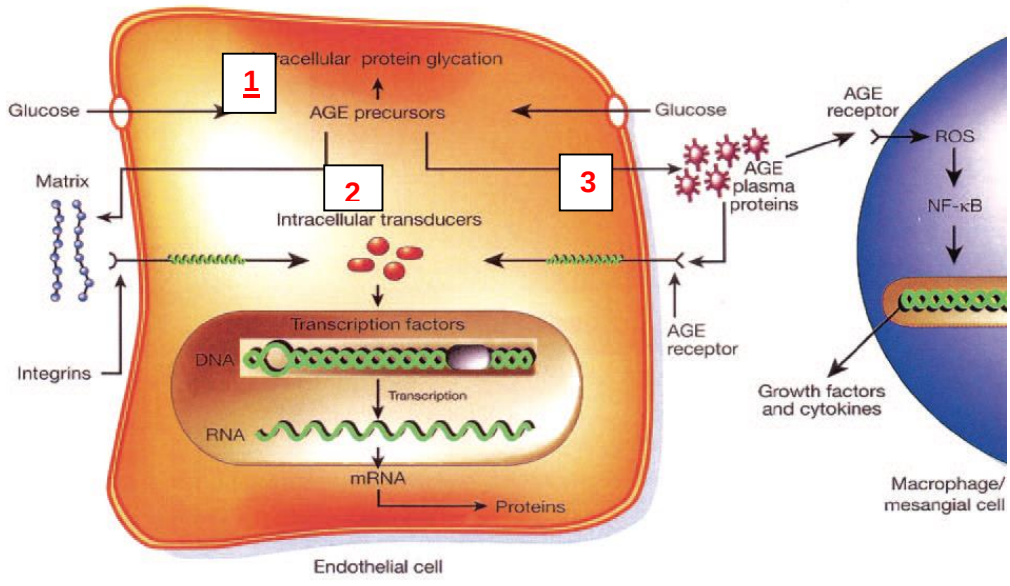

Figure 2.3: Increased production of AGE precursors and its pathologic consequences (Brownlee, 2005)

2.1.1.3. Activation of protein kinase c

Persistent activation of several PKC isoforms mediates tissue injury caused by diabetes-induced reactive oxygen species (ROS). This results in the enhanced *de novo* synthesis of DAG (Giacco and Brownlee, 2010). Intracellular hyperglycaemia increases the amount of DAG produced through the reduction of glycerol-3-phosphate (Brownlee, 2001). The availability of glycerol-3-phosphate is increased because ROS inhibits the activity of the glycolytic enzyme. GAPDH catalyzes the conversion of glyceraldehyde- 3-phosphate to D-glycerate 1,3-bisphosphate (Morris *et al.,* 2013). Hyperglycaemia may activate PKC isoforms indirectly through both ligation of AGE receptors and increased activity of the polyol pathway by increasing reactive oxygen species (Brownlee, 2001; Morris *et al.,* 2013; Blake and Trounce, 2014).

2.1.1.4. Increased flux through the hexosamine pathway

The shunting of excess intracellular glucose into the hexosamine pathway might also cause several manifestations of diabetic complications. Figure 2.4. shows that the over-modification of glucosamine to serine and threonine residues causes pathologic changes in gene expression such as increased modification of Sp1 (transcription factor) which leads to an increase in expression of the transforming growth factor-β1 (TGF-α and TGF-β1) and plasminogen activator inhibitor-1 (PAI-1), both of which have negative effects on blood vessels of diabetic patients (Brownlee, 2001, 2005; Morris *et al.,* 2013; Blake and Trounce, 2014).

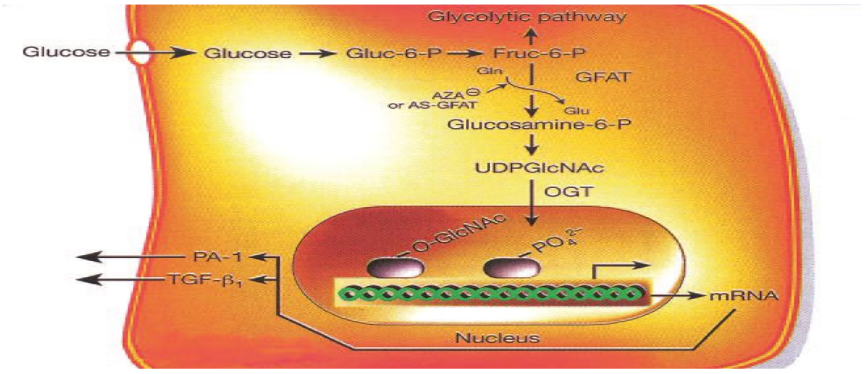

Figure 2.4.: Hyperglycaemia increases flux through the hexosamine pathway (Brownlee, 2005)

Human body cells that are unable to reduce the glucose transport are damaged by hyperglycaemia (Brownlee, 2005), through the overproduction of superoxide ions in the mitochondria. The cells undergo oxidative stress and eventually tissue destruction (Brownlee, 2001, 2005; Cade, 2008; Giugliano *et al.,* 2008; Giacco and Brownlee, 2010).

2.2. Diabetic Wounds

The skin provides a barrier against a range of biochemical and biological disturbances (Tobin, 2005; Kong, 2011) and provides protection to the immune system through cellular and humoral components such as lymphocytes, macrophages and lymphatic vessels (Tobin, 2005). Sometimes the skin is an indicator of internal complications including diabetes mellitus (Argyropoulos *et al.,* 2016). Diabetes is one of the major causes of lower limb amputations in various parts of the world (Sibidla, 2014; Dreifke *et al.,* 2015) and foot ulceration and infection are the main factors that lead to amputation. Diabetic wounds are examples of chronic wounds because they are caused by endogenous mechanisms associated with disorders that compromise the skin's integrity (Sabale *et al.,* 2012). Diabetic wounds are characterized by several pathological complications such as neuropathy (damaged nerves), peripheral vascular disease (poor blood flow) and foot ulceration (Priyadarshini *et al.,* 2013; Durgad *et al.,* 2014)

Diabetic foot ulcers are the predominant diabetic wounds that have been classified and categorized into 3 types namely: neuropathic diabetic foot ulcer, ischaemic diabetic foot ulcer, neuroischaemic diabetic foot ulcer (International Best Practice Guidelines (IBPG), 2013; Alavi *et al.,* 2014). Neuropathy (Figure 2.5.) is characterized by sensory loss, pink and granulating wound bed surrounded by a callus. Ischaemia (Figure 2.6.) is characterized by pain, necrosis, pale and sloughy wound bed with poor granulation and delayed healing (Boulton, 2005). Neuroischaemic (Figure 2.7.) is characterized by sensory loss and necrosis IBPG, 2013).

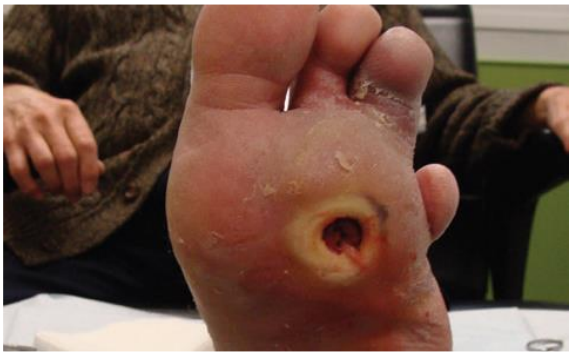

Figure: 2.5. Neuropathic Diabetic foot ulcer (IBPG, 2013)

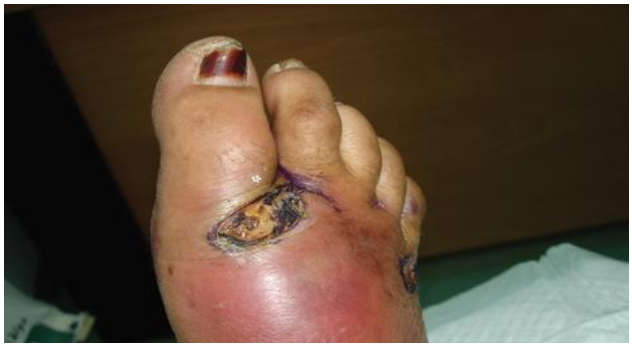

Figure: 2.6. Ischaemic DFU (IBPG, 2013)

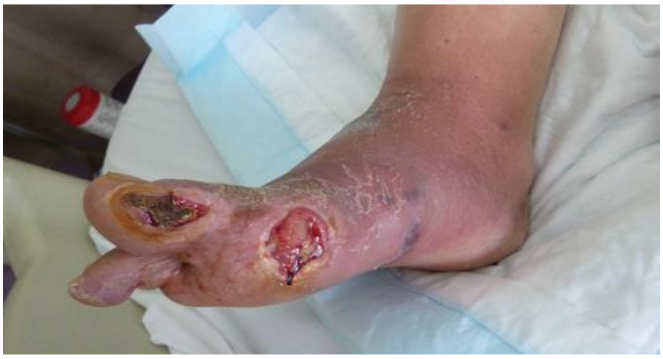

Figure: 2.7. Neuroischaemic DFU (IBPG, 2013)

2.3. Wound Healing Process

Wound healing is a process of repair following injury to the skin. The normal wound healing process consists of coagulation, inflammation, migration/ proliferation and remodeling phases (Grice *et al*., 2010). It requires the interaction of many cells as well as growth factors and enzymes (Blakytny and Jude, 2006). After tissue injury, fibrin plug is formed to re-establish homeostasis (Moura *et al.,* 2013). Platelets aggregate at the wound site to release different growth factors that lead to vasodilation and increased permeability of the nearby blood vessels. The growth factors are also responsible for recruiting neutrophils and monocytes (Blakytny and Jude, 2006). Monocytes and neutrophils are regarded as inflammatory cells produced to prevent infection and they have an important role in new tissue formation (Blakytny and Jude, 2006; Moura *et al.,* 2013). During re-epithelialization, keratinocytes and fibroblasts proliferate to construct the extracellular matrix (ECM) that will enhance wound closure. The collagenous matrix replaces the ECM with the formation of new blood vessels via a process called angiogenesis. The angiogenesis stops when the wound area is completely filled with new granulation tissue (Moura *et al.,* 2013). The last phase of wound healing is characterized by the degradation of the previously formed granulation tissue and dermis regeneration (Guo and Di Pietro, 2010; Moura *et al.,* 2013).

2.3.1. Impaired wound healing

Chronic wounds are characterized by a common sequence of processes that impair wound healing such as (i) prolonged inflammatory phase, (ii) persistent infection (formation of antibiotic resistant bacteria) and(iii) inability of dermal and epidermal cells to respond to respiratory stimuli (Bertestanu *et al*., 2013).

Wound healing may also be impaired by altered chemotaxis of the macrophage and neutrophils as well as prolonged inflammation (Guo and Di Pietro, 2010; Moura *et al*., 2013). These defects are caused by the impaired expression of growth factors (Moura *et al*., 2013).The compromised function of the neutrophils and macrophages makes the wounds of diabetic patients prone to bacterial infections (Akhi *et al.,* 2015).

Literature reports that the delay in diabetic wound healing is caused by the excessive and persistent activity of metalloproteinases (MMPs) or lower levels of MMP inhibitors

(Blakytny and Jude, 2006; Moura *et al.,* 2013). The healing may also be delayed by reduced supply of oxygen to the wound area which cause an imbalance between oxygen reactive species (Guo and Di Pietro, 2010). Free radicals are biologically toxic to the human body; their activity could result in severe destruction of extracellular matrix, cellular DNA and recurrent ulcers (Blakytny and Jude, 2006): It has been reported that abnormalities in Nitric oxide (a free radical produced by the nitric oxide synthase (NOS)) activity contribute to impaired wound healing process (Blakytny and Jude, 2006; Moura *et al.,* 2013).

Chronic wounds are a worldwide health burden and the delayed wound healing process is often associated with an elevated concentration of bacteria present in the wound (Han *et al*., 2011) and the interaction between the host immune system and the colonizing bacteria determines the clinical outcome of the wound (Mclnnes *et al.,* 2014; Bessa *et al.,* 2013).

2.4. Wound bacterial infections

Wound infection is the successful invasion, proliferation of one or more microorganisms within the sterile body tissues and sometimes pus formation serves as a sign of wound infection (Mohammed *et al*., 2013a). Wound bacterial infection occurs in three stages namely: (i) The bacterial adherence onto skin, (ii) Microbial community establishment (iii) Biofilm maintenance (Walcott *et al.,* 2008).

Bacteria use different mechanisms to attach onto the host extracellular matrix. Some bacteria inject effector proteins into host cells (see Figure 2.8) (Walcott *et al.,* 2008) while others rely on adhesin interactions with the host cell receptor (Ryan *et al.,* 2010). Macrophages and neutrophils (host first line defense) have receptors that identify pathogen-associated molecular patterns (PAMP) and in turn activate the phagocytes to clear out the single cell bacteria. Once the bacteria have adhered, the host's immunity may be unable to overcome the infection without medical intervention (Walcott *et al.*, 2008; Ryan *et al.,* 2010).

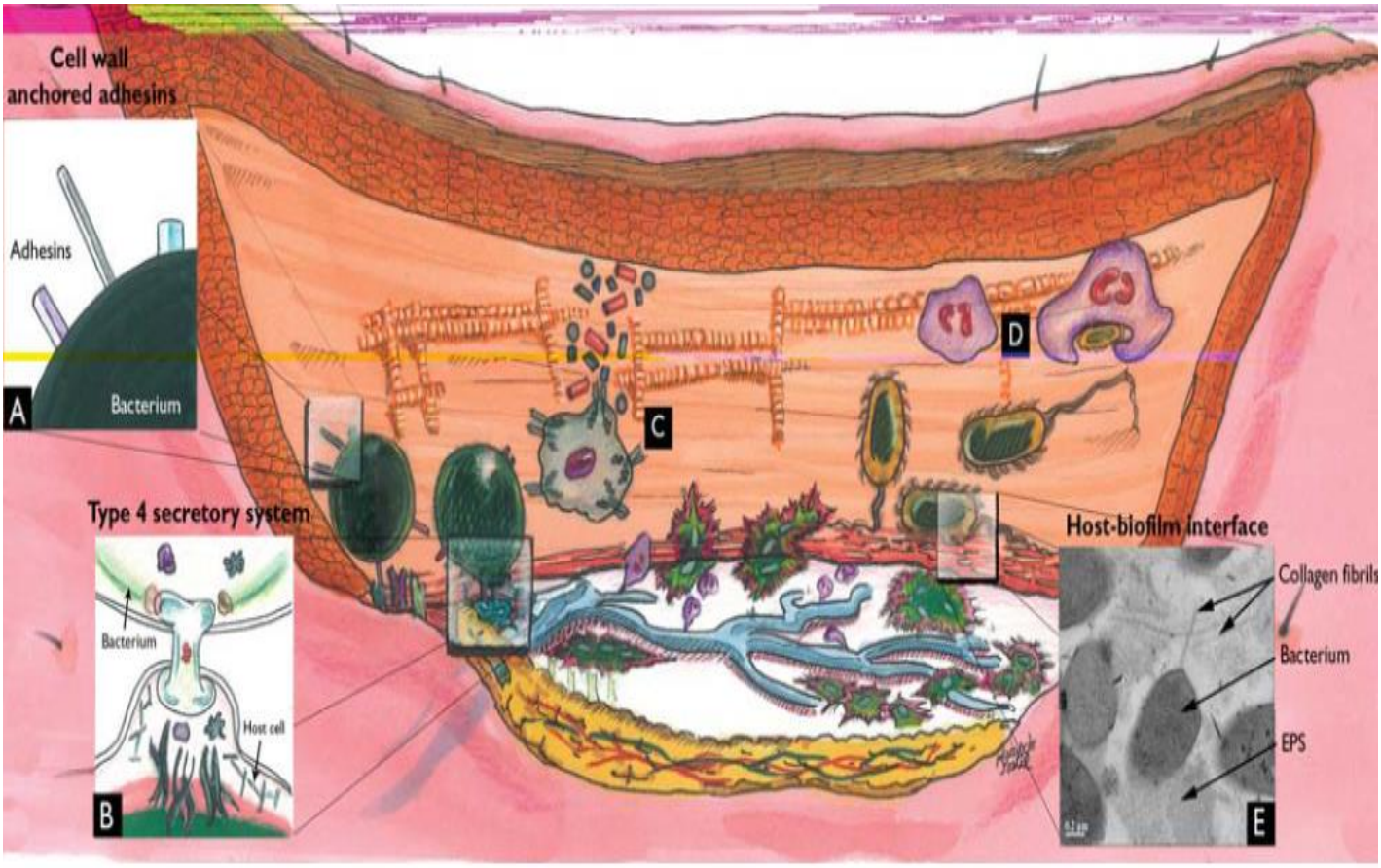

Figure 2.8: A diagrammatic presentation of the bacterial adherence onto skin, first stage in wound infection (Walcott *et al.,* 2008)

The microbial community establishment stage is the second stage of the infection (Figure 2.9.), whereby the attached bacteria invades wound tissue. The bacteria selectively organizes within the basement membranes of vessels in the wound bed (Walcott *et al.,* 2008; Rajpaul, 2015), forming barriers that are uncontrollable to white blood cells (leukocytes), opsonisation and all other innate immune strategies (O'mahony *et al.,* 2008).

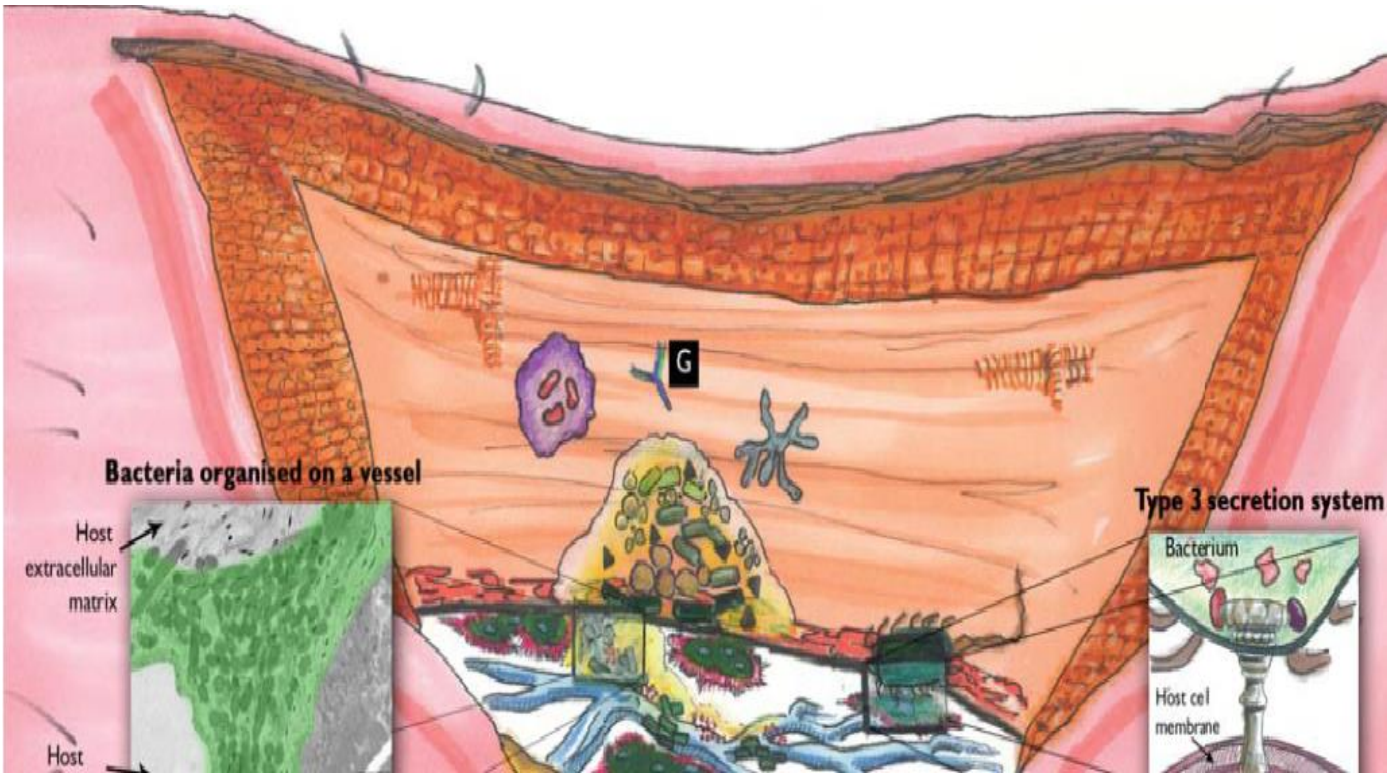

Figure 2.9.: A diagrammatic presentation of the microbial community establishment stage in wound infection (Walcott *et al.,* 2008)

In the last stage of the infection, the bacteria maintain a sustainable inflammatory niche by manipulating the host immune response (Walcott *et al.,* 2008). Most bacteria possess virulence factors that kill neutrophils (Figure 2.10.). The dying neutrophils release a large amount of elastase into the surrounding environment (Trengove *et al.,* 1999; Walcott *et al.,* 2008; Widgerow, 2011; McCarty *et al.,* 2012).The neutrophil-derived elastase degrades the interleukin-8 receptor (CXCR-I) which is responsible for inducing chemotaxis to macrophages and neutrophils by causing them to migrate to the site of infection. The fragments of the CXCR-I receptor stimulate Toll-like receptor-2 (TLR2) on dendritic cells and macrophages to produce a massive release of pro-inflammatory cytokines (Trengove *et al.,* 1999; Walcott *et al.,* 2008). The action of pathogen-associated molecular patterns (PAMP) and fragments of CXCR-I work through a nuclear factor kappa B pathway (NF-KB) to release pro-inflammatory cytokines (Hayden and Ghosh, 2008). Inflammatory cytokine affects the wound environment by stimulating the production of matrix metalloproteinases (MMPs). Elevated levels of MMPs in the granulation tissue

contribute to the chronicity of wounds (Trengove *et al.,* 1999; Blakytny and Jude, 2006; Moura *et al.,* 2013). The pro-inflammatory cytokines create gaps in the endothelial cells of the capillaries and allow neutrophil to migrate through the vessel wall into the wound bed. This completes a sustainable cycle in which bacteria cause excessive neutrophil migration and persistent inflammation (Walcott *et al.,* 2008)

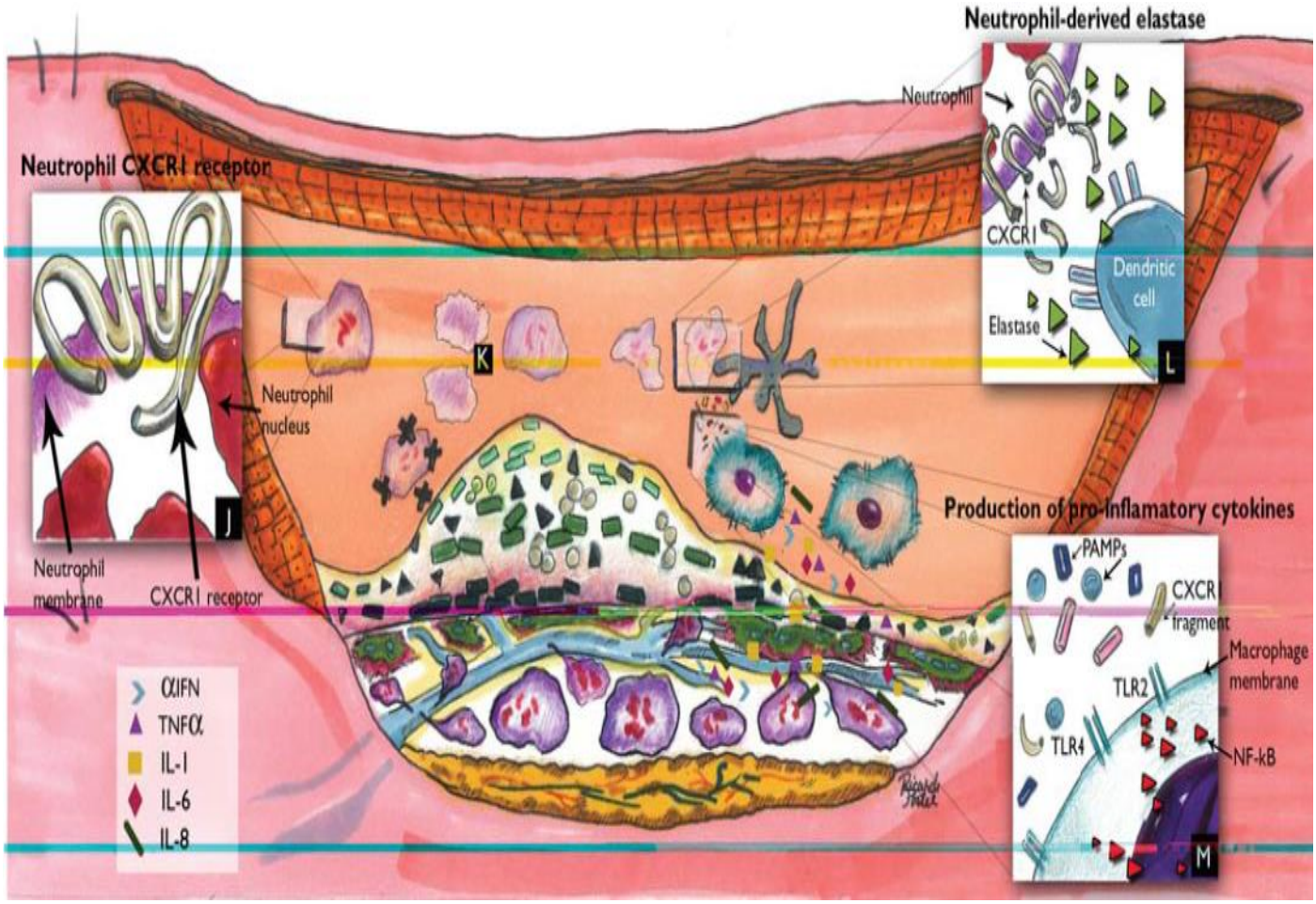

Figure 2.10.: A diagrammatic presentation of the biofilm maintenance stage in wound infection (Walcott *et al.,* 2008).

Chronic wounds may contain a mixture of aerobic and anaerobic bacteria (Roberts and Simon, 2012). The pathogenic and non-pathogenic microorganisms colonize the wounds and interact to cause infection (Peleg *et al,* 2010). Some bacteria have adopted mutualistic, synergistic and competitive antagonistic relationships that facilitate their existence on epithelial surfaces (Solomkin *et al.,* 2004; Peters *et al.,* 2012). These

relationships are relevant to human health because they can have a significant impact on the outcome and etiology of the bacterial infection (Stacy *et al.,* 2014; Wigneswaran *et al.,* 2016).

Bacteria exist in two forms in the skin flora: planktonic state and biofilm state (Han *et al.,* 2011; Vickery *et al.,* 2014). In these states, the bacteria may differ significantly in characteristics such as morphology, mode of communication and metabolism (Han *et al.,* 2011; Rajpaul 2015). Chronic wound biofilms create an environment different from the planktonic state that allows the bacteria to adhere and aggregate (Han *et al.,* 2011).The planktonic bacteria are easily destroyed by the immune system (Griswold, 2012).

Biofilms are complex sessile polymicrobial communities embedded in a self-secreted exo-polysaccharide matrix and typically exist as micro-colonies (Han *et al.,* 2011; Griswold, 2012; Rajpaul, 2015). They quickly develop a glue encasement (a glycocalyx), which is a thick complex carbohydrate that cements the bacteria to the host (Griswold, 2012). Bacteria in a biofilm state grow slowly and this may lead to decreased uptake of the drug and other physiologic changes that impair the effectiveness of antimicrobial agents (Siddiqui and Bernstein, 2010; Griswold, 2012 Vickery *et al.,* 2014). The biofilm extracellular polymeric substance (EPS) act as a barrier that further prevents the antibiotics from penetrating the plasma membrane to lyse the bacteria (Han *et al.,* 2011; Seth *et al.,* 2012; Vickery *et al.,* 2014), thus giving the biofilm a chance to initiate stress responses and expression of efflux pumps (Han *et al.,* 2011).

The microorganisms that are reportedly involved in diabetic wound infection include Gram-positive cocci such as the *Staphylococci, Streptococci, Enterococci* and Gram-negative bacilli such as *Enterobacteriaceae* species (Grice *et al.,* 2010; Roberts and Simon*,* 2012). *Staphylococcus aureus* is the predominant pathogen in diabetic wound infections (Joseph and Lipsky 2010; Akhi *et al.,* 2015).

Some diabetic wound infections are caused by *Pseudomonas* species. These microorganisms can produce a variety of toxins and proteases and have the ability to resist phagocytosis initiated by the host defense system, which on the other hand, is already compromised in diabetic patients. *P. aeruginosa* is one of the microorganisms

that have been associated with high antimicrobial resistance (Sivanmaliappan and Sevanan, 2011). Organisms such as species of *Klebsiella* and *Proteus* have also been commonly associated with diabetic wound infection. They were once susceptible to a wide range of antibiotics, however, they are becoming resistant to these antibiotics (Joseph and Lipsky*,* 2010; Akhi *et al*., 2015).

2.4.1. Treatment of Diabetic Wounds Infection

The treatment of diabetic ulcers consists of 3 basic components: debridement, offloading and infection control (Kruse and Edelman, 2006; Wu *et al.,* 2007).Other forms of management/treatment include pharmacological agents such as anticonvulsants, anti-depressants, anti-arrhythmic for the relief of pain but there are side effects that have been reported (Bril *et al.,* 2011). Debridement consists of the removal of necrotic tissue; this is necessary to decrease the risk of infection, however, it does not heal the ulcer (Wu *et al.,* 2007). Infection control includes treating the wounds with oral antibiotics such as cephalexin, amoxicillin, clindamycin (Kruse and Edelman, 2006), however, with increasing antibiotic resistance these antibiotics are becoming ineffective (Bloom *et al.,* 2015).

2.5. Antibiotic resistance

Antibiotics comprise the most commonly used therapeutic drugs against bacterial infections worldwide (Leekha *et al.,* 2011; Lin *et al.,* 2015) and have been a successful form of treatment. However, their efficacy has been compromised by the emergence of antibiotic-resistant pathogens (Lin *et al.,* 2015).The increase in misuse and mismanagement of antibiotics by human activities creates an environment for the selection of resistance against antibiotics (Gullberg *et al.,* 2011; Laxmnarayan *et al.,* 2011; Mohammed *et al.,* 2013; Gottrup *et al.,* 2014; Essack *et al.,* 2016). Antibiotic resistance results in longer duration of illness, high mortality rates and increased cost for alternative treatment (Laxmnarayan *et al.,* 2011; Essack *et al.,* 2016). Prolonged hospitalization and antibiotic therapy can also alter the sensitivity profile of the bacteria thus producing a greater pathogenic effect (Roberts and Simon, 2012)

Mechanisms of antibiotic resistance are mainly categorized into two groups; intrinsic (natural) and acquired resistance. Acquired resistance is caused by changes in the genetic make-up of the microorganism and can be transferred from one organism to another through conjugation or mating (Kiser *et al.,* 2011).This includes the horizontal gene transfer carried on transmissible plasmids (Suleman and Meyer, 2012). The intrinsic mechanisms of resistance include the production of drug–inactivating enzymes, alteration of drug targets and altered drug uptake also known as effluxing (Kiser *et al.,* 2011; CLSI, 2012; Lin *et al.,* 2015). There has been an emerging antibiotic resistance to Gram-negative bacteria due to the impermeability of the cell wall (Kiser *et al.,* 2011; Kamel *et al.,* 2014; Akhi *et al*., 2015). These microorganisms have been associated with the threatening emergence of multidrug- resistance (MDR) (Wellington *et al.,* 2013) especially in the hospital setting (Rossolini *et al.,* 2014). Some MDR strains possess the ability to spread in the clinical setting through cross-infection which may compromise the overall treatment in patients (Rossolini *et al.,* 2014). Therefore, it is important to assess the microbiological profiles in diabetic wounds as part of antimicrobial stewardship programs that are tackling antibiotic resistance.

2.6. Herbal Treatment

Herbal medicine is used by 75-80% of the population in the treatment of various infections in most developing countries (Ahmad and Wajid, 2013). Medicinal herbs or extracts (alone or in combination) have been applied topically to treat wounds (Ayyanar and Ignacimuthu, 2009; de Wet *et al.,* 2013). Research has been extended to the evaluation of the antibacterial activities of some plants' extracts in diabetic wound healing (Carrington *et al.,* 2012). Plants like *Justicia secunda* (Carrington *et al.,* 2012) and *Caesalpinin sappan* (which inhibited *S. aureus* and methicillin resistant *staphylococcus aureus* (MRSA) isolated from a diabetic wound (Temrangsee *et al.,* 2011) have been studied. *Chamaesyce fendleri*, *Platycarpha glomerata, Carpobrotus dimidiatus* and *Jatropha zeyheri* are commonly used by Zulu traditional healers in the management of wounds and other soft skin related infections (Zobolo and Mkabela, 2006; Luseba *et al.,* 2007; Lall and Kishore *et al.,* 2014).

Platycarpha glomerata (Figure 2.11a.) is a rhizomatous herb that belongs to the family of Asteraceae (Funk and Koekemoer, 2011). The roots of *Platycarpha glomerata* are traditionally used to ward off evil spirits around the home. They are also used as diuretic, enema, topically for itches, pains, burns, boils, eye problems, joint pain, sores and skin condition (Philander, 2011). *Platycarpha glomerata* leaves are used for steaming painful legs, treating wounds and preventing visitors from fighting on premises during a ceremony or ritual (Zobolo and Mkabela, 2006). Little has been reported about the chemistry of this genus but *Platycarpha glomerata* has been documented to possess five thiophenes and diol that were isolated from the rhizome; two germacranolides isolated from the aerial parts (Funk and Koekemoer, 2011).

Chamaesyce fendleri also known as *Euphorbia hirta* (Figure 2. 11b) is a perennial herb from the family of Euphorbiaceae. It is traditionally used to treat poison ivy, breast injuries, livestock snakebite, and also applied to cuts as a hemostatic (Vistal, 1952; Kumar *et al*., 2010).

Carpobrotus dimidiatus (Figure 2.11c), from the family Mesembryanthemaceae, is used as a remedy for many ailments such as dressing burns using the leaf juice (Fawole *et al.,* 2010; Lall and Kishore, 2014). The leaf extract and pulp are used to make antiseptic (Watt and Breyer-Brandwijk, 1962). The *Carpobrotus* species display phytochemicals such as flavonoids, tannins, alkaloids, phytosterols and aromatic acids (Van der Watt and Pretorius, 2001).

Jatropha zeyheri (Figure 2.11d) which belongs to the family of Euphorbiaceae with various traditional medicinal uses (Aiyelaagbe *et al.,* 2007) is a perennial herb with simple, sparsely branched stems and a thick rootstock (Archer and Victor, 2005). *J. zeyheri* has shown to produce some secondary metabolites such as saponins, tannins, cardiac glycosides and flavonoids that have anti- inflammatory and antibacterial activities (Mongalo *et al.,* 2013).The rhizome is used to heal wounds and boils (Archer and Victor, 2005).

A **B**

C **D**

Figure: 2.11.: (a) *Platycarpha glomerata* (b) *Chamaesyce fendleri* (c) *Carpobrotus dimidiatus* (d) *Jatropha zeyheri*

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CHAPTER THREE

ISOLATION AND IDENTIFICATION OF BACTERIA RECOVERED FROM WOUNDS OF DIABETIC PATIENTS

Abstract

Diabetic wound infections still remain a health concern, therefore, correct identification of bacteria is essential in monitoring the spread of the infections as well as in administering the correct treatment. This study focused on isolating and identifying bacteria present in diabetic wounds of hospitalized patients in northern KwaZulu-Natal and assessing the distribution patterns of the bacteria in wounds. The wound specimen were collected and swabbed onto selective and differential media. The bacteria identities were presumptively ascertained through biochemical characterization (Gram-stain, catalase test, oxidase test and API) and then confirmed through 16S rDNA sequencing. A total of 42 isolates were recovered from 83% of the patients sampled from the three participating hospitals (X, Y, and Z). Gram-negative bacilli from *Enterobacteriaceae* were predominant followed by *Staphylococci spp* and *Enterococcus faecalis* with 43% polymicrobial cases from hospital Z and 29% from hospital X. Distribution of some opportunistic pathogens and nosocomially-acquired pathogens were also observed across the patients. The adverse effects associated with the recovered bacteria in diabetic wounds pose a serious health concern and preventive measures should be taken.

Keywords: Diabetic complications, wounds, bacteria, infection

3.1. Introduction

Literature abounds with reports of bacterial flora on human skin (Kloos and Musselwhite, 1975**;** Christensen and Bruggemann, 2014). This predisposes patients to an increased risk of being infected by bacteria that are free-living on the skin (Hirsch *et al.,* 2008; Kong, 2011; Ak *et al.,* 2016). However, the type and quantity of the microorganisms serve as an indication of the wound infection (Bowler *et al.,* 2001). Diabetic wound infection is one of the main chronic complications of diabetes with life-threatening adverse effects in healthcare (Hirsch *et al.,* 2008; Kamel *et al.,* 2014). The increased blood glucose impairs blood flow, leukocyte function, and chemotaxis of the neutrophils and macrophages (Hirsch *et al.,* 2008; Collins and Toiba, 2010). Other factors such as surgical procedures, hospitalization and prolonged antibiotic therapy may predispose patients to infection (Siddique and Bernstein, 2010). Infection is driven by the pathogenicity and virulence of the bacteria (Siddique and Bernstein, 2010; Sahay, 2013; Bessa *et al.,* 2013), as some bacteria become more virulent in the presence of high glucose (Sahay, 2013). Diabetic wound infections are normally polymicrobial (Sivanamaliappan and Sevanan, 2011), and this can further compromise the host cell function (Bowler *et al.,* 2001).

Accurate identification of polymicrobial bacterial species present in an infection is important in determining whether the isolate is causing a genuine infection or whether it is colonizing or contaminating the wound site (Woo *et al.,* 2008; Rogers *et al.,* 2009). Routine analysis of wound specimen normally involves the use of traditional culture methods such as selective and differential agar media to culture the anaerobic and aerobic bacteria (Bowler *et al.,* 2001). The organisms are classified by means of similarities and differences based on their phenotypic characteristics such as cell appearance, cell shape, size and pigmentation (Woo *et al.,* 2008; Oates *et al.,* 2012; Perry and Freydiere, 2007; Kiser *et al.,* 2011). Gram stains, biochemical tests (catalase and oxidase) and controlled growth conditions are required for definitive grouping of bacteria (Perry and Freydiere, 2007; Woo *et al.,* 2008). Biochemical tests demonstrate the ability of test organisms to degrade specific substrates such as carbohydrates, amino acids, and other organic molecules. Other biochemical tests involve the ability of an organism to grow in the presence of a single nutrient source (Kiser *et al.,* 2011). The major role played by routine analysis of bacteria in wound care is the appropriate use of antimicrobial

agents. However, it is essential to correctly identify the microbes clinically to help eliminate healthcare burdens (Rhoads *et al.,* 2012; Bjarnsholt, 2013).

It has become more difficult to identify polymicrobial bacterial species present in an infection through culture methods (Rogers *et al.,* 2009). However molecular diagnostic techniques have provided much evidence that most chronic wounds are polymicrobial (Dowd *et al.,* 2011). Culture-based techniques often fail to identify fastidious bacteria that are important in diagnosis (Kong, 2011; Scharschmidt and Fischbach, 2013) and they may underestimate microbial diversity (Oates *et al.,* 2012) while culture-independent methods are able to detect bacterial species that were omitted by culture-based techniques (Wigneswaran *et al.,* 2016). The ability to characterize bacteria using 16S ribosomal RNA (rRNA)-based phylogenies has enabled a faster way of identifying bacteria and elucidating the role of bacterial pathogens in the development of infectious diseases (Wigneswaran *et al.,* 2016). The 16S rDNA sequencing surveys only a portion of the microbial genome that encodes the 16S rRNA subunit (Janda and Abbott, 2002). This molecular technique determines the nucleotide sequence of ribosomal RNA from various bacteria in order to assess their relative position in the evolutionary order (Kolbert and Persing, 1999; Mignard and Flandrois, 2006; Sontakke *et al*., 2009), thereby grouping bacterial isolates into taxonomic and phylogenetic groups based on their genetic composition (Janda and Abbott, 2002). The significance of 16S rRNA is that it is present in all prokaryotic cells with conserved and variable sequence regions evolving at different rates, making it suitable for bacteria identification (Srinvasan *et al.,* 2015).

The assessment of the bacteria present in wounds is essential. It provides antibiotic therapy guide that can help manage and prevent amputations and improve the quality of life (Akhi *et al.,* 2015). To the best of the researcher's knowledge, South Africa (and indeed the Northern KwaZulu-Natal region) has been minimally represented in similar studies. It is hoped that this study will provide the necessary and essential literature in this particular field.

3.2. MATERIALS AND METHODS

This section gives a brief description of the materials and methods used in the study. The details of the preparation of the reagents/media and the methodologies are presented in Appendices A and B, respectively. Table 3.1 presents the list of materials used in the study.

Table 3.1: The list of materials

Name of material	Supplier
Mannitol Salt agar	Merck
MacConkey agar (without / with crystal violet)	Merck
Nutrient agar	Merck
Nutrient broth	Merck
Sterile sampling swabs (with Amies media)	Merck
Sterile cotton inoculating swabs	Deltalab
Hydrogen peroxide	Sigma
API 20E test kit (Biomerieux)	Polychem
API 20 Staph test kit (Biomerieux)	Polychem
API 20 Strep test kit (Biomerieux)	Polychem
API 20 NE test kit (Biomerieux)	Polychem
Microscope (Primo star)	Zeiss
Autoclave	Rexmed
Incubator	Scientific
Laminar flow	Labotec
Ethanol	Sigma
Eppendorf tubes	Merck
Barritt's reagent A (VP1)	Sigma
Barritt's reagent B (VP2)	Sigma
Nitrate reagent A	Sigma
Nitrate reagent B	Sigma
TDA reagent (Tryptophan Deaminase reagent)	Sigma

Zinc dust	Sigma
Kovacs oxidase reagent (tetra-methyl-*p*-phenylenediamine dihydrochloride)	Sigma
Potassium hydroxide pellets	Sigma
James Reagent (for Indole test)	Sigma
Mineral oil	Sigma
Ninhydrin	Sigma
Zym A reagent	Sigma
Zym B reagent	Sigma
Crystal violet	Sigma
Iodine	Sigma
Safranin	Sigma

3.2.1. Wound Specimen Collection

This study was carried out after approval (UZREC 171110-030 PGM 2015/195) from the ethics committee of the University of Zululand was obtained (see Appendix C). The full cooperation of the patients was duly obtained (see Appendix C). The wound specimens of 18 hospitalized diabetic patients (diagnosed by medical doctors to be diabetic; 22% male and 78% female) were collected from three different rural-based Northern KZN hospitals in 2015-2016 (See appendix F). Hospital X is a district healthcare facility which provides services to the rural community while Hospital Y is a district healthcare center which provides health care services to neighboring healthcare institutions. Hospital Z is a cost effective regional hospital which provides high safety standards. The demographic data of patients such as age, gender, and ethnic group were recorded prior to sampling (Bahashwan and El Shafey; 2013). The medical doctors were responsible for swabbing the wounds after washing them with sterile saline and sterile cotton pads. Sterile swabs were introduced to the base of the wound and then subsequently inserted in Amies transport media at 4° C to maintain the specimen during transportation to the University of Zululand's biochemistry laboratory (Gadepalli *et al.,* 2006**;** Kamel *et al*., 2014).

3.2.2. Specimen Isolation

The spread plate method described by Ørskov (1922) was used to inoculate the specimen from the swabs onto the primary media containing plate's namely nutrient agar, mannitol salt agar and MacConkey agar exclusively. The plates were incubated at 37°C for 24-48hours, after which successive quadrant streak technique was used to purify the colonies. Pure colonies were kept on nutrient agar plates at 4°C and 20% glycerol stocks at -80°C (Mohammed *et al*., 2013b; Kamel *et al.,* 2014).

3.2.3. Identification of the Isolated Bacteria

Isolates were presumptively identified using Gram-staining, (Gram, 1884) morphological characterization (colony shape, size, pigmentation, etc.) according to the methods of Kiser *et al.,* (2011). Standard biochemical tests such as catalase (McLeod and Gordon, 1923) and oxidase (Gordon and McLeod, 1928) were carried out followed by the presumptive identification of bacteria using Analytical Profile Index (API) test kits namely; API 20 Staph, API 20 Strep, API 20E*,* API 20NE according to the manufacturer's instructions (Biomerieux S.A). The confirmation of the bacteria identities was done using PCR by amplifying the 16S rDNA and analyzing the sequenced products through BLAST Search (NCBI). The PCR products were visualized through the agarose gel (Altschul *et al.,* 1997). The 16S rDNA sequencing was carried out by Inqaba Biotec, Pretoria, South Africa. See Appendix B for PCR primer sequences. Referenced bacterial strains (ATCC) were used as positive controls where necessary.

3.2.4. Statistical Analysis

Variants were analysed using Graphpad prism version 6 to determine the one way ANOVA, two way ANOVA, means and standard deviations.

3.3. RESULTS

3.3.1. Sample Collection

A Total of 7 patients from hospital X, 4 patients from hospital Y and 7 patients from Hospital Z participated in the study. The classification of the patients sampled are listed in table 3.2

Table 3.2: General Classification of the patients

Variables	Mean (%)
Age (years)	66.6
Male gender	22.2
Female gender	77.8
Wound site (Lower limb)	94.4
(Other body parts)	5.6

3.3.2. Isolation and Presumptive identification of bacteria

The presumptive identities obtained from API were confirmed by amplifying the 16S rDNA of each isolate using PCR and the purified sequence products obtained were compared with those present in the GeneBank using BLAST. Tables 3.3 to 3.5 show the characteristics of the isolates (the presumptive and the confirmed identities through 16S rDNA/ BLAST analysis). The observed differences are highlighted in blue. The observed phenotypic differences indicate the anomalies between culture-dependent techniques and 16S rDNA sequencing.

Table 3.3. The characteristics and presumptive identities of bacterial isolates from hospital X

Code of bacteria	GPM	Gram-stain	Morphology	Oxidase test	Catalase test	Presumptive ID	API Identification	BLAST Report
***Pat A1**	m-f	Gram+ **Gram-**	cocci **Bacilli**	Positive **Negative**	Positive	***Micrococcus***	*Kocuria varians*	***Proteus mirabilis (SS1)***
Pat A2	Non l-f	Gram -	Bacilli	Negative	Positive	***Enterobacteriaceae***	*Proteus mirabilis*	*Proteus mirabilis (P3)*
***Pat B1**	L-f	Gram- **Gram+**	Coccobacill **Bacilli**	Positive	Positive	**Non-*E***	*Sphingomonas paucimobilis*	***Bacillus spp (NE3)***
***Pat B2**	L-f	Gram-	Bacilli	Positive **False Positive**	Positive **Slow-Positive**	**Non-*E***	*Rhizobium radiobacter*	***Klebsiella pneumoniae (NE2)***
***Pat B4**	M-f	Gram+ **Gram-**	Cocci **Bacilli**	Negative	Negative **Slow-Positive**	***Enterococcus***	*Aerococcus viridans*	***Klebsiella pneumoniae (A1)***
***Pat B5**	M-f	Gram+ **Gram-**	Cocci **Bacilli**	Positive **Negative**	Negative **Positive**	***Enterococcus***	*Globicatella sanguinis*	***Proteus mirabilis (E3)***
***Pat B6**	L-f	Gram -	Bacilli	Positive **False-Positive**	Positive	**Non-*E***	*Burkholderia cepacia*	***Klebsiella pneumoniae (B1)***

***Pat C1**	non L-F	Gram -	Bacilli	Positive	Positive	**Non-*E***	*Burkholderia cepacia*	***Acintobacter baumannii (B2)***
***Pat D1**	L-f	Gram -	Bacilli	Positive **Negative**	Positive	**Non-*E***	*Aeromonas hydrophila*	***Escherichia coli (NEE6)***
***Pat D2**	M-f	Gram+	Cocci **Bacilli**	Negative	Negative **Positive**	***Enterococcus***	*Aerococcus viridans1*	***Corynebacterium striatum (A3)***
***Pat E1**	Non L-f	Gram-	Bacilli	Positive **Negative**	Positive	**Non-*E***	*Ochrobactrum anthropi*	***Morganella morganii (NE1)***
Pat F1	Non M-f	Gram +	Cocci	Negative	Positive	***Micrococcus/ Staphylococcus***	*Staphylococcus epidermidis*	*Staphylococcus epidermidis (S5)*
Pat F2	Non M-f	Gram+	Cocci	Negative	Positive	***Micrococcus/ Staphylococcus***	*Staphylococcus xylosus*	***Staphylococcus aureus (S4)***
***Pat F3**	Red colonies	Gram +	Cocci pairs/ chains	Negative	Negative	***Enterococcus***	*Streptococcus porcinus*	***Enterococcus faecalis (ST2)***
***Pat F4**	Red colonies	Gram+	Cocci cluster	Negative	Negative	***Enterococcus***	*Aerococcus viridans1*	***Enterococcus faecalis (A4)***
Pat G	No growth	-	-	-	-			

Table 3.4. The characteristics and presumptive identities of bacterial isolates from hospital Y

Code of bacteria	GPM	Gram stain	Morphology	Oxidase test	Catalase test	Presumptive ID	API Identification	Blast Report
Pat A1	Non L-f	Gram-	Bacilli	Positive	Positive	**Non-*E***	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa (NP3)*
Pat A2	Non L-F	Gram-	Bacilli	Negative	Positive	***Enterobacteriaceae***	*Proteus mirabilis*	*Proteus mirabilis (P2)*
Pat B1	No growth	-	-	-	-	**-**		
***Pat C1**	Non-M-f	Gram+	Cocci **Coccobacilli**	Negative	Negative		*Enterococcus durans*	***Desemzia incerta (E1)***
***Pat C2**	Non L-f	Gram+ **Gram-**	Cocci **Bacilli**	Negative **Positive**	Negative **Positive**	**Non-*E***	*Enterococcus faecium*	***Janthinobacterium* spp (E2)**
Pat D1	Non-L-f	Gram-	Bacilli	Negative	Positive	***Enterobacteriaceae***	*Proteus mirabilis*	*Proteus mirabilis (P4)*
***Pat D2**	Non M-f	Gram+ **Gram-**	Cocci **Bacilli**	Positive **Negative**	Positive		*Kocuria varians*	***Proteus mirabilis (SS2)***

Table 3.5. The characteristics and presumptive identities of bacterial isolates from hospital Z

Code of bacteria	GPM	Gram-stain	Morphology	Oxidase test	Catalase test	Presumptive ID	API Identification	Blast Report
***Pat A1**	Non L-f	Gram- **Gram+**	Bacilli **Cocci**	Negative	Positive	***Enterobacteriaceae***	*Proteus vulgaris*	***Enterococcus faecalis (P1)***
***Pat A2**	Non L-f	Gram-	Bacilli	Positive **Negative**	Positive	**Non- *E***	*Vibrio alginolyticus*	***Escherichia coli (NE4)***
***Pat A4**	Non M-f	Gram+	Cocci	Negative	Negative **Positive**	***Enterococcus***	*Aerococcus viridans 1*	***Staphylococcus aureus (A2)***
***Pat A5**	M-F	Gram+	Cocci	Negative	Negative	***Enterococcus***	*Streptococcus porcinus*	***Enterococcus faecalis (ST1)***
***Pat B1**	Non L-f	Gram- **Gram+**	Bacilli	Positive	Positive	***Pseudomonas***	*Pseudomonas aeruginosa*	***Bacillus* spp (NP1)**
***Pat B2**	Non L-f	Gram- **Gram+**	Bacilli	Positive	Positive	***Pseudomonas***	*Pseudomonas aeruginosa*	***Bacillus* spp (NP2)**
Pat C1	Non L-f	Gram-	Bacilli	Negative	Positive	***Enterobacteriaceae***	*Citrobacter koseri*	*Citrobacter koseri (PP1)*
***Pat C2**	M-f	Gram+ **Gram-**	Cocci **Bacilli**	Negative	Positive	***Staphylococcus***	*Staphylococcus xylosus*	***Proteus mirabilis (S3)***
Pat D1	M-F	Gram+	Cocci	Negative	Positive	***Staphylococcus***	*Staphylococcus spp*	*Staphylococcus aureus (S6)*
***Pat D2**	L-F	Gram-	cocci **Bacilli**	Negative	Positive	***Enterococcus***	*Aerococcus viridans*	***Klebsiella oxytoca (A5)***
Pat E1	M-f (golden	Gram+	Cocci	Negative	Positive	***Micrococcus/ Staphylococcus***	*Staphylococcus aureus*	*Staphylococcus aureus (S1)*
Pat E2	M-f (white)	Gram+	Cocci	Negative	Positive	***Staphylococcus***	*Staphylococcus aureus*	**N/S 4**

Pat E3	L-f	Gram-	Bacilli	Positive	Positive	**Non-*E***	*Aeromonas hydrophila/ caviae*	**N/S 3**
***Pat E4**	Non L-f	Gram-	Bacilli	Positive **False-Positive**	Negative **Positive**	**Non-*E***	*Aeromonas hydrophila/ caviae*	***Klebsiella pneumoniae (NEE3)***
Pat E5	L-f	Gram-	Bacilli	Negative	Negative	***Enterobacteriaceae***	*Klebsiella oxytoca*	*Klebsiella oxytoca (PP2)*
***Pat F1**	Non L-f	Gram-	Bacilli	Positive **Negative**	Positive	**Non-*E***	*Aeromonas hydrophila/ caviae*	***Enterobacter xiangfangensis (NEE2)***
Pat F2	M-f	Gram+	Cocci	Negative	Positive	***Micrococcus/ Staphylococcus***	*Staphylococcus aureus*	***Staphylococcus sciuri (S2)***
Pat F3	L-f	Gram-	Bacilli	Positive	Positive	**Non-*E***	*Rhizobium radiobacter*	**N/S 1**
Pat F4	L-f	Gram-	Bacilli	Positive	Positive	**Non-*E***	*Aeromonas sobria hydrophila/ caviae*	**N/S 2**
***Pat F5**	Non L-f	Gram-	Bacilli	Positive **False-Positve**	Negative	**Non-*E***	*Aeromonas hydrophila*	***Klebsiella pneumoniae (NEE4)***
***Pat F6**	Non L-f	Gram-	Bacilli	Positive **Negative**	Positive	**Non-*E***	*Aeromonas hydrophila*	***Enterobacter xiangfangensis (NEE5)***
Pat G 1	No growth	-	-	-	-			

Key: n/s – Not sequenced, Non-E- non –*Enterobacteriaceae*, GPM- Growth on Primary Media, * (code) indicates that there were some anomalies among the biochemical tests, presumptive ID and the Blast report

A total of 42 isolates were recovered from 15 (83%) patients; no isolates were obtained from the wounds of 3 (17%) patients. Isolates recovered from hospital X were 15 (36%), while those from hospital Y and hospital Z were 6 (14%) and 21 (50%) respectively. Figure 3.1. Shows the overall distribution pattern of the isolates from the three hospitals

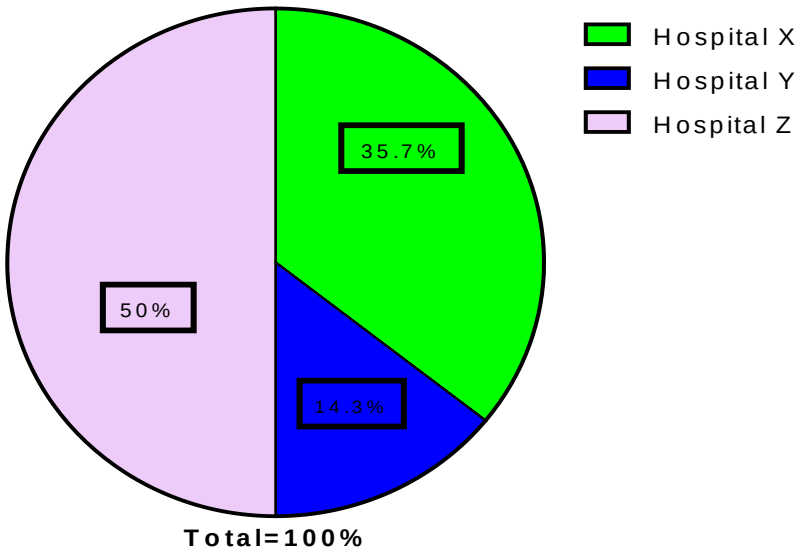
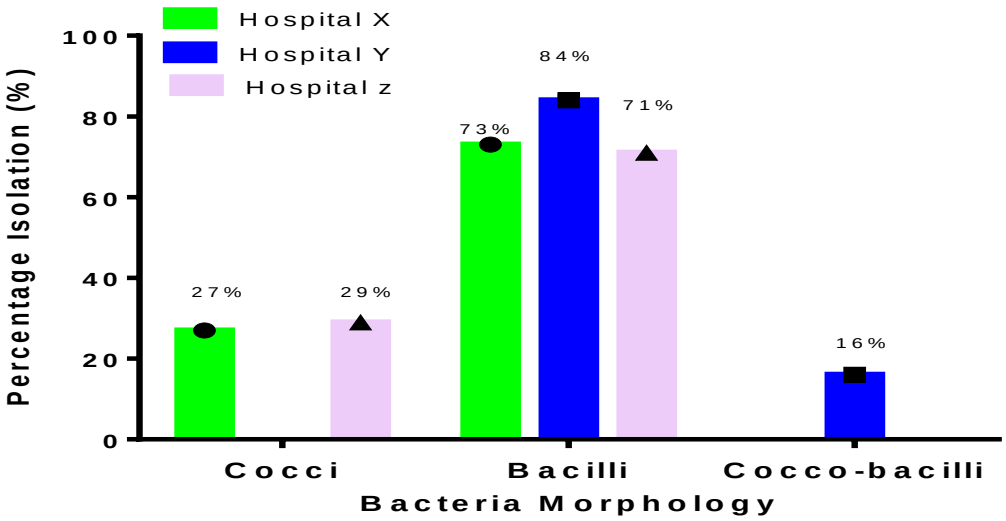


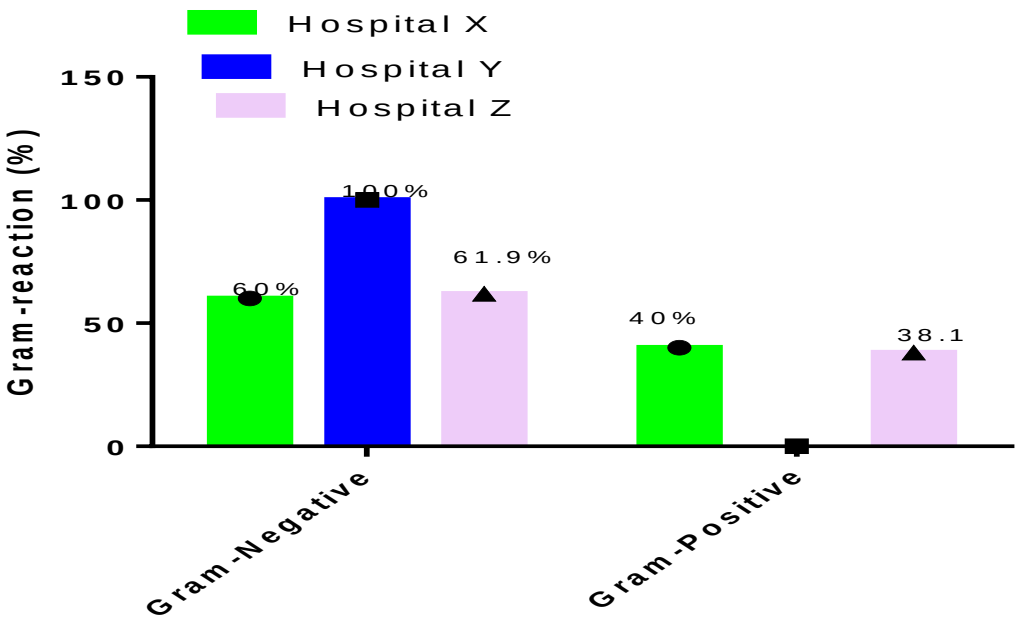
Figure 3.1: The overall isolation percentage across the different hospitals.

The isolates classified according to their microscopic morphology during the Gram-staining (figure 3.2) revealed that Hospital Y had more bacilli isolates (83%) compared to the other hospitals. Cocci isolates were predominant at hospital Z (29%) while no such isolates were recorded from hospital Y where only a cocco-bacillus was recovered. Figure 3.3 shows how much of the Gram-positives were isolated in comparison with Gram-negatives.



(Data was subjected to 95% Confidence interval analysis)

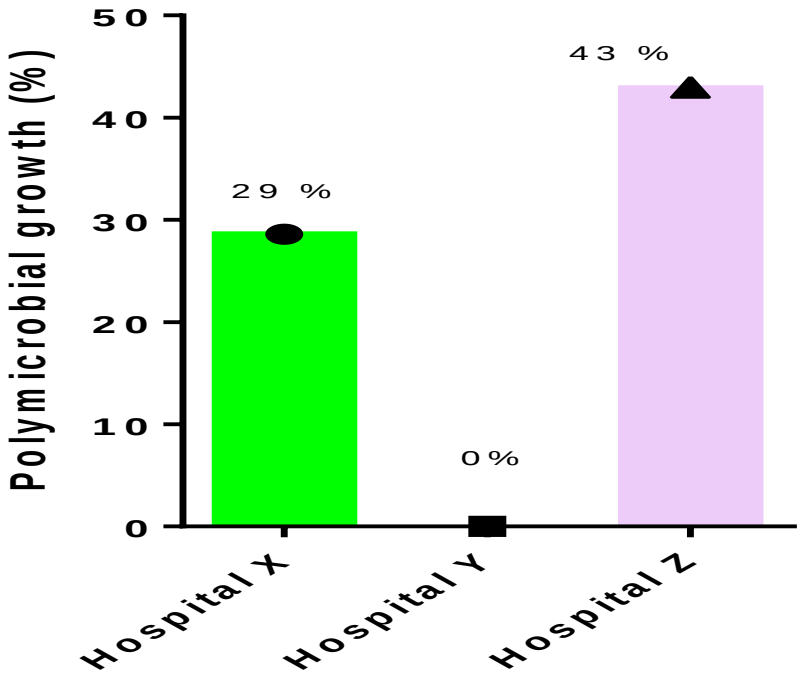
Figure 3.2: The different bacteria morphologies isolated from the hospitals



(Data shown to be significantly different through one-way Anova ****, P < 0, 0001)

Figure 3.3: The Gram-reaction of the isolates

Some wounds were colonized by several types of bacteria; 29 % and 43% of the wounds from hospital X and hospital Z respectively were polymicrobial (more than 3 isolates recovered) as indicated in figure 3.4. No polymicrobial growth was evident from hospital Y patients.

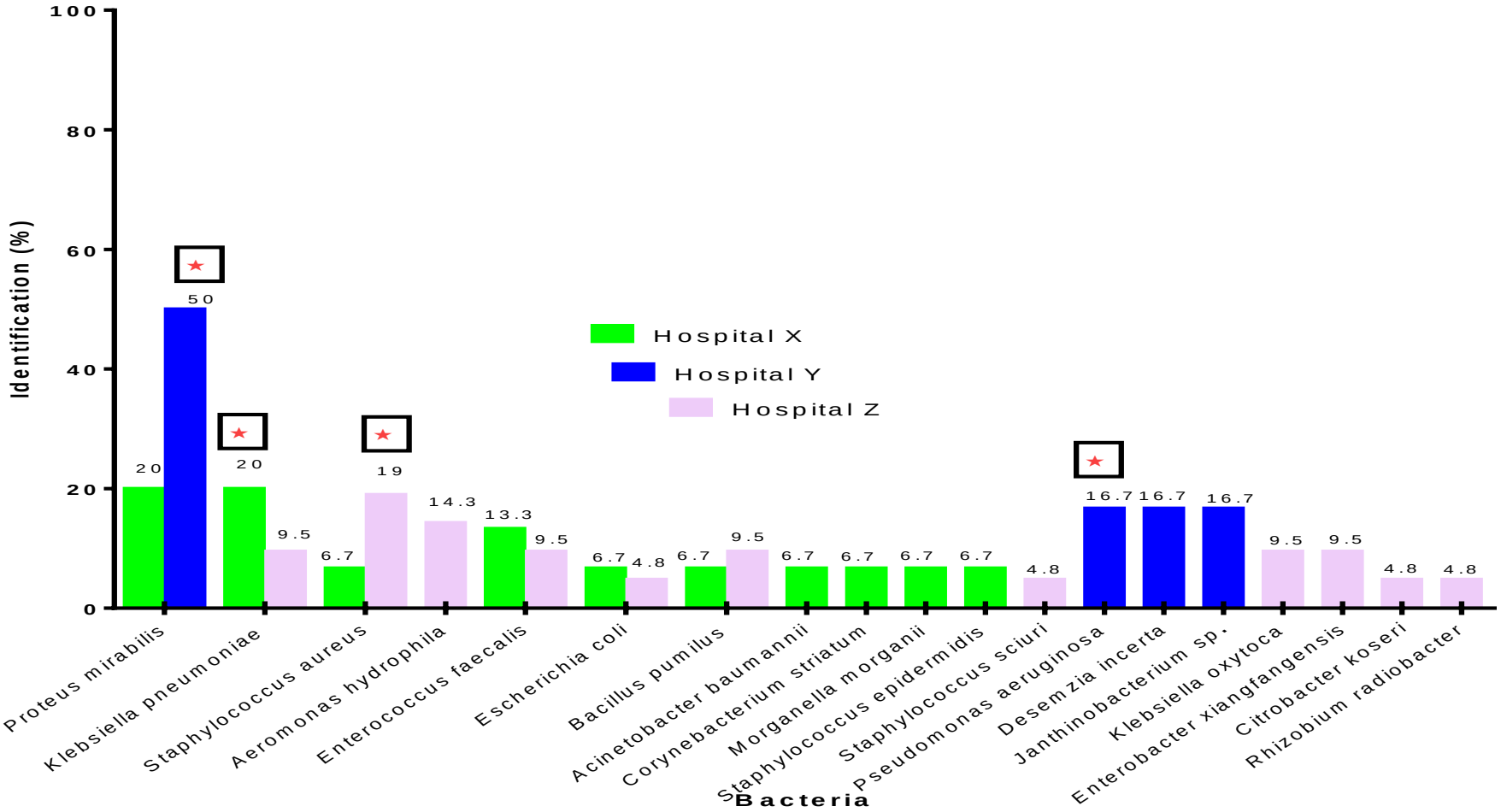


(Data was subjected to 95% Confidence interval analysis)

Figure 3.4.: The occurrence of polymicrobial growth in the different wounds of hospitalized patients

3.3.3. The Distribution Patterns of the Bacteria Species

The Gram-negative bacilli from *Enterobacteriaceae* such as *Proteus mirabilis* (20%) and *Klebsiella pneumoniae* (20%) were the predominant bacteria species from hospital X. *Staphylococcus aureus* (19%) was mostly recovered at hospital Z while *Proteus mirabilis* (50%) was common at hospital Y, as shown in figure 3.5. A few skin commensals such as *Corynebacterium striatum, Staphylococcus epidermidis* were also recovered. Greater species diversity was observed in the wounds of patients from hospital Z. Two species of *Klebsiella* were recovered (*Klebsiella pneumoniae* and *Klebsiella oxytoca*).



(Data shown to be significantly different through two-way Anova **, P-value = 0.0013)

Figure 3.5. The Distribution of bacteria isolates recovered from the hospitals under study

3.4. Discussion

Diabetic wound infections are a global challenge, compromising the quality of life, especially in developing countries (Kihla *et al.,* 2014; Mihai *et al.,* 2014; Akhi, 2015). In this study, bacteria were recovered from the wounds of 83% of the sampled diabetic patients. Samples confirmed the prevalence of bacteria in the wounds of diabetic patients in agreement with Nelson *et al.,* (2013) and Dunyach-Remy *et al.,* (2016). In 94% of the cases, the wounds were in the lower limbs and this has been attributed to vascular permeability that causes impaired blood supply to the peripheries during a diabetic state (Noor *et al.,* 2015).The wounds were also noted mostly in the elderly (> 60 years) whose immune system is already compromised due to ageing (Murphy and Frick, 2012), thereby increasing the risk of bacterial infections (Perim *et al.,* 2015)

Biochemical tests are solely based on phenotypic properties of bacteria which are shared by most species (Janda and Abbott, 2002; Perry and Freydiere, 2007; Kiser *et al.,* 2011). As a result misidentification is common, which can also account for the anomalies observed in tables 3.3- 3.5 whereby culture-based methods of identification (API) misinterpreted some of the results which were confirmed as different by the 16S rDNA analysis. Several studies have reported that antimicrobial therapy may affect the bacterial cell wall without killing the bacteria and may lead to altered cell morphology. Thus misidentification is common especially in the Gram-stain (Stone and Steele, 2009; Thairu *et al.,* 2014). Gram-viable bacteria stain opposite their true Gram-reaction thus limiting the use of the Gram-stain in bacterial identification (Stone and Steele, 2009). Catalase and oxidase tests play a crucial role in enzyme-based methods of identification. However, some bacteria contain enzymes different from catalase or cytochrome oxidase c that alter these particular reactions thereby giving false results (Janda and Abbot, 2002; Woo *et al*., 2008; Rhoads *et al*., 2012). Therefore the 16S rDNA results were preferred in this study because they accurately identified bacteria isolates and unculturable strains, giving a better understanding of bacteria etiology in infections (Woo *et al.,* 2008; Wigneswaran *et al.,* 2016).

The Gram-negative bacilli were the most recovered from the patients' wounds in all three hospitals (figure 3.3), supporting what has been reported by Kamel *et al.,* (2014) and Akhi *et al.*, (2015) that most diabetic wounds are colonized by Gram-negative

bacilli. The wounds were monomicrobial in 76% of the cases, which closely associates them with mild diabetic wound infections (Bowler *et al.,* 2001; Benwan *et al.,* 2012). Polymicrobial wounds on the other hand are inclined to severe infections (Peters *et al.,* 2012; Akhi *et al.,* 2015) and were noted in 24% of the cases in the study. In severe infections, there is an increased risk of biofilm formations which in turn delays wound healing (Han *et al.,* 2011) due to the impaired host defense (Griswold, 2012), decreased uptake of treatment drug by biofilms (Vickery *et al.,* 2014) and microbial synergy between less invasive and virulent bacteria (Landis, 2008), leading to longer hospital stays and in extreme cases limb amputations (Xavier *et al.,* 2014) which affect the quality of life.

The results of the study showed microbial diversity in diabetic wounds ranging from skin commensals, opportunistic pathogens, true pathogens and nosocomial-acquired microorganisms which all play a role in the wound etiology (Leung *et al.,* 2014; Emeka *et al.,* 2015; Holt *et al.,* 2015). Severe wound infections have been reported to be linked to facultative anaerobic and aerobic bacteria such as *S. aureus, S epidermidis, Enterococci* spp, *Pseudomonas* spp, *Escherichia* coli (James *et al.,* 2008; Xavier *et al.,* 2014; Akhi *et al.,* 2015), which were also recovered in some patients in this study. *Proteus mirabilis* was the most predominant isolate in the study associated with both nosocomial and community acquired infections (Rozalski *et al.,* 2012) and which can cause infections in different body sites (Feglo *et al.,* 2010). It also occurs in moist environments and immuno-compromised hosts along with *E. coli, Enterobacter spp and Klebsiella* spp which were also recovered in the study (Mordi and Momoh, 2009; Kwiecinska-Pirog *et al.,* 2013; Mahon *et al.,* 2015). Through its virulence factors such as fimbriae and flagella, it can adhere onto epithelial tissue and cause infection (Jacobsen and Shirtliff, 2011; Rozalski *et al.,* 2012).

Factors that contribute to the severity of diabetic wound infections include virulence and pathogenicity which can be attributed to some of the isolates recovered in this study such as *P. aeruginosa* and *S. aureus.* The latter have been reported to produce virulence factors that are destructive in the wound healing process. *P. aeruginosa* possesses virulence factors such as exoproteases, siderophores, exotoxins, hydrogen cyanide and pyocyanin that attack host defenses and impair wound healing (Leung *et al.,* 2014) while *S. aureus* possesses factors such as coagulase, catalase and clumping that play a role in infection (Bessa *et al.,* 2013) mainly in immuno-

compromised individuals such as diabetic individuals (Mahon *et al.,* 2015). *S. aureus* has a role in deepening and spreading infections in body tissue by damaging the host cell membranes and causing cell lysis (Dunyach-Remy *et al.,* 2016) which can also be attributed to diabetic wounds.

Staphylococcus sciuri (coagulase-negative) is among the recovered identities in this study which has been implicated in hospital and community acquired infections (Coimbra *et al.,* 2011; Nemeghaire *et al.,* 2014; Emeka *et al.,* 2015). The two species of *Klebsiella* identified in this study are frequently responsible for nosocomial infection in humans and greatly impact immunocompromised hosts (Lowe *et al.,* 2012; Holt *et al.,* 2015). This emphasizes the threat that they pose to public health. Although little has been reported about the virulence of *K. pneumoniae* (Leung *et al.,* 2014), three distinct phylagroups (Kp I, Kp II, Kp III) have been defined and all are implicated in human infections (Holt *et al.,* 2015).

The presence of bacteria alone is not indicative of infection (Bader, 2008), however, most bacteria recovered in the study have been reported to have debilitating effects in immunocompromised hosts. Therefore, the recovery of such bacteria in diabetic patients' wounds is a serious health concern which calls for necessary measures especially in a hospital setting.

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CHAPTER FOUR

THE ANTIBIOTIC SUSCEPTIBILITY PATTERNS OF BACTERIA RECOVERD FROM DIABETIC WOUNDS

Abstract

Antimicrobial susceptibility testing of bacteria recovered from diabetic wounds remains an important way of controlling infection and monitoring the emergence of antibiotic-resistant bacteria. Early diagnosis of microbial resistance patterns is aimed to help institute the appropriate antibacterial therapy and improve the adverse effects of diabetic wound infection. This study focused on determining the phenotypic resistance patterns of the bacteria isolated from the diabetic wounds of patients in three northern KwaZulu-Natal hospitals (X, Y and Z). Antimicrobial disk diffusion assay was performed on 42 bacterial isolates using antibiotics from different classes. Multidrug resistant patterns were noted among different bacterial groups such as Enterococci (83%), Enterobacteriaceae (55%), Non-Enterobacteriaceae (50%), Staphylococci (43%) and Gram-positive rods (33%). The bacteria conferred resistance to penicillin (100%), ampicillin (91%), cefepime (60%), ceftazidime (55%) and gentamicin (52%). Hospital X's bacteria were found to be most resistant to erythromycin (80%) and ciprofloxacin (70%), while hospital Z's bacteria were most resistant to vancomycin (50%) and penicillin (50%), with Hospital Y's bacteria showing the most resistance to imipenem (45%). The Gram-negative bacilli (*Enterobacteriaceae),* were predominant in the study with 86% of *Proteus mirabilis* resistant to imipenem*. Klebsiella spp* and *Escherichia coli* also exhibited resistance to important antibiotics treatment. The noted antibiotic resistance patterns are a concern to public health.

Keywords: Diabetic wounds, bacteria, antibiotics, resistance

4.1. Introduction

Despite the role played by antibiotics in managing bacterial infections, antibiotic resistance has become a serious international health drawback compromising the safety of the population (National Department of Health (NDH) 2015; Essack *et al.,* 2016). Antibiotic resistance occurs when bacteria becomes insensitive to a drug to which it was once susceptible (Dantas and Sommer, 2012; WHO, 2014). Resistance is the result of bacteria mutation and selection pressure from the irrational use of antibiotics (Laxminarayan *et al.,* 2013; Vuotto *et al.,* 2014; Ventola, 2015; Shahi and Kumar, 2016). The World Health Organization (WHO) has reported that, globally, there are very high statistics of antibiotic resistance and they continue to rise (WHO, 2014) and threaten the success of medical intervention at all levels of health care (Grundmann *et al.,* 2011). South Africa has made efforts to tackle antibiotic resistance by introducing several infection prevention and control training programs countrywide; however, these programs have not been implemented in some healthcare facilities (Suleman and Meyer, 2012) especially in deep-rural areas. The South African surveillance data verified that there is increasing resistance in all major infection causing bacteria (NDH, 2015).

Gram-negative bacteria are a major therapeutic challenge causing severe infections in hospital settings (Perez *et al.,* 2014; Robert *et al.,* 2014; Rossolini *et al.,* 2014). A number of critically ill patients particularly with sepsis are treated with antibiotics but clinical outcomes are not improving due to the emergency of multidrug-resistant bacteria that limit the choice for therapy (Tumbarello *et al.,* 2012; Roberts *et al.,* 2014). The resistance conferred by bacteria such as *Enterobacteriaceae* (*Escherichia coli, Klebsiella, Enterobacter, Proteus*) and *Pseudomonas* may be due to the production of different β-lactamase enzymes that have activity against penicillin, 1st, 2nd, 3rd generation cephalosporin which has magnified antibiotic resistance in hospital settings, mainly in patients with wounds (Thenmozhi *et al.,* 2014). *Proteus mirabilis* is predominant in clinical settings (Tumbarello *et al.,* 2012; Adamus-Bialek *et al.,* 2013).

Diabetics with non-healing and slow healing wounds are prone to antibiotic-resistant bacterial infections due to inappropriate use of antibiotics and frequent hospitalization. Diabetics also suffer from peripheral arterial diseases which may lead to poor penetration of antibiotics into the lower limb tissues and promote selection of resistant

bacterial strains (Kandemir *et al.,* 2007; Zubair *et al.,* 2011; Shahi *et al.,* 2013; NDH, 2015). Some bacterial strains have the ability to spread in clinical settings and cause cross infection (Rossolini *et al.,* 2014) which makes healthcare settings an emergence point of resistant bacterial phenotypes (Torok *et al.,* 2012). In some cases, this has resulted in worse outcomes such as death of patients (WHO, 2014).

In addition, bacteria can be intrinsically resistant to antibiotics through inherent structural and functional characteristics which result in absence or reduced access of the drug to its target (Dantas and Sommer, 2012; Murali *et al.,* 2014; Blair *et al.,* 2015; Bloom *et al.,* 2015). For example, most diabetic wounds are inhabited by bacteria in a biofilm form which has been reported to limit antibiotic diffusion (Dantas and Sommer, 2012). These bacteria are favored by invasive procedures or contaminating substrates in wound care that make them proliferate (Grundmann *et al.,* 2011). Bacteria can acquire resistance through horizontal gene transfer carried on plasmids, transposons and integrons by conjugation or mating and mutation (Suleman and Meyer, 2012; Murali *et al.,* 2014; Blair *et al.,* 2015; Shahi and Kumar, 2016). Horizontal gene transfer has played a major role in transmission of the β-lactam antibiotics that has major contribution to resistance (Davies and Davies, 2010). Diabetic wounds are thus increasingly inhabited by antibiotic-resistant bacteria (Bowler *et al.,* 2012; Mohammed *et al.,* 2013; Essack *et al.,* 2016).

Antimicrobial susceptibility testing is essential in providing treatment regimes that will help reduce morbidity and mortality in patients infected by bacteria (Ataee *et al.,* 2012; Arena *et al.,* 2015). Antimicrobial susceptibility testing *in vitro* is also used to characterize multidrug resistant bacteria (Godebo *et al.,* 2013). The common microbiological method is the Kirby-Bauer disk diffusion method that determines the sensitivity or resistance of pathogenic bacteria to various antimicrobial agents (Defale *et al.,* 2016). The data obtained is important for compilation of surveillance reports on antimicrobial resistance which serve as guidance in antimicrobial therapy (Arena *et al.,* 2015; Leekha *et al.,* 2011).

Chapter 3 of this dissertation reported the isolation and characterization of 42 bacteria from the wounds of hospitalized diabetic patients. In this chapter the antimicrobial susceptibility profiles of the bacterial isolates were evaluated.

4.2. Materials and Methods

The details of the materials and methods used in the isolation and characterization of the bacteria have been described in chapter 3.

4.2.1 Growth media

Mueller-Hinton broth and Mueller-Hinton agar (media) were purchased from Merck (biolab).

4.2.2 Antibiotics

All the antibiotic discs were supplied by Polychem (OXOID). The 9 antibiotics represented different antibiotic classes as shown in Table 4.1.

Table 4.1. List of antibiotics purchased

Antibiotic Class	Antibiotics
β-lactams	Penicillin (10 units)
	Ampicillin (10 µg)
	Ceftazidime (30 µg)
	Cefepime (30 µg)
	Imipenem (10 µg)
Glycopeptides	Vancomycin (30 µg)
Aminoglycosides	Gentamicin (10 µg)
Fluoroquinolones	Ciprofloxacin (5 µg)
Macrolides	Erythromycin (15 µg)

4.2.3 Antibiotic Susceptibility Test

Antibiotic susceptibility testing of the bacterial isolates was done according to the Bauer *et al.* (1966) method as recommended by the Clinical Laboratory Standard Institute (CLSI) (2013). The test isolates were grown on Mueller-Hinton broth overnight for 18-24 hours at 37°C after which turbidity of the suspension was adjusted to match 0.5 McFarland's standard. A volume of 100 µl of the diluted suspension was inoculated into Mueller-Hinton agar containing plates and streaked with sterile swabs. Antibiotic disks were tested against different bacterial groups such as Staphylococci (1), Enterococci (2), Gram-negative bacilli from *Enterobacteriaceae (3),* non-*Enterobacteriaceae* Gram-negative bacilli (4) and Gram-positive rods (5). The antibiotic disks were evenly placed on plates aseptically and incubated for 18-24 hours overnight at 37°C. The choices and interpretive standards of antibiotics were based on the CLSI (2014) guideline. The zones of inhibition of the bacteria were measured and classified into three groups: Resistant, Intermediate and Susceptible. (See Appendix E). Multidrug-resistant patterns were assessed whereby bacteria identities resistant to two or more classes of antibiotics were recorded as multi-drug resistant (Godebo *et al.,* 2013).

4.2.4. Statistical Analysis

Variants were analysed using one-way Anova and two-way Anova through Graphpad prism version 6, whereby graphs and figures were constructed.

4.3. Results

4.3.1. Antibiotic susceptibility test

The five groups of bacteria showed varying susceptibility patterns as shown in Table 4.2. Multidrug resistance patterns were noted among the different groups; Enterococci (83%), Enterobacteriaceae (55%), Non-Enterobacteriaceae (50%), Staphylococci (50%) and the Gram-positive rods (33%).

Table 4.2: The antibiotic sensitivity profile of the bacteria identities

Code	***IDENTITIES***	***Antibiotic Sensitivity Profiling***								
		AMP	**CIP**	**CAZ**	**VA**	**P**	**IMP**	**CN**	**E**	**F-EP**
	Staphylococci Group (n=7)									
S1	*Staphylococcus aureus*	**-**	**S**	**-**	**I**	**R**	**-**	**S**	**S**	**-**
S2	*Staphylococcus sciuri*	**-**	**I**	**-**	**I**	**R**	**-**	**R**	**I**	**-**
A2	*Staphylococcus aureus*	**-**	**S**	**-**	**R**	**R**	**-**	**S**	**I**	**-**
S4	*Staphylococcus aureus*	**-**	**R**	**-**	**I**	**R**	**-**	**R**	**I**	**-**
S6	*Staphylococcus aureus*	**-**	**S**	**-**	**I**	**R**	**-**	**S**	**S**	**-**
N/S 4	*Staphylococcus aureus*	**-**	**S**	**-**	**I**	**R**	**-**	**S**	**S**	**-**
S5	*Staphylococcus epidermidis*	**-**	**R**	**-**	**I**	**R**	**-**	**R**	**R**	**-**
	Enterococci Group (n=6)									
A3	*Corynebacterium striatum*	**R**	**R**	**-**	**R**	**R**	**-**	**S**	**R**	**-**
A4	*Enterococcus faecalis*	**R**	**R**	**-**	**R**	**R**	**-**	**R**	**R**	**-**
St1	*Enterococcus faecalis*	**S**	**R**	**-**	**I**	**R**	**-**	**R**	**I**	**-**
ST2	*Enterococcus faecalis*	**R**	**R**	**-**	**I**	**R**	**-**	**R**	**R**	**-**
P1	*Enterococcus faecalis*	**R**	**I**	**-**	**R**	**R**	**-**	**R**	**R**	**-**
E1	*Desemzia incerta*	**R**	**S**	**-**	**S**	**R**	**-**	**I**	**I**	**-**
	Enterobacteriaceae Group (n=20)									
P2	*Proteus mirabilis*	**R**	**S**	**R**	**-**	**-**	**S**	**S**	**-**	**R**
P3	*Proteus mirabilis*	**R**	**S**	**R**	**-**	**-**	**R**	**R**	**-**	**R**
P4	*Proteus mirabilis*	**R**	**I**	**S**	**-**	**-**	**R**	**R**	**-**	**R**
S3	*Proteus mirabilis*	**I**	**S**	**R**	**-**	**-**	**R**	**S**		**R**
SS1	*Proteus mirabilis*	**R**	**S**	**R**	**-**	**-**	**R**	**S**	**-**	**R**
SS2	*Proteus mirabilis*	**I**	**S**	**R**	**-**	**-**	**R**	**R**	**-**	**R**
E3	*Proteus mirabilis*	**R**	**S**	**R**	**-**	**-**	**R**	**R**	**-**	**R**
PP1	*Citrobacter koseri*	**R**	**S**	**S**	**-**	**-**	**S**	**S**	**-**	**I**
PP2	*Klebsiella oxytoca*	**R**	**S**	**S**	**-**	**-**	**I**	**S**	**-**	**S**
A5	*Klebsiella oxytoca*	**R**	**I**	**R**	**-**	**-**	**R**	**R**	**-**	**R**

A1	*Klebsiella pneumoniae*	**R**	**S**	**R**	**-**	**-**	**I**	**R**	**-**	**R**
NEE3	*Klebsiella pneumoniae*	**R**	**I**	**R**	**-**	**-**	**S**	**R**	**-**	**R**
NE2	*Klebsiella pneumoniae*	**R**	**S**	**S**	**-**	**-**	**S**	**S**	**-**	**S**
NEE4	*Klebsiella pneumoniae*	**R**	**S**	**S**	**-**	**-**	**S**	**S**	**-**	**S**
B1	*Klebsiella pneumoniae*	**R**	**S**	**I**	**-**	**-**	**S**	**S**	**-**	**S**
NE1	*Morganella morganii*	**R**	**R**	**S**	**-**	**-**	**S**	**R**	**-**	**S**
NE4	*Escherichia coli*	**R**	**R**	**R**	**-**	**-**	**I**	**R**	**-**	**R**
NEE6	*Escherichia coli*	**R**	**R**	**I**	**-**	**-**	**S**	**R**	**-**	**R**
NEE2	*Enterobacter xiangfangensis*	**R**	**S**	**S**	**-**	**-**	**S**	**S**	**-**	**S**
NEE5	*Enterobacter xiangfangensis*	**R**	**I**	**R**	**-**	**-**	**I**	**R**	**-**	**R**
	Non-Enterobacteriaceae bacilli Group (n=6)									
NS 1	*Aeromonas hydrophila*	**R**	**S**	**S**	**-**	**-**	**S**	**S**	**-**	**S**
NS 2	*Aeromonas hydrophila*	**R**	**S**	**S**	**-**	**-**	**S**	**S**	**-**	**S**
NS 3	*Rhizobium radiobacter*	**R**	**S**	**S**	**-**	**-**	**S**	**S**	**-**	**S**
NP3	*Pseudomonas aeruginosa*	**R**	**S**	**S**	**-**	**-**	**R**	**R**	**-**	**S**
E2	*Janthinobacterium sp.*	**R**	**R**	**R**	**-**	**-**	**R**	**R**	**-**	**R**
B2	*Acinetobacter baumannii*	**R**	**I**	**R**	**-**	**-**	**S**	**R**	**-**	**R**
	Gram-Positive rod Group (n=3)									
NE3	*Bacillus sp.*	**R**	**S**	**R**	**-**	**-**	**S**	**S**	**-**	**R**
NP1	*Bacillus sp*	**R**	**S**	**R**	**-**	**-**	**S**	**R**	**-**	**R**
NP2	*Bacillus sp*	**R**	**I**	**R**	**-**	**-**	**I**	**I**	**-**	**R**

Keys: S-Susceptible, I-Intermediate, R-Resistant, AMP- Ampicillin, CIP-Ciprofloxacin, CAZ-Ceftazidime, VA-Vancomycin, P-Penicillin, IMP-Imipenem, CN-Gentamicin, E-Erythromycin, F-EP- Cefepime, **-** means not tested n- number of isolates tested

Table 4.3 shows the percentage resistance observed for each bacteria group against an individual antibiotic. Resistance percentages of 100% were noted among the Staphylococci and Enterococci group against penicillin, while non-Enterobacteriaceae and the Gram-positive rods exhibited 100% resistance against ampicillin. The highest resistance was observed against β-lactams in all groups.

Table 4.3. The resistance of different bacteria groups against antibiotics

Bacteria Group	Antibiotic	% Resistance
Staphylococci	Ciprofloxacin	33
	Vancomycin	17
	Penicillin	100
	Gentamicin	50
	Erythromycin	17
Enterococci	Ampicillin	83
	Ciprofloxacin	67
	Vancomycin	50
	Penicillin	100
	Gentamicin	67
	Erythromycin	67
Enterobacteriaceae	Ampicillin	95
	Ciprofloxacin	15
	Ceftazidime	55
	Imipenem	35
	Gentamicin	55
	Cefepime	65
Non-Enterobacteriaceae	Ampicillin	100

	Ciprofloxacin	17
	Ceftazidime	33
	Imipenem	33
	Gentamicin	50
	Cefepime	33
Gram-Positive Rods	Ampicillin	100
	Ciprofloxacin	0
	Ceftazidime	100
	Imipenem	0
	Gentamicin	67
	Cefepime	100

Table 4.4 below shows the overall susceptibility patterns of each individual antibiotic against all the bacteria across the different groups. It is of interest that a 100% and 91% resistance against penicillin and ampicillin was noted respectively.

Table 4.4: The overall antibiotic susceptibility patterns

Mode of Action	Antibiotic class	Antibiotics	Resistance (%)	Intermediate (%)	Susceptibility (%),
Inhibition of peptidoglycan synthesis	β-Lactam	Ampicillin (n=35)	**91**	**6**	**3**
		Penicillin (n=12)	**100**	**0**	**0**
		Ceftazidime (n=29)	**55**	**7**	**38**
		Cefepime (n=30)	**60**	**3**	**37**
		Imipenem (n=30)	**33**	**17**	**50**
Inhibition of the DNA gyrase and topoisomerase IV activity	Fluoroquinolones	Ciprofloxacin (n=42)	**24**	**19**	**57**
Disruption of the peptidoglycan cross-linkage	Glycopeptides	Vancomycin (n=12	**33**	**58**	**9**
Inhibition of the protein synthesis at the 30S ribosomal unit	Aminoglycosides	Gentamicin (n=42)	**52**	**5**	**43**
Inhibition of the protein synthesis at the 50S ribosomal unit	Macrolides	Erythromycin (n=12)	**42**	**42**	**16**

Keys: n- number of organisms tested, Mode of action (CLSI, 2012)

Antibiotic resistance was further evaluated in each of the hospitals under study to obtain the percentage resistance of the bacteria recovered against each individual antibiotic and the results are presented in Figure 4.1. Bacteria from Hospital X showed the highest resistance patterns against erythromycin (80%) and ciprofloxacin (70%), while bacteria from Hospital Z showed the highest resistance at 50% against vancomycin and penicillin. Hospital Y bacteria was most resistant to imipenem (45%). The data shown to be significantly different (P-value <0, 0001)

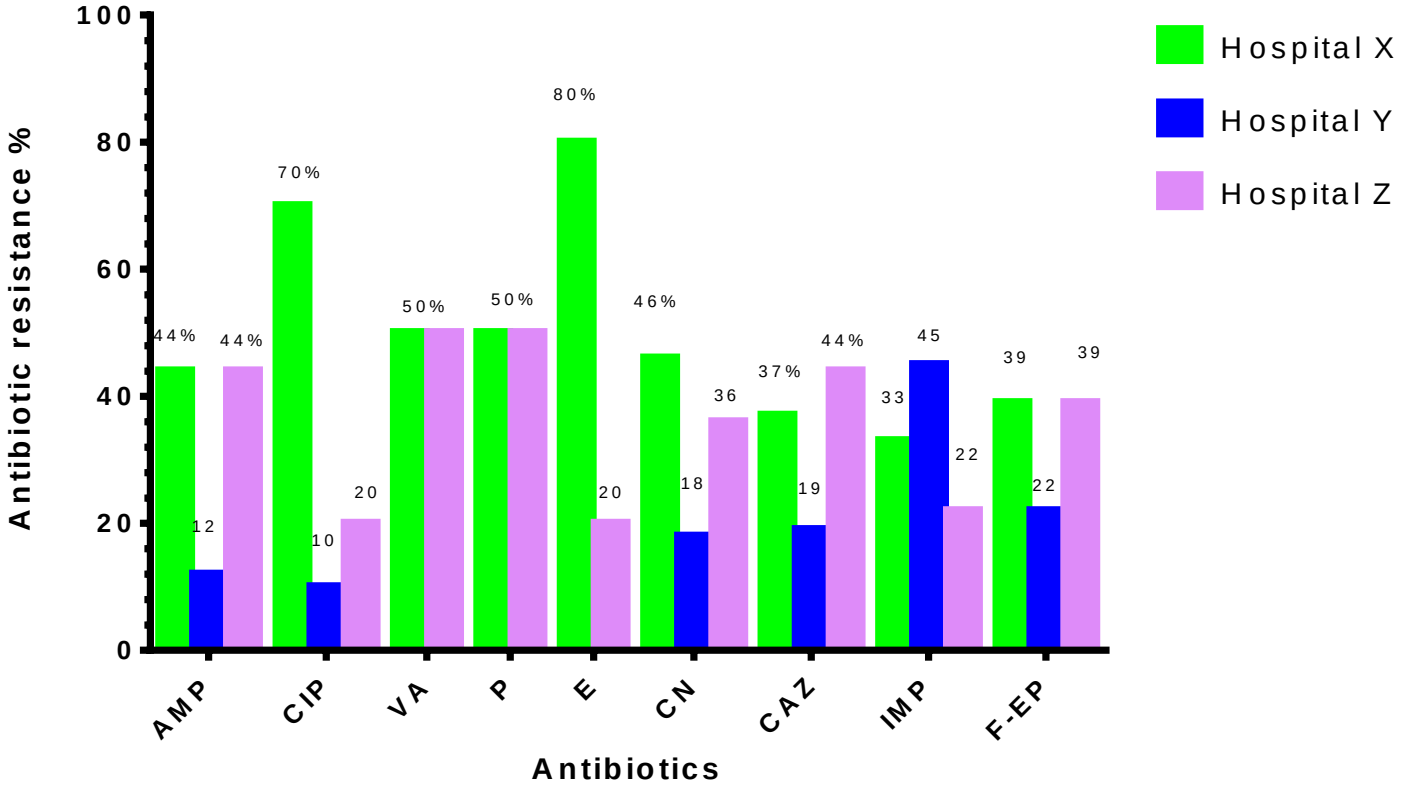

Figure 4.1: Antibiotic resistance percentages (for each antibiotic) against bacteria from the different hospitals

4.4. Discussion

The burden of antibiotic-resistant bacteria has become common in diabetic patients with wounds (Zubair *et al.,* 2011; Shahi *et al.,* 2013). Multidrug resistance patterns were noted in the study with the highest percentage being among the *Enterococci* group (table 4.2). The *Enterococci* spp have been reported to be resistant to multiple antibiotics in clinical settings (Miller *et al.,* 2014; Daniel *et al.,* 2015), with various resistant mechanisms being reported against all antimicrobial agents in clinical practice (Miller *et al.,* 2014). The other clinical challenge of antibiotic resistance is the decline in efficacy of antibiotic treatment; with the dry pipeline of developing new antibiotic agents which threatens the provision of prompt treatment (Cars *et al.,* 2011; Magiorakos *et al.,* 2012; Shahi and Kumar, 2016).

The bacteria were mostly resistant to β-lactam (penicillin, ampicillin, cefepime and ceftazidime) and aminoglycoside (gentamicin) (table 4.3 and 4.4). The observed resistance may be exhibited by bacterial cell walls (β-lactam) and ribosomal proteins (gentamicin). β-lactams are bactericidal and inhibit the cell wall synthesis of different organisms (CLSI, 2012). Penicillin is most active against Gram-positives while ampicillin is an excellent broad-spectrum antibiotic (Kiser *et al.,* 2011). Cephalosporins and carbapenems are active against Gram-negatives (Shaikh *et al.,* 2015), however, in this study most bacteria were resistant to these antibiotics. Bacteria can protect themselves against β-lactams through alteration in penicillin binding proteins (PBPs) that reduce the affinity of β-lactams to the sites of action and the production of β-lactamases that are able to hydrolyze the β-lactam ring (Zhou *et al.,* 2015). The frequent use of β-lactams as the first line of treatment has played a major role in the development of antibiotic resistant bacteria (El-Sokkary *et al.,* 2015). As a result, infections caused by β-lactam resistant organisms have increased over the years (Shaikh *et al.,* 2015). To make matters worse, penicillin is still being prescribed without considering its effects on resistance (Zeng and Lin, 2013).

The declining effectiveness of antibiotics is believed to be also driven by mismanagement of antibiotics (Laxminarayan *et al.,* 2016) in hospitals where most infection causing bacteria are isolated (NDH, 2015). In this study, Hospital X was implicated in resistance

against ciprofloxacin, a broad spectrum bactericidal antibiotic that is a last line of defense in antibiotic treatment (Jacoby, 2005; Kiser *et al.,* 2011) and erythromycin, which is considered bacteriostatic and targets gram-positives (Kiser *et al.,* 2011; Liwu and Jaka, 2015). Resistance to quinolones can be a severe clinical problem due to their importance in health (Fasugba *et al.,* 2015). Therefore, the use of ciprofloxacin needs to be well monitored. Bacteria resistant to ciprofloxacin can mediate resistance through mutations that alter the drug targets or reduce drug accumulation and develop plasmids that protect bacterial cells from the lethal effect of quinolones (Jacoby, 2005; Beceiro *et al.,* 2013; Fasugba *et al.,* 2015), while erythromycin and vancomycin can be resisted through methylation of bacterial ribosomes altered cell-wall precursors respectively (Liwu and Jaka, 2015), which all compromise the available treatment and increase the burden of antibiotic resistance.

Enterobacteriaceae showed varying susceptibility patterns, with 55% resistance to antibiotics. It has been reported to be resistant to a number of antibiotics through the production of β-lactamases (Wellington *et al.,* 2013; Thenmozhi *et al.,* 2014; Shaikh *et al.,* 2015; Adesoji *et al.,* 2016). All *Proteus mirabilis* (predominant) isolates were shown to be susceptible to ciprofloxacin while 86% were resistant to imipenem. Imipenem resistance has been reported to be natural in *P. mirabilis* (Tsai *et al.,* 2015). Imipenem is normally the last line of treatment in extended-spectrum β-lactamase producing organisms (Thenmozhi *et al.,* 2014; Tsai *et al.,* 2015). Some multidrug resistant strains of *P. mirabilis* produce these enzymes (Tumbarello *et al.,* 2012; El-Sokkary *et al.,* 2015). Among the *Klebsiella* and *Escherichia coli* species, multidrug resistant patterns were observed against β-lactams, ciprofloxacin and gentamicin in which resistance has been previously reported by Iredell *et al.* (2015). The ability of *Klebsiella* species to rapidly spread in the hospital environment contributes to their resistance which has been closely associated with the production of extended-spectrum β-lactamases (ESBL), mostly mediated by plasmids (Marques *et al.,* 2011; Chaudhary and Payasi, 2013). In 2011 *K. pneumoniae* was documented with the most extreme cases of multidrug resistant bacterial infection in South Africa (NDH, 2015). This emphasizes the importance of routine susceptibility testing before treatment is prescribed (Murali *et al.,* 2014).

The resistance observed on skin commensals; *Staphylococcus epidermidis* and *Corynebacterium striatum,* is normally experienced in patients receiving treatment through antibiotics, especially the broad-spectrum (Andersson, 2004) such as ciprofloxacin and gentamicin. *Enterococcus faecalis,* also regarded as normal skin flora (Mengeloglu *et al.,* 2011), showed multidrug resistant patterns with one species *(Enterococcus faecalis (A4)),* showing resistance to all test antibiotics. The resistance shown by the normal skin flora poses a threat to other sensitive bacteria and patients (Anderson, 2004; Cogen *et al.,* 2008). Kiser *et al.* (2011), reported that susceptibility testing of bacteria from an anatomic site for which they are found should be avoided because results may encourage the physician to treat normal conditions. The problem is that skin microflora has become resistant to the current treatment (Christensen and Bruggemann, 2014; Hahn *et al.,* 2016) as evidenced by their multidrug-resistant patterns recorded in the study. Treatment in immune compromised patients such as diabetics where bacteria are most likely to become pathogenic is now a challenge.

The findings show that it is important to evaluate possible resistance patterns of bacteria since some bacterial strains have become resistant to current treatment. The resistance conferred by Gram-negative bacilli and some skin commensals pose a threat to antibiotics used in the treatment of wound infection and may lead to a public health crisis.

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CHAPTER 5

THE SUSCEPTIBILTIY OF ANTIBIOTIC-RESISTANT BACTERIA ISOLATED FROM DIABETIC WOUNDS TO SOME MEDICINAL PLANT EXTRACTS

Abstract

The prevalence of antibiotic resistant bacteria in diabetic wounds has become a challenge that has motivated the search for new antimicrobial compounds present in plants. Plants such as *Platycarpha glomerata, Chamaesyce fendleri, Carpobrotus dimidiatus* and *Jatropha zeyheri* have been used by traditional healers in the management of skin related infections, making leads for alternative treatment of diabetic wound infections. The plants were first screened for the presence of phytochemicals. The dichloromethane, acetone and water extracts of the plants were separately screened for antibacterial activities through the agar-well diffusion assay against bacteria isolated from diabetic wounds. The bacteria that were found to be sensitive to the diffusion assay were subjected to micro-broth dilution assay to determine the minimal inhibitory concentration and minimal bactericidal concentration of the extracts. Most of the thirteen bacteria isolates tested were resistant to the plants extracts. However, zones of inhibition ranging from 11±0.1-22±0.6 mm, were observed with the acetone extract of *P. glomerata* and the aqueous and acetone extracts of *C. fendleri*. The MIC values ranged from 10- 20 mg/ml and MBC values at 5- > 20 mg/ml. The observed antibacterial activities could be attributed to the presence of phytochemicals in the plants which could be explored as lead compounds in the treatment of diabetic wound infections.

Keywords: diabetic wounds, bacteria, antibiotic resistance, alternative treatment, plants

5.1. Introduction

Diabetic wounds, like all open wounds, are often infected with bacteria (Sharma *et al.,* 2013; Akhi *et al.,* 2015), which are reportedly becoming resistant to antibiotics (Alzahrani *et al.,* 2011; Sandeep *et al.,* 2015). First-line penicillin-based antibiotics have become ineffective to bacteria such as Gram-negative bacilli, Gram-positive cocci and some skin microflora (Bessa *et al.,* 2013; Akhi *et al.,* 2015; Shahi and Kumar, 2016; Holmes *et al.,* 2016). The increasing challenge of antibiotic resistance and absence of effective antimicrobial agents is a worldwide concern (Baym *et al,* 2016; Holmes *et al.,* 2016). The previous chapters (chapters 3 and 4) of this dissertation described the antibiotic resistance profile of bacteria isolated from wounds of hospitalized diabetic patients. In the search for newer antimicrobial agents, plants and their metabolites have become a great source of novel biomolecules (Barreto *et al.,* 2014 Ngueda and Shey, 2014). Use of phytochemicals in medicine has several advantages which include fewer side effects, better patient tolerance and affordability when compared with synthetic antibiotics (Tabassum and Hamdani, 2014). Phytochemicals such as tannins, flavonoids and saponins have been reported to possess wound healing activities and antimicrobial properties which can have an advantage in wound treatment (Budovsky *et al.,* 2015; Saini *et al.,* 2016).

The use of medicinal plants for treatment of skin related infections is very common in many rural areas (De Wet *et al.,* 2013; Mabona and Van Vuuren, 2013). Plants such as *Platycarpha glomerata, Chamaesyce fendleri, Carpobrotus dimidiatus* and *Jatropha zeyheri* have been used in skin related infections and documented to possess healing activities (Zobolo and Mkabela, 2006; Luseba *et al.,* 2007; Lall and Kishore *et al.,* 2014). Zobolo and Mkabela (2006), reported that *Platycarpha glomerata* leaves are used for steaming painful legs and treating wounds, while *Chamaesyce fendleri* is applied to cuts as a hemostatic (Vistal, 1952; Kumar *et al*., 2010), *Carpobrotus dimidiatus* leaf sap is used in wound dressing as an antiseptic (Fawole *et al.,* 2010; Lall and Kishore, 2014) and *Jatropha zeyheri* rhizome is used to heal wounds and boils (Archer and Victor, 2005). In this section, the antibacterial activity of medicinal plant extracts was investigated, as the

World Health Organization encourages the use of medicinal plants after they have been scientifically validated for safe use (Vargas *et al.,* 2014).

5.2. Materials and Methods

Most of the materials and chemicals used in this section have already been listed in chapters 3 and 4.

5.1. Materials

Materials	Supplier
Ciprofloxacin powder	Polychem
Iodo-nitro-tetrazolium chloride (INT)	Polychem
Dimethlysulphoxide (DMSO)	Polychem
Sodium azide	Sigma
96-well microtitre plates	Merck
Acetone	Polychem
Dichloromethane	Polychem
Hydrochloric acid	Merck
Mayer's reagent	Fluka
Dragendorff's reagent	Fluka
Ferric Chloride	Fluka
Chloroform	Polychem
Sulfuric acid	Polychem

5.2.2. Plant collection

The herb of *C*. *fendleri* and leaves of *C. dimidiatus* were collected from the University of Zululand, KwaDlangezwa campus, while the rhizome of *P .glomerata* and herb of *Jatropha zeyheri* were collected from Oribi and KwaMhlabuyalingana respectively in KwaZulu-Natal, South Africa. All the plants were identified and confirmed by the staff of the department of Botany in the University of Zululand. Voucher specimen were prepared and kept at the University herbarium

5.2.3 Phytochemical screening

Qualitative phytochemicals screening was done to test for saponins, flavonoids, terpenoids, tannins, phenolics, and alkaloids (Sofowara, 1984). Color change or precipitation was taken as evidence of the presence of phytochemicals. (See Appendix B.

5.2.4 Extraction

The plants materials (*C. fendleri, J. zeyheri* and *P. glomerata)* were milled to powder. *P. glomerata* powder was firstly defatted using hexane to reduce the high wax content of the plant. The milled plants were separately extracted (1:5 w/v) in different solvents (dichloromethane, acetone and water). The mixture was placed on a mechanical shaker at 100 rpm for 24 hours at room temperature, after which the extracts were filtered through the Whatman No 1 filter paper. The freshly collected *C. dimidiatus* leaves were chopped into small pieces and extracted with acetone, dichloromethane and water separately. Sodium azide was added to the aqueous extracts. The extracts were filtered through Whatman No 1 filter paper. All the organic solvent extracts were concentrated using the rotary evaporator and then air dried to a constant weight while the aqueous extracts were air-dried. The extracts were weighed and kept in the refrigerators at 4^0C until needed (Mongalo *et al.*, 2013).

5.2.5. Test microorganisms

The randomly selected 13 test bacteria were isolated from diabetic patients' wounds as described in chapter 3 and their antibiotic resistance patterns evaluated as reported in chapter 4.The bacteria included *Bacillus spp (NE3), Klebsiella pneumoniae (NE2), Klebsiella pneumoniae (A1), Escherichia coli (NEE6), Corynebacterium striatum (A3), Proteus mirabilis (P2), Desemzia incerta (E1), Janthinobacterium spp (E2), Enterococcus faecalis (P1), Escherichia coli (NE4), Staphylococcus aureus (A2), Enterococcus faecalis (ST1)* and *Citrobacter koseri* (PP1)

5.2.6. Susceptibility test

The plant extracts were tested for antibacterial activity using the agar-well diffusion method described by Oribi *et al*. (1996). The test microorganisms were grown in Mueller-Hinton broth (see appendix A for the preparation of broth) overnight at 37°C after which their turbidity was adjusted to match 0.5 McFarland standard prior to use. The spread plate method was used to inoculate the test organisms onto Mueller-Hinton agar plates using sterile swabs. Test wells were bored using the 1ml sterile pipette tip wider ends. A volume of 100 µl of the extracts (20 mg/ml) dissolved in 10% dimethlysulphoxide (DMSO) were added to the respective test wells. A concentration of 20 µg/ml ciprofloxacin was used as positive control while sterile distilled water was used as a negative control and 10% DMSO as a solvent control. The plates were kept on the bench for 1 hour to allow the diffusion of the antimicrobials and then incubated for 18-24 hours at 37°C, after which the diameters of the zones of inhibition were measured in millimeters (Rojas *et al.,* 2006).The tests were done in triplicates

5.2.7. Minimum inhibitory concentration (MIC) determination.

The MIC's of the extracts against susceptible bacteria were determined using the micro broth dilution assay. Test organisms were standardized to match the 0.5 McFarland standard. A 100 µl volume of double strength Mueller-Hinton broth was introduced into each of the wells in a 96 well microtitre plate. The extracts at a starting concentration of 20 mg/ml were added on the A wells and serially diluted from A-H to yield different test concentrations. The solvent control was 10% DMSO and 20 µg/ml ciprofloxacin was used as a positive control. The plates were incubated overnight at 37°C. To determine the MIC 40 µl of 0.2 mg/ml Iodo-nitro-tetrazolium chloride (INT) was added to each well. After the addition of INT, a pinkish color (visualized by the naked eye) indicated microbial growth. The living bacteria, through reduction reactions, convert INT to red formazan. The MIC was recorded as the lowest concentration of the extract that prevented the appearance of visible growth of the bacteria after 18- 24 hours of incubation (Mongalo *et al.,* 2013).

5.2.8. Minimum bactericidal concentration (MBC) determination.

The MBC was determined from the MIC micro broth dilution assay where a volume of 15 µl from each well that did not show growth after 18-24 hours of incubation was sub-cultured by spot inoculating onto fresh Mueller-Hinton agar plates. The plates were incubated for 24- 48 hours after which the number of colonies were counted. The MBC was defined as the lowest concentration of the extract killing more than or equal to 99.9% of the inoculums compared with the initial viable counts (Sudjana *et al*., 2009, Penduka *et al.,* 2011).

5.2.9 Statistical Analysis

The Microsoft office excel 2013 version for windows program was used to determine the means and standard deviations.

5.3. Results

5.3.1. Phytochemical screening

The results of the qualitative phytochemical screening of the four selected traditional medicinal plants are displayed in Table 5.1.All the plants were found to possess saponins, terpenoids and tannins. *P. glomerata* and *J. zeyheri* apparently contained more saponins.

Table 5.2: Phytochemical screening of the selected medicinal plants

Phytochemicals	C. fendleri	P. glomerata	C. dimidiatus	J. zeyheri
Saponins	+	++	+	++
Tannins	+	+	+	+
Terpenoids	+	+	+	+
Alkaloids	+	-	+	+
Phenolics	-	+	+	+
Flavonoids	+	-	-	+

Keys: + denotes presence; - denotes absence; ++ denotes presence in large quantity

5.3.2. Susceptibility test

The bacteria isolated from hospitals X, Y and Z (see chapter 3) were tested for their sensitivity to selected medicinal plant extracts. The results are presented in table 5.2., 5.3, 5.4 and 5.5. It was observed that the microorganisms were resistant to the plant extracts. Yet, the acetone and aqueous extracts of *C. fendleri* (table 5.2) exhibited zones of inhibition against *D incerta* and *E coli*, respectively. Table 5.3 indicates that some *P. glomerata* extracts showed zones of inhibition. The acetone extracts were active against *E coli, C koseri,* and *P mirabilis*, while the DCM extracts inhibited the growth of *Janthinobacterium* spp (11± 0.7 mm), that is resistant to ciprofloxacin. It was observed that the *J. zeyheri* root extracts had no antibacterial activity (at the concentrations tested) on the 13 isolated bacteria (table 5.4). None of the *C. dimidiatus* extracts exhibited antibacterial activity against the isolated bacteria (table 5.5.)

Table 5.3. The zones of inhibition (mm) of *C. fendleri* crude extracts against selected bacteria isolated from diabetic wounds

Bacteria	Acetone extract (20 mg/ml)	Dichloromethane (20 mg/ml)	Aqueous extract (20 mg/ml)	Ciprofloxacin (20 µg/ml)	10 % DMSO
Bacillus spp (NE3)	0	0	0	27.8 ± 5.8	0
Klebsiella pneumoniae (NE2)	0	0	0	35.8 ± 6.2	0
Klebsiella pneumoniae (A1)	0	0	0	32.8 ± 5.5	0
Escherichia coli (NEE6)	0	0	0	34.5 ± 5.3	0
Corynebacterium striatum (A3)	0	0	0	22.5 ± 2.9	0
Proteus mirabilis (S3)	0	0	0	32.3 ± 8.6	0
Desemzia incerta (E1)	18.0 ± 1.7	0	0	0	0
Janthinobacterium spp (E2)	0	0	0	0	0
Enterococcus faecalis (P1)	0	0	0	0	0
Escherichia coli (NE4)	0	0	13.0 ± 0.7	31.5 ± 3.8	0
Staphylococcus aureus (A2)	0	0	0	33.9 ± 8.2	0
Enterococcus faecalis (ST1)	0	0	0	0	0
Citrobacter koseri (PP1)	0	0	0	34.3 ± 5.4	0

Note: The results are recorded as mean± standard deviation of the three replicates.

Table 5.4: The zones of inhibition (mm) of *P. glomerata* rhizome crude extracts against bacteria isolated from diabetic wounds

Bacteria	Acetone (20 mg/ml)	Dichloromethane (20 mg/ml)	Aqueous (20 mg/ml)	Ciprofloxacin (20 µg/ml)	10% DMSO
Klebsiella pneumoniae (A1)	0	0	0	32.8 ± 5.5	0
Bacillus spp (NE3)	0	0	0	27.8 ± 5.8	0
Klebsiella pneumoniae (NE2)	0	0	0	35.8 ± 6.2	0
Escherichia coli (NEE6)	20.0 ± 1.0	0	0	34.5 ± 5.3	0
Corynebacterium striatum (A3)	0	0	0	22.5 ± 2.9	0
Desemzia incerta (E1)	0	0	0	0	0
Janthinobacterium spp (E2)	0	11 ± 0.7	0	0	0
Enterococcus faecalis (P1)	0	0	0	0	0
Escherichia coli (NE4)	0	0	0	31.5 ± 3.8	0
Staphylococcus aureus (A2)	0	0	0	33.9 ± 8.2	0
Enterococcus faecalis (ST1)	0	0	0	0	0
Citrobacter koseri (PP1)	22.0 ± 0.6	0	0	34.3 ± 5.4	0
Proteus mirabilis (S3)	18.0 ± 1.2	0	0	32.3 ± 8.6	0

Note: The results are recorded as mean ± standard deviation of the three replicates.

Table 5.5: The antibacterial activity of *J. zeyheri* root crude extracts (zones of inhibition in mm) against bacteria isolated from diabetic wounds

Bacteria	Acetone (20 mg/ml)	Dichloromethane (20 mg/ml)	Aqueous (20 mg/ml)	Ciprofloxacin (20 µg/ml)	10% DMSO
Klebsiella pneumoniae (A1)	0	0	0	32.8 ± 5.5	0
Bacillus spp (NE3)	0	0	0	27.8 ± 5.8	0
Klebsiella pneumoniae (NE2)	0	0	0	35.8 ± 6.2	0
Escherichia coli (NEE6)	0	0	0	34.5 ± 5.3	0
Corynebacterium striatum (A3)	0	0	0	22.5 ± 2.9	0
Desemzia incerta (E1)	0	0	0	0	0
Janthinobacterium spp (E2)	0	0	0	0	0
Enterococcus faecalis (P1)	0	0	0	0	0
Escherichia coli (NE4)	0	0	0	31.5 ± 3.8	0
Staphylococcus aureus (A2)	0	0	0	33.9 ± 8.2	0
Enterococcus faecalis (ST1)	0	0	0	0	0
Citrobacter koseri (PP1)	0	0	0	34.3 ± 5.4	0
Proteus mirabilis (S3)	0	0	0	32.3 ± 8.6	0

Note: the results are recorded as mean ± standard deviation of the three replicates.

Table 5.6: The zones of inhibition (mm) of *C. dimidiatus crude* extracts against bacteria isolated from diabetic wounds.

Bacteria	Acetone (20 mg/ml)	Dichloromethane (20 mg/ml)	Aqueous (20 mg/ml)	Ciprofloxacin (20 µg/ml)	10% DMSO
Klebsiella pneumoniae (A1)	0	0	0	32.8 ± 5.5	0
Bacillus spp (NE3)	0	0	0	27.8 ± 5.8	0
Klebsiella pneumoniae (NE2)	0	0	0	35.8 ± 6.2	0
Escherichia coli (NEE6)	0	0	0	34.5 ± 5.3	0
Corynebacterium striatum (A3)	0	0	0	22.5 ± 2.9	0
Desemzia incerta (E1)	0	0	0	0	0
Janthinobacterium spp (E2)	0	0	0	0	0
Enterococcus faecalis (P1)	0	0	0	0	0
Escherichia coli (NE4)	0	0	0	31.5 ± 3.8	0
Staphylococcus aureus (A2)	0	0	0	33.9 ± 8.2	0
Enterococcus faecalis (ST1)	0	0	0	0	0
Citrobacter koseri (PP1)	0	0	0	34.3 ± 5.4	0
Proteus mirabilis (S3)	0	0	0	32.3 ± 8.6	0

Note: The results are recorded as mean ± standard deviation of three replicates.

5.4.1 MIC and MBC

The MIC and MBC results of the susceptible bacteria are as shown in table 5.6. The MIC values of the plant extracts ranged from 10 - 20 mg/ml while that of ciprofloxacin ranged from 0.625 - 5 µg/ml. The MBC of the plant extracts were >20 indicating that the observed inhibitory activities were more bacteriostatic than bactericidal. The MBC of ciprofloxacin ranged between 5 - >20 µg/ml.

Table 5.7: The Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) values of the plant extracts (mg/ml) and of Ciprofloxacin (µg/ml) against susceptible bacteria.

Bacteria	Plant extracts	MIC of the plant extracts	MBC of the plant extracts	MIC of ciprofloxacin	MBC of ciprofloxacin
Escherichia coli (NEE6)	*P. glomerata* (acetone)	10	>20	2.5	5
Desemzia incerta (E1)	*C. fendleri* (acetone)	20	>20	R	-
Janthinobacterium spp (E2)	*P. glomerata* (DCM)	20	>20	R	-
Escherichia coli (NE4)	*C. fendleri* (aqueous)	20	>20	5	>20
Citrobacter koseri (PP1)	*P. glomerata* (acetone)	20	>20	1.25	>20
Proteus mirabilis (S3)	*P. glomerata* (acetone)	10	>20	0.625	10

Note: R- denotes resistant at a maximum concentration of 20 µg/ml., – denotes not determined

5.5. Discussion

Medicinal plants-based treatment of various diseases has a long and extended history. However, a lot of people still have misconceptions about the use of traditional medicine in medical care (Carmona and Pereira, 2013). It is therefore necessary to scientifically evaluate traditional medicinal plants in a bid to improve their efficacy and medicinal use (Jager, 2003; Vargas *et al.,* 2014). The traditional use and the antibacterial activities of the plants in this study have been reported by a number of researchers (Zobolo and Mkabela, 2006; Kumar *et al.,* 2010; Lall and Kishore, 2014; Mongalo *et al.,* 2013). It is however apparent that some multidrug-resistant bacteria isolated from diabetic wounds of hospitalized patients were also resistant to the plant extracts in this study. The ability of bacteria to develop resistance to herbal drugs is not well documented (Vadhana *et al.,* 2015), but Khan *et al.,* (2009) observed that clinical isolates such as *E.coli* and *K. pneumoniae* were resistant to plant crude extracts. The resistance of Gram-negative bacteria may be attributed to the lipid or protein composition of their outer membrane which may also be associated with high levels of antimicrobial effluxing (Delcour, 2009; Chethana *et al.,* 2013; Zhou *et al.,* 2015). The outer membrane enables the bacteria to tolerate toxic environments better by changing their permeability to antibacterial agents (Corona and Martinez, 2013; Iredell *et al.,* 2015). Plant crude extracts may be weak antimicrobial agents because of dilutions of active components or antagonistic activities among the plant metabolites (Balouiri *et al.,* 2016). Therefore crude extracts may show better activity when used in synergy with other plant extracts or antibiotics (Tiwari *et al.,* 2015), as some plant extracts have been able to reverse penicillin-resistance when used in combination (Chethana *et al*., 2013). This notwithstanding, the antibacterial activity observed on some of the crude extracts of *C fendleri* and *P glomerata* (Tables 5.2 and 5.3) which were active against antibiotic-resistant *Escherichia coli (NE4 and NEE6), Citrobacter koseri (PP1), Proteus mirabilis (S3), Janthinobacterium spp (E2)* and *Desemzia incerta (E1)* indicates that the plants possess constituents that have potential use in antibiotic resistance studies; this also validates the use of the plants in wound healing in folklore. *Janthinobacterium spp* is non-pathogenic to humans (Tabor-Godwin *et al.,* 2009) but it can be a reservoir of antibiotic resistant genes (Valdes *et al.,* 2015),which is a cause of concern considering that some of the wounds were

polymicrobial. In such instances, antibiotic resistance genes may be transferred to the pathogenic bacteria. *Desemzia incerta* which cause foot odor (Stackebrandt *et al.,* 1999), can be eliminated through the use of medicinal plant crude extracts.

The presence of various phytochemicals may serve as an indication of biological activity, be it antioxidant, antibacterial or anti-inflammatory (Saini *et al.,* 2016), that could help curb diabetic wound infections (Alo *et al.,* 2012; Sharma *et al.,* 2013). Tannins (Saini *et al.,* 2016) and saponins (Soetan *et al.,* 2006; Karimi *et al.,* 2011) have been reported to exhibit antimicrobial activity. Their presence in the plants (Table 5.1) could have contributed to the observed antibacterial activities. It is worth noting that wound healing does not only occur through bacterial infection control, but also through other mechanisms such as free radical scavenging and antioxidants activities. Research has shown that flavonoids and saponins (Saini *et al.,* 2016) can help regenerate lost tissue and induce healing (Thakur *et al.,* 2011).This may account for the folklore use of the studied medicinal plants in wound healing despite their limited antibacterial activities *in-vitro*.

The isolation and characterization of active components of crude extracts could lead to the discovery of potent drugs against antibiotic resistant microorganisms.

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CHAPTER SIX

6.1. General Discussion

The presence of bacteria in diabetic wounds along with clinical symptoms such as erythema, pain, tenderness and pus (Bessa *et al.,* 2013) may play a role in delayed wound healing (Percival *et al.,* 2010; Benwan *et al.,* 2012; Bessa *et al.,* 2013; Akhi *et al.,* 2015). In this study there was a high prevalence of bacteria in diabetic patients' wounds. Bacteria was recovered in 83% of the sampled patients. This high prevalence is a cause for concern as bacteria can produce toxins that attack the immune system and cause necrosis and sepsis (Jones, 2004), which ultimately delay wound healing. The negative effects of delayed wound healing are prolonged infection control therapy and hospital stays that may culminate in limb amputations (Siddique and Bernstein, 2010; Roberts and Simon, 2012). 94% of the wounds in this study were located in the lower limbs.

The API and 16S rDNA analysis results matched 24 % of the time. Molecular based techniques such as the 16S rDNA have highly conserved and variable regions that enable them to identify bacteria efficiently and accurately (Woo *et al.,* 2008; Srinivasan *et al.,* 2015) but can be relatively complex, costly (Marlowe and Bankowski, 2011) and inaccessible in resource limited settings (Britton *et al.,* 2016). In resource limited clinical settings such as the rural hospitals under study, expertise in diagnostics is inadequate. This may contribute to reduced improvement in patients' conditions and control of bacterial infections (Caliendo *et al.,* 2013). In South Africa, a number of experienced health care workers migrate from the public to the private sector. This has adversely affected national healthcare since only 15% of the country's population can afford private healthcare services (Ashmore, 2013). Lack of expertise may compromise correct identification of bacteria as well as treatment of bacteria infected wounds. The misidentification of Gram-positive and Gram-negative bacteria may lead to incorrect antibiotic use and contribute to antibiotic resistance (Iredell *et al.,* 2015; Shahi and Kumar, 2016).

Antibiotic resistance was noted in bacteria across all the three hospitals with the highest resistance being recorded against penicillin (100%), ampicillin (91%)) which are first line

of treatment (El-Sokkary *et al.,* 2015; Laxminarayan *et al.,* 2016). This limits treatment options as second line treatment drugs also have become insensitive to bacteria as observed in this study. Multidrug resistant patterns of 83% were recorded for Enterococcus group (*Enterococcus faecalis, Corynebacterium striatum and Desemzia incerta*) and 55% for Enterobacteriaceae *(Proteus mirabilis, Klebsiella spp, Escherichia coli)* which was the most predominant. The prevalence of *Enterobacteriaceae spp* in the study may pose a public health crisis if not controlled. These bacteria are commonly associated with a number of infections and resistance worldwide (Wellington *et al.,* 2013; Thenmozhi *et al.,* 2014; Shaikh *et al.,* 2015; Adesoji *et al.,* 2016).The observed resistance to antibiotic treatment can significantly impact infections that normally do not require hospitalization, such as enteric and urinary tract infections (Guentzel *et al.,* 1996). Resistance to antibiotics by skin commensals such as *Staphylococcus epidermidis* and *Corynebacterium striatum* as observed in this study poses selection pressure on antibiotic usage (Shahi and Kumar, 2016).

The crisis of antibiotic resistance has made it necessary for researchers to scientifically validate traditional medicinal plants as an alternative (Ahmad and Wajid, 2013; de Wet *et al.,* 2013; Budovsky *et al.,* 2015) especially in rural areas where plant-based medication are affordable and readily available at local level (Tabassum and Hamdani, 2014). Despite being widely documented as having medicinal properties against skin related infections (Zobolo and Mkabela, 2006; Kumar *et al.,* 2010; Lall and Kishore, 2014; Mongalo *et al.,* 2013) the studied plants (*Chamaesyce fendleri, Platycarpha glomerata, Carpobrotus dimidiatus* and *Jatropha zeyheri*), exhibited limited antibacterial activities against the bacteria isolates. This can be attributed to the fact that some crude plant extracts contain metabolites that work antagonistically to dilute and suppress antibacterial potentials (Abubakar, 2009; Balouiri *et al.,* 2016). It is therefore essential to isolate and characterize active components in the crude extracts that exhibit antibacterial activities. It is also worth noting that wound healing is a combination of physiological processes such as tissue regeneration that can be induced by saponins and flavonoids (Saini *et al.,* 2016; Thakur *et al.,* 2011). Therefore the presence of phytochemicals such as saponins and flavonoids in plants that have been studied can be an indication of the importance of these plants in wound healing.

6.2. Conclusion

The recovery of bacteria in diabetic wounds is a major threat to the wound healing process and public health in general. This is further compounded by resistance patterns observed in current first-line antibiotic treatment. The prevalence of drug resistant bacteria in wounds calls for alternative medicinal approaches to eliminate bacteria and improve public health.

6.3. Limitations of the Study

- Few patients were obtained for sampling
- Surface swabbing was used and that only provided bacteria at the surface of the wounds
- Two selective and differential media were used for isolation in the study.

6.4. Future works

- To determine the potential of bacteria species to form biofilms.
- To determine the presence of antibiotic resistance genes responsible for the observed resistance and virulence genes that may facilitate infection in the studied bacteria.
- Isolation and characterization of bioactive compounds in the plants.

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APPENDIX

APPENDIX A (Reagent preparation)

A1. Mueller-Hinton broth

A 23 g of broth powder was dissolved in 1 litre of distilled water. The prepared broth was sterilized for 15 minutes using autoclave through high pressure saturated steam at 121°C.

A2. Mueller-Hinton agar

A 38 g of agar powder was suspended in 1 litre distilled water and allowed to stand for 15 minutes. The prepared agar was sterilized using autoclave for 15 minutes at 121°C. The agar was allowed to cool and then aseptically poured onto the petri plates. About 10 ml of agar was poured on each plate. The plates were allowed to solidify on the laminar flow cabinet and stored in the fridge.

A3. Nutrient broth

A 16 g of broth powder was suspended in 1 litre of distilled water, mixed well and dispensed into final vials. The prepared broth was sterilized using autoclave for 15 minutes through high pressure saturated steam at 121°C.

A4. Nutrient agar

A 31 g of agar powder was mixed with 1 litre of distilled water. The prepared agar was sterilized using autoclave for 15 minutes at 121°C. The agar was allowed to cool to 45-50°C and then aseptically poured onto the petri plates. About 10ml of agar was poured on each plate. The plates were allowed to solidify on the laminar flow cabinet and stored in the fridge.

A5. MacConkey agar (without crystal violet)

A 50 g of agar powder was weighed and mixed with 1 litre of distilled water. The mixture was boiled and stirred until completely dissolved. The prepared agar was autoclaved at 121°C for 15 minutes. The agar was allowed to cool to about 45- 50°C before it was poured onto petri dishes aseptically. About 10 ml of agar was poured on each plate. The plates were allowed to solidify on the laminar flow cabinet and stored at 4° C refrigerator.

A6. Mannitol salt agar 120 g of agar powder was suspended in 1 litre of distilled water. The mixture was boiled and stirred until dissolved completely. The prepared agar was sterilized using autoclave for 15 minutes at 121°C. The agar was cooled to about 45-50 °C and poured onto petri dishes which were allowed to solidify and kept in the fridge at 4°C.

A7. Gram-stain reagents

(a) Crystal violet

About 5 g of crystal violet powder was added to 50 ml of 95 % ethanol to make solution A. 2 g of ammonium oxalate was added to 200 ml of distilled water to make solution B. Solution A was mixed with solution B to obtain a crystal violet staining reagent in a total volume of 250 ml.

(b) Gram's Iodine

0.83 g of iodine and 1.65 g of potassium iodide were added to 250 ml of sterile distilled water and vortexed till iodine was dissolved.

(c) Decolorizing agent

A volume of 125 ml of 95 % ethanol was mixed with 125 ml of acetone to obtain a ratio of 1:1 ethanol-acetone mixture.

(d) Counterstain

0.63 g of safranin was added to 25 ml of ethanol and mixed with 225 ml of distilled water to make a mixture of 250 ml.

A8. 3 % Hydrogen peroxide (H_2O_2)

A volume of 1.5 ml of H_2O_2 was added to 48.5 ml of sterile distilled water to make a total volume of 50 ml.

A9. 1% tetra-methyl-p-phenylenediamine dihydrochloride (Kovács oxidase reagent)

0.8 g of Kovács oxidase reagent was added to 80 ml of sterile distilled water and refrigerated in a dark bottle.

A10. Ciprofloxacin

0.02 g of the antibiotic was mixed with 1ml sterile distilled water and refrigerated at 4°C.

A11. 10% DMSO

A volume 100 µl of DMSO was mixed with 900 µl of distilled water to make a volume of 1 ml.

A12. 0.85 % Saline

0.43 g of sodium chloride (NaCl) was mixed with 50 ml of distilled water.

APPENDIX B (detailed method)

B1. Isolation

Swab specimen collected from patients were inoculated onto different agar medium such as MacConkey agar and Mannitol salt agar using a spread plate method to obtain bacterial growth. Different quadrants were picked and a 4-way streak method was performed to obtain pure colonies and the inoculating loop was sterilized on flame and allowed to cool thoroughly before streaking each quadrant on the agar plate. The cooled loop was passed back and forth through the inoculum in the first quadrant several times to ensure bacterial growth. The first quadrant was at least one quarter of the plate and lines were close together. The plate was rotated and quadrant two was streaked by passing the sterile loop through the edge of quadrant one and streaking the rest of the area. The same procedure was repeated till quadrant four was streaked. The plates were incubated for 24-48 hours overnight at 37°C.

B2. Gram Stain

Gram stain was done according to Gram (1884) with some modifications. Four Gram staining reagents were used namely crystal violet as a primary stain; Gram's iodine as a mordant stain; decolorizing agent 1:1 ethanol-acetone mixture and safranin as a counter stain. The smear was prepared in a glass slide from an 18- 24 hour old pure culture. The smear was covered with a few drops of crystal violet for about one minute then it was washed off with water. The smear was immediately treated with few drops of Gram's iodine and allowed to act for about a minute and it was rinsed off with water. The ethanol-acetone mixture was added and quickly washed off with water in less than 30 seconds because prolonged or under-decolorization could mislead in identifying the Gram-reaction. A few drops of safranin were added on the slide and after a minute washed off with water. Excess water was removed using tissue paper, air-dried and heat-fixed before observations were done on a microscope. The lower objectives (x4, x10, x40) were used to project the well-stained field and oil immersion objective was used to view the slide saturated with a small drop of immersion oil. Figure C1, C2, C3, shows the images that

were taken at (x100) oil immersion objective. The Gram reaction and morphology of the bacteria was observed and recorded. The Gram reaction was confirmed using 3 % potassium hydroxide (KOH) and KOH was emulsified onto the slide and a loopful of the bacterial culture that is 18- 24 hours old was transferred onto the slide. The suspension was mixed slowly lifting up the loop to observe for any string formation as a release of DNA.

B3. Catalase test

A well-isolated 18-24 hour old culture from nutrient agar was prepared. A sterile microscope slide was placed in the petri dish to limit catalase aerosols. A sterile wooden toothpick was used to collect a small amount of the organism from the bacterial culture and placed onto the microscope slide. Sterile pipette tips were used to pipette 1 drop of 3 % hydrogen peroxide (H_2O_2) onto the microscope slide without mixing. The petri dish was covered with a lid to limit aerosols and bubble formation was observed. A positive reaction was recorded for immediate bubble formation and no bubble formation was recorded as a negative reaction for catalase test.

B4. Oxidase test

A small piece of filter paper was soaked in 1 % Kovács oxidase reagent and allowed to dry. A sterile wooden toothpick was used to pick a well- isolated colony from a18-24 hour old bacterial culture grown on nutrient agar and rubbed onto the treated filter paper. Color changes were observed. A positive reaction was noted when color changed to dark purple within 5-10 seconds. A delayed oxidase positive reaction was recorded when color changed to purple within 60- 90 seconds which was also considered as positive. The negative reaction was noted when color did not change or took longer than 2 minutes to turn purple.

B5. ANALYTICAL PROFILE INDEX

API 20 NE™

API 20 NE was used for the identification of non-fastidious Gram-negative rods which do not belong to the *Enterobacteriaceae* family. The incubation box (tray and lid) was prepared. About 5 ml of sterile distilled water was distributed in the bottom of the tray to create a humid atmosphere. The specimen number was recorded on the elongated flap of the tray and the strip was removed from its individual packaging and placed in the incubation box. The inoculum was prepared by transferring 1-4 colonies (18-24 hour old) from a well isolated agar plate into 0.85 % physiological saline without additives. The suspension was prepared to match 0.5 McFarland and was immediately used after preparation. The bacterial suspension was inoculated onto the strip from test NO_3 to PNPG using the same sterile pipette and formation of bubbles at the base of the tubes was avoided. The API AUX medium was mixed with 200 µl of the saline bacterial suspension and the mixture was filled into the tubes and cupules of the test [GLU] to [PAC]. The mineral oil was added to the underlined tests (GLU, ADH, and URE) to form a convex meniscus. The incubation box was closed and incubated at 29° C for 24 hours. After the incubation the strip was read. All spontaneous reactions were recorded (GLU, ADH, URE, ESC, GEL and PNPG) based on the table B1. The assimilation test were covered to protect against airborne contamination while reading the NO_3 and TRP test. For the NO_3 test; one drop of NIT 1 and one drop of NIT 2 reagents were added to the NO_3 cupule. The reaction was recorded after five minutes. A red color indicated a positive reaction while colorless indicated negative results. To validate the reduction of the nitrates to nitrite and N_2 gas to nitrogen, about 2-3 mg of Zinc dust was added and a red color indicated negative results for the nitrate reduction after five minutes. For the TRP test; one drop of James reagent was added and immediate changes were observed. A pink color indicated a positive reaction. The bacterial growth was observed for the assimilation tests. An opaque cupule was recorded as positive and a transparent cupule was recorded as negative for the reaction. The scores were calculated and API web kit V8.0 was used to identify the isolates using a 7-digit profile obtained from score calculation of positive tests in the different groups. When identification gave low

discrimination, unacceptable or doubtful profile for another 24 hours at 29°C, reincubation was done. Before the incubation, the NO_3 and TRP test cupule were cleared by removing reagents and covering the cupule with mineral oil.

Table B1: The interpretation of results for API 20 NE

Test	Reaction	
	Negative	**Positive**
NO_3	Colorless	Pink-red, colorless (Zinc)
TRP	Colorless/pale green/yellow	Pink
GLU	Blue-green	Yellow
ADH	Yellow	Orange/pink/red
URE	Yellow	Orange/pink/red
ESC	Yellow	Grey/brown/black
GEL	No pigment diffusion	Diffusion and black pigment
PNPG	Colorless	Yellow
GLU	Transparent	Opaque
ARA	Transparent	Opaque
MNE	Transparent	Opaque
MAN	Transparent	Opaque
NAG	Transparent	Opaque
MAL	Transparent	Opaque
GNT	Transparent	Opaque
CAP	Transparent	Opaque
ADI	Transparent	Opaque
MLT	Transparent	Opaque
CIT	Transparent	Opaque
PAC	Transparent	Opaque

API 20 E™

The bacteria was first isolated and identified to belong to the culture of *Enterobacteriaceae* or non- fastidious Gram-negative rods. The strip was set by preparing the incubation box (tray and lid) and distributing about 5 ml of sterile distilled water. The strain was labelled onto the tray. The strip was removed from its packaging and placed in the incubation box. The inoculum was prepared by transferring a single well isolated colony (18-24 hour old) into 5 ml sterile saline. The bacterial suspension was emulsified to achieve a homogenous mixture. The suspension was immediately used after preparation. The strip was inoculated with the bacterial suspension. The strip was tilted to prevent the formation of bubbles. For the tests [CIT], [VP] and [GEL] the tube and cupule were filled. The tests ADH, LDC, ODC, H_2S and URE were over laid with mineral oil to create anaerobiosis after adding the bacterial suspension and incubated for 18-24 hours at 36°C. After the incubation period, the strip was read according to the table B2. If three or more tests were positive, all reactions were recorded on the result sheet, however, if the number of positive tests were less than three, the strip was reincubated for another 24 hours before the additional reagents were added. For the TDA test one drop of TDA reagent was added. A positive reaction was indicated by a reddish-brown color. For the VP test, one drop of VP 1 and VP2 reagents was added. A red or pink color indicated a positive reaction before 10 minutes. If the color appeared slightly pink after 10 minutes, the reaction was considered negative. A drop of James reagent was added to the IND test and a pink color indicated a positive test. The table below shows how some reactions were recorded. The identification was obtained with the numerical profile by calculating the scores in the result sheet and adding the values corresponding to positive reactions within each group. A seven-digit profile number was obtained for 20 tests on API and oxidase test which was performed exclusively. The numerical profile was searched using APIweb™ identification software V 5.0.

Table B2: The interpretation of results for API 20 E

Test	Reaction	
	Negative	**Positive**
ONPG	Colorless	Yellow
ADH	Yellow	Red-orange
LDC	Yellow	Red-orange
ODC	Yellow	Red-orange
CIT	Pale green-yellow	Blue-green-blue
H_2S	Colorless- greyish	Black deposit
URE	Yellow	Red-orange
TDA	Yellow	Reddish-brown
IND	Colorless-pale yellow green	Pink
VP	Colorless-pale pink	Pink-red
GEL	No diffusion	Diffusion of a black pigment
GLU	Blue/ blue-green	Yellow
MAN	Blue/ blue-green	Yellow
INO	Blue/ blue-green	Yellow
SOR	Blue/ blue-green	Yellow
RHA	Blue/ blue-green	Yellow
SAC	Blue/ blue-green	Yellow
MEL	Blue/ blue-green	Yellow
AMY	Blue/ blue-green	Yellow
ARA	Blue/ blue-green	Yellow

API STAPH

The incubation box (tray and lid) was prepared. About 5ml of sterile distilled water was distributed into the wells of the tray to create a humid atmosphere. The strain was recorded on the elongated flap of the tray. The strip was removed from the packaging and placed into the incubation box. An isolate from Mannitol salt agar that was identified to be catalase positive was subcultured on nutrient agar. A culture of 18-24 hour old was

used to prepare a bacterial suspension with API staph medium. The homogenous bacterial suspension was prepared to match 0.5 McFarland and used immediately after preparation. The microtubes were slightly under-filled with bacterial suspension. ADH and URE microtubes were overlaid with mineral oil to ensure anaerobiosis. The incubation box was closed and incubated at 36°C for 18-24 hours. The VP, NIT, and PAL tests were added to specific reagents to develop the reactions after incubation. A drop of VP 1 and VP 2 reagents were added on the VP test. The reaction was monitored for about 10 minutes and a violet-pink color indicated a positive reaction while pale pink or light pink indicated a negative reaction. A drop of NIT 1 and NIT 2 reagents were added on the NIT test microtube and the reaction was monitored for 10 minutes. A positive reaction was indicated by a red color and a negative reaction by a colorless- light pink color. ZYM A and ZYM B reagents were added for the PAL test and monitored for 10 minutes. Yellow indicated a negative reaction while violet indicated a positive reaction. The remaining reactions were interpreted through the Table B3. The identification was obtained with the numerical profile on the API web identification software (V5.0).

Table B3: The reading of results for API 20 STAPH

Test	Reaction	
	Positive	**Negative**
0	_	Red
GLU	Yellow	Red
FRU	Yellow	Red
MNE	Yellow	Red
MAL	Yellow	Red
LAC	Yellow	Red
TRE	Yellow	Red
MAN	Yellow	Red
XLT	Yellow	Red
MEL	Yellow	Red
NIT	Red	Colorless-light pink
PAL	Violet	Yellow

VP	Violet-pink	Colorless-light pink
RAF	Yellow	Red
XYL	Yellow	Red
SAC	Yellow	Red
MDG	Yellow	Red
NAG	Orange-red	Yellow
ADH	Red-violet	Yellow

API STREP

The incubation box and strip was prepared as previously described. The inoculum was prepared from a 24 hour old culture that was identified to be a cocci, Gram-positive, catalase-negative. The inoculum was suspended into 5mls of sterile distilled water. The suspension with a turbidity greater than 4 McFarland standard was prepared and used immediately. 100 µl of the suspension was distributed from VP- LAP tests. For the ADH test, the tube was only filled. About 0.5 ml of the suspension was mixed with API strep medium and the mixture was distributed to the tests RIB-GLYG. Mineral oil was overlaid on the ADH to GLYG tests. The lid was replaced and incubated at 36° C for 4- $4^{1/2}$ hours to obtain the first reading and the second reading was obtained after 24 hours. Certain tests required specific reagents to develop reactions. A drop of VP1 and VP2 was added in the VP test. Two drops of 2% ninhydrin were added in the HIP test while ZYM A and ZYM B were added in the tests PYRA, βGUR, αGAL, βGAL, PAL and LAB. All reactions were read after 10 minutes. Reincubation was necessary if the API web gave results such as low discrimination, unacceptable profile, doubtful profile, invalid identification. After a 24 hour incubation the ESC, ADH and RIB to GLYG tests were read. The tests were interpreted according to the table below (Table B4). The scores were calculated and the numerical profile was loaded on the API web software V8.0 for identification.

Table B4: The reading of results for API 20 STREP

Test	Reaction	
	Positive	**Negative**
VP	Pink/ Red	Colorless
HIP	Dark blue/ violet	Colorless
ESC	**4 hours:** Grey/Black	Colorless
	24 hours: Black	Pale grey
PYRA	Orange	Colorless/ pale orange
αGAL	Violet	Colorless
βGUR	Blue	Colorless
βGAL	Violet	Colorless
PAL	Violet	Colorless
LAP	Violet	Colorless/ pale violet
ADH	Red	Yellow
RIB	**4 hours:** Orange/ yellow	Red
	24 hours: Yellow	Red/ orange
ARA	**4 hours:** Orange/ yellow	Red
	24 hours: Yellow	Red/ orange
MAN	**4 hours:** Orange/ yellow	Red
	24 hours: Yellow	Red/ orange
SOR	**4 hours:** Orange/ yellow	Red
	24 hours: Yellow	Red/ orange
LAC	**4 hours:** Orange/ yellow	Red
	24 hours: Yellow	Red/ orange
TRE	**4 hours:** Orange/ yellow	Red
	24 hours: Yellow	Red/ orange
INU	**4 hours:** Orange/ yellow	Red
	24 hours: Yellow	Red/ orange

B6. 16S Gene Sequencing

The DNA was obtained from the cultures using Zymo Research fungal/bacterial DNA kit™. The 16S target region was amplified using DreamTaq™ DNA polymerase and the primers shown in table B5. The PCR products were gel extracted using Zymoclean™ gel DNA recovery kit and sequenced in the forward and reverse directions on the ABI PRISM™ 3500xl Genetic analyser. The sequence products were purified using Zymo Research-96 DNA sequencing clean-up kit and analysed using CLC Main Workbench 7 followed by a BLAST search (NCBI).

Table B5: 16S Primers sequences

Name of Primers	Target	Sequence (5' to 3')
16S-27F	16S rDNA sequence	AGAGTTTGATCMTGGCTCAG
16S-1492R	16S rDNA sequence	CGGTTACCTTGTTACGACTT

B7. Phytochemical screening

Test for alkaloids: 0.5 g of plant extract was mixed with 5 ml of 1% Hydrochloric acid. The mixture was stirred on the steam bath and filtered; 1 ml of filtrate was mixed with Mayer's reagent and another 1 ml of the filtrate was mixed with Dragendorff's reagent and the turbidity or precipitation indicated the presence of alkaloids.

Test for tannins: 5 g of plant extract was mixed with 10 ml of water and the mixture was stirred and filtered. 2 ml of filtrate was mixed with 0.1 %$Fecl_3$ solution (few drops).Blue-black, green or blue-green precipitate (precipitate) was an indication of the presence of tannins.

Test for saponins: 0.5 g of plant powder was boiled with10 ml of water and filtered. The filtrate was allowed to cool and shaken vigorously. The mixture was allowed to stand 15-20 minutes. The froth indicated the presence of saponins.

Test for terpenoids: 0.5 g of plant material was mixed with 2 ml of chloroform, and 3 ml of concentrated sulfuric acid was carefully added to form a layer. A reddish-brown coloration of the interface was taken as evidence of the presence of terpenoids.

Test for flavonoids: Ferric Chloride test: 1 g of extract was mixed with 10 % of ferric chloride. A dark brown or dirty brown precipitate indicated the presence of flavonoids.

APPENDIX C (Ethics)

FORM OF CONSENT OF PATIENTS WITH DIABETIC WOUNDS

Researchers

Wendy Mthembu, Dr D. Penduka, Prof A.M. Zobolo and Prof A.R Opoku

Institution

University of Zululand

Department

- Department of Biochemistry and Microbiology
- Department of Botany

Research Project

This survey is interested in finding hospitalized patients with diabetic wounds from which wound specimen would be collected at Ngwelezane hospital and further studies would be carried out on the microorganisms recovered. This study is for academic purposes.

Please take note of the following:

You are under no obligation to be sampled if you do not feel comfortable in participating in the study. Interviewees' identity will not be revealed to the public.

I hereby confirm that I understand the content of this document and the nature of research project and I voluntarily agree to participate in the above mentioned project.

Signature of interviewee **Date**

…… ……..

IFOMU LOKUCELA IMVUME YOKUCWANINGA IZIGULI EZINEZILONDA ZASHUKELA

Umcwaningi

[1,2]Wendy Mthembu, [1]uDokotela D. Penduka, [2]uProfesa A.M. Zobolo, uProfesa A.R. Opoku

Isikhungo Semfundo

INyuvesi yakwaZulu

Umnyango

- [1]Umnyango wakwa Biochemistry ne Microbiology
- [2]Umnyango weZezitshalo

Ucwaningo

Lolu cwaningo luhlose ukuthola iziguli ezinezilonda zashukela esibhedlela saseNgwelezane. Ezilondeni sidinga ukuthola amagciwane abangela lezilonda ukuthi zingapholi. Lolu Cwaningo luyingxenye yezemfundo.

Qaphela lokhu okulandelayo:

Awuphoqelekile ukuba ingxenye yalolu cwaningo uma ungakhululekile ngako. Obambe iqhaza kulolucwaningo akasoze adalulwe empakathini.

Ngiyaqinisekisa ukuthi ngiyakuqonda okuqukethweyileli pheshana kanye nohlelo lwalolucwaningo ngalokho ngiyavuma ukuba yingxenye yalo.

Igama lobambe iqhaza **Usuku**

……. **……**

UNIVERSITY OF ZULULAND
RESEARCH ETHICS COMMITTEE
(Reg No: UZREC 171110-030)

RESEARCH & INNOVATION
Website: http://www.unizulu.ac.za
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ETHICAL CLEARANCE CERTIFICATE

Certificate Number	**UZREC 171110-030 PGM 2015/195**							
Project Title	**Isolation and characterization of bacteria recovered from wounds of hospitalized diabetic patients in Northern KwaZulu Natal**							
Principal Researcher/ Investigator	W Mthembu							
Supervisor and Co-supervisor	Prof AR Opoku		Prof AM Zobolo & Dr. D Penduka					
Department	Biochemistry & Microbiology							
Nature of Project	Honours/4th Year		Master's	x	Doctoral		Departmental	

The University of Zululand's Research Ethics Committee (UZREC) hereby gives ethical approval in respect of the undertakings contained in the above-mentioned project proposal and the documents listed on page 2 of this Certificate.

Special conditions: (1) The Principal Researcher must report to the UZREC in the prescribed format, where applicable, annually and at the end of the project, in respect of ethical compliance.
(2) Documents marked "To be submitted" (see page 2) must be presented for ethical clearance before any data collection can commence.

The Researcher may therefore commence with the research as from the date of this Certificate, using the reference number indicated above, but may not conduct any data collection using research instruments that are yet to be approved.

Please note that the UZREC must be informed immediately of

- Any material change in the conditions or undertakings mentioned in the documents that were presented to the UZREC
- Any material breaches of ethical undertakings or events that impact upon the ethical conduct of the research

Classification:

Data collection	Animals	Human Health	Children	Vulnerable pp.	Other
X		X			

Low Risk	Medium Risk	High Risk
		X

The table below indicates which documents the UZREC considered in granting this Certificate and which documents, if any, still require ethical clearance. (Please note that this is not a closed list and should new instruments be developed, these would require approval.)

Documents	Considered	To be submitted	Not required
Faculty Research Ethics Committee recommendation	X		
Animal Research Ethics Committee recommendation			X
Health Research Ethics Committee recommendation	X		
Ethical clearance application form	X		
Project registration proposal	X		
Informed consent from participants			X
Informed consent from parent/guardian			X
Permission for access to sites/information/participants	X		
Permission to use documents/copyright clearance			X
Data collection/survey instrument/questionnaire			X
Data collection instrument in appropriate language		Only if necessary	
Other data collection instruments		Only if used	

The UZREC retains the right to

- Withdraw or amend this Certificate if
  - Any unethical principles or practices are revealed or suspected
  - Relevant information has been withheld or misrepresented
  - Regulatory changes of whatsoever nature so require
  - The conditions contained in this Certificate have not been adhered to
- Request access to any information or data at any time during the course or after completion of the project

The UZREC wishes the researcher well in conducting the research.

Professor Nokuthula Kunene
Chairperson: University Research Ethics Committee
12 November 2015

CHAIRPERSON
UNIVERSITY OF ZULULAND RESEARCH ETHICS COMMITTEE (UZREC)
REG NO: UZREC 171110-30
12 -11- 2015
RESEARCH & INNOVATION OFFICE

APPENDIX D (Images)

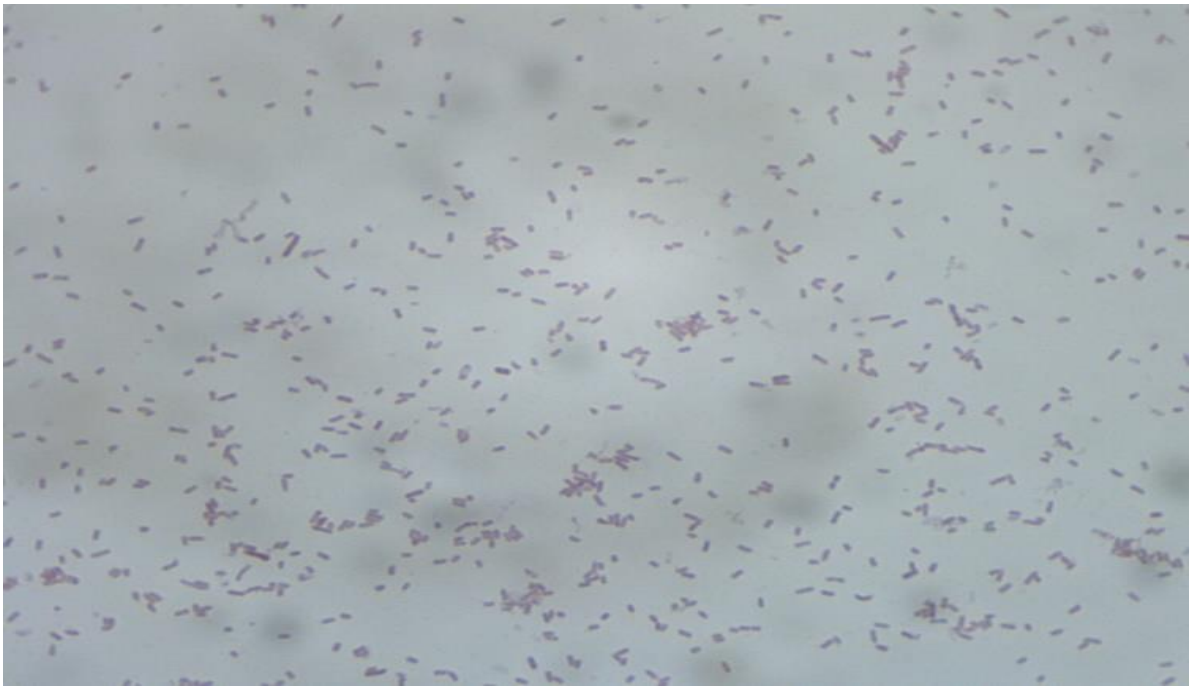

Figure D1: An image of a Gram-negative bacilli (*Proteus mirabilis)*

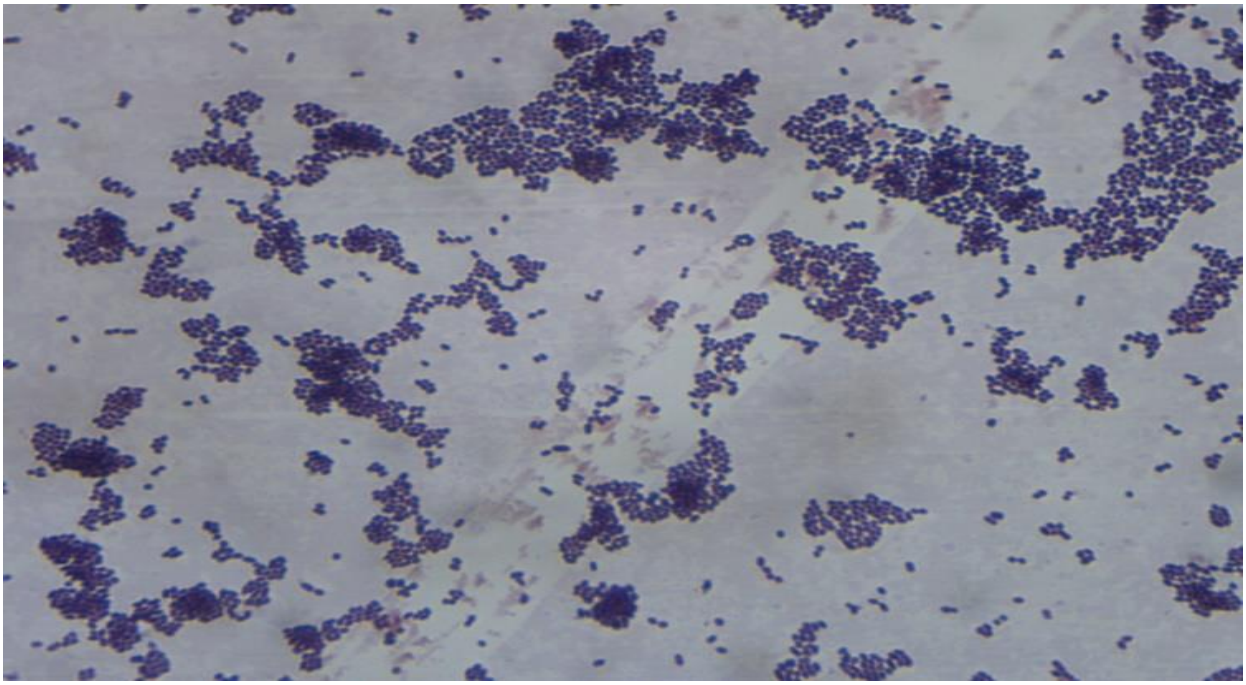

Figure D2: An image of a Gram-positive cocci (*Staphylococcus aureus)*

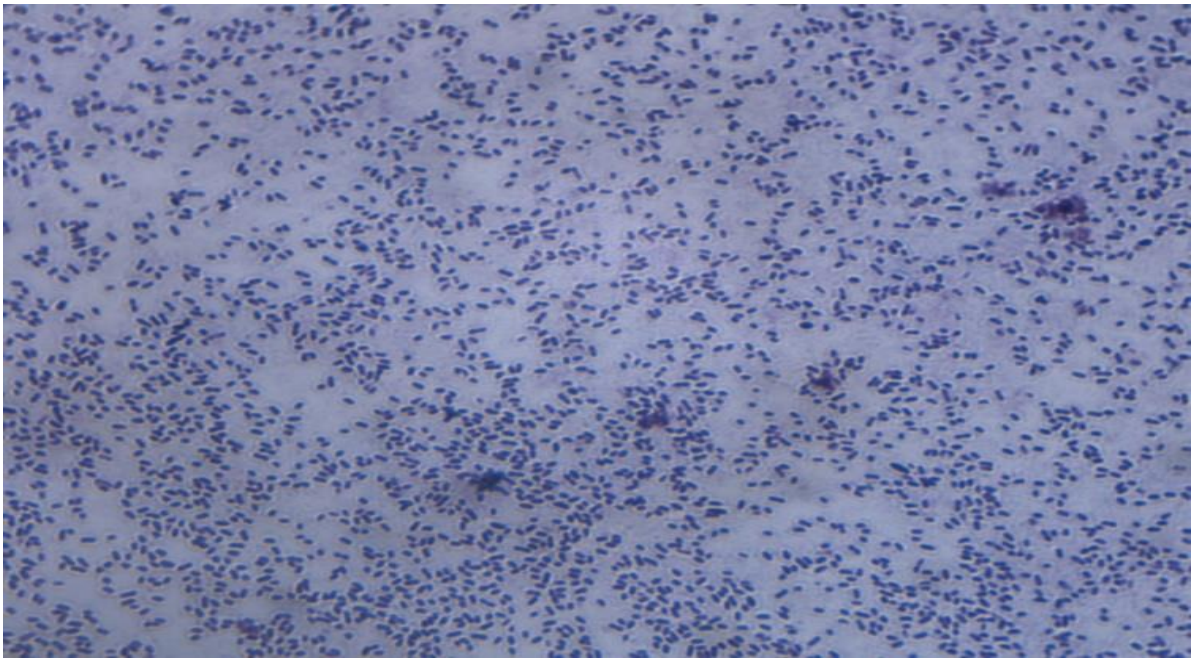

Figure D3: An image of a Gram-positive bacilli (*Bacillus pumilus)*

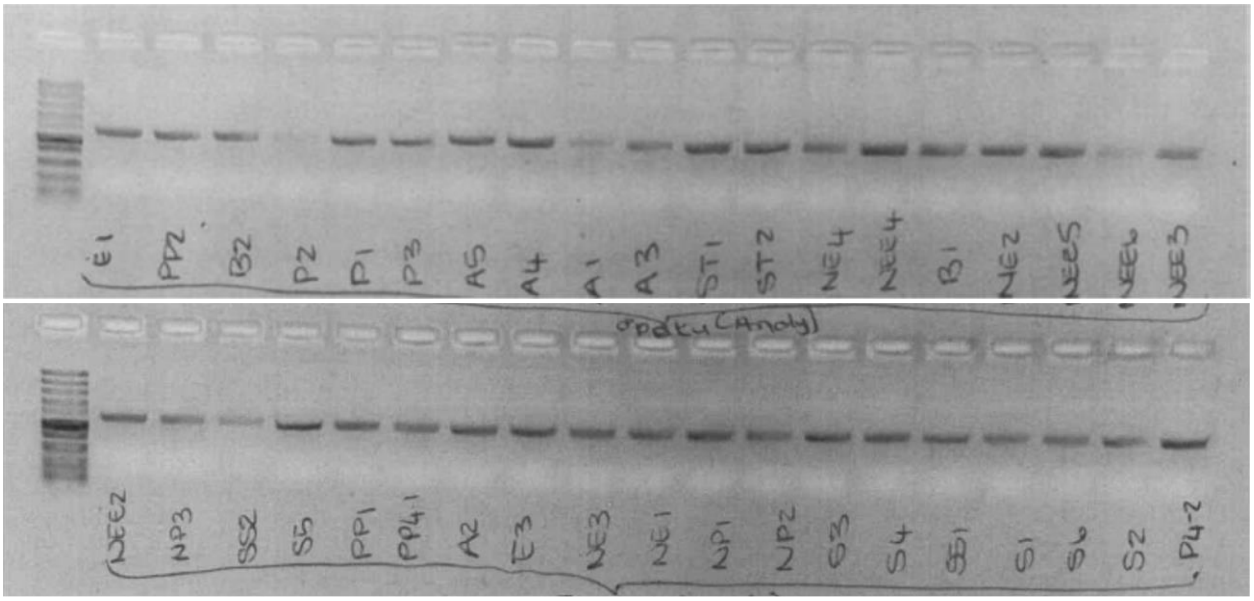

Figure D4: A photographic image of an agarose gel indicating the amplification of the 16S target region

APPENDIX E (Antibiotic susceptibility interpretive standards)

Table E.1: Antibiotic specific disk content and interpretive standards against the different bacterial species

Bacteria Group/ Antibiotics	Disk Content	Zone Diameter		
		S	I	R
Enterobacteriaceae				
Ampicillin	10 µg	***≥ 17***	***14-16***	***≤ 13***
Gentamicin	10 µg	***≥ 15***	***13-14***	***≤ 12***
Ciprofloxacin	5 µg	***≥21***	***16-20***	***≤15***
Imipenem	10 µg	***≥ 23***	***20-22***	***≤19***
Ceftazidime	30 µg	***≥21***	***18-20***	***≤17***
Cefepime	30 µg	***≥ 25***	***19-24***	***≤18***
Non-Enterobacteriaceae and Gram-positive rods				
Gentamicin	10 µg	***≥15***	***13-14***	***≤12***
Ciprofloxacin	5 µg	***≥21***	***16-20***	***≤13***
Ceftazidime	30 µg	***≥18***	***15-17***	***≤14***
Cefepime	30 µg	***≥18***	***15-17***	***≤14***
Imipenem	10 µg	***≥19***	***16-18***	***≤15***
Staphylococci				
Penicillin	10 units	***≥ 29***	***-***	***≤28***
Vancomycin	30 µg	***≥ 17***	***15-16***	***≤14***
Erythromycin	15 µg	***≥23***	***14 -22***	***≤13***
Gentamicin	10 µg	***≥ 15***	***13-14***	***≤12***
Ciprofloxacin	5 µg	***≥21***	***16-20***	***≤15***
Enterococci				
Penicillin	10 units	***≥15***	***-***	***≤14***
Ampicillin	10 µg	***≥17***	***-***	***≤16***
Vancomycin	30 µg	***≥17***	***15-16***	***≤14***
Erythromycin	15 µg	***≥23***	***14 -22***	***≤13***
Ciprofloxacin	5 µg	***≥21***	***16-20***	***≤15***

APPENDIX F (The map of the hospitals studied)

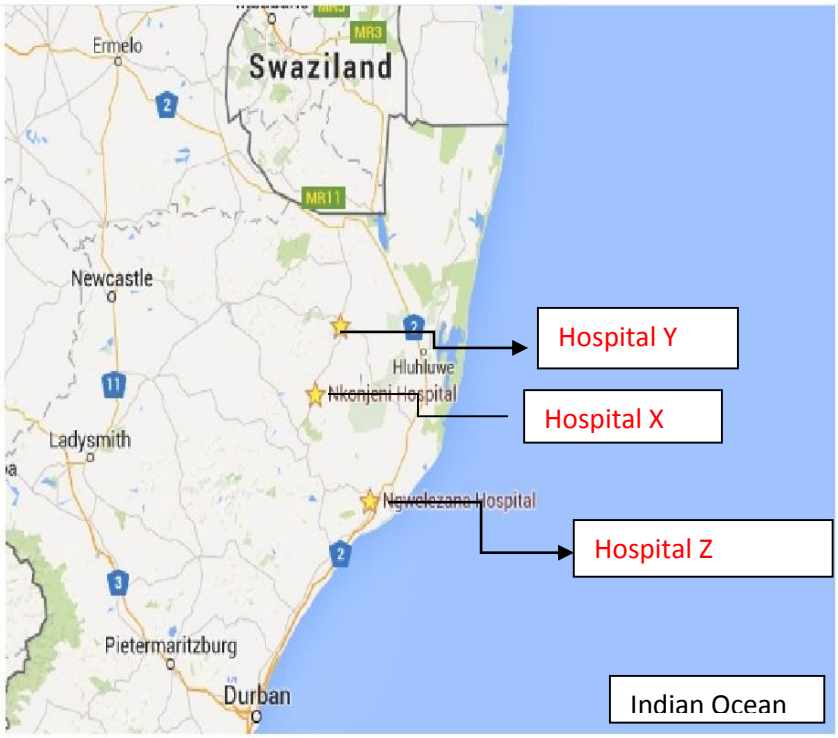

Figure F1: A Map showing the study area. Keys:*(Pictures obtained from Google Earth, 2015)