ISOLATION, SENSITIVITY PATTERNS AND MOLECULAR CHARACTERISATION OF BACTERIAL ISOLATES FROM INFECTED DIABETIC FOOT ULCERS IN PATIENTS AT KENYATTA NATIONAL HOSPITAL.

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DECLARATION

I declare that this is my original work and has not, to the best of my knowledge, been presented anywhere else.

This thesis is submitted in partial fulfillment for the award of Master of Science Degree in Tropical and Infectious Diseases at the University of Nairobi.

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DEDICATION

To Hellen and Richard, my parents, Jack and Peninah, my siblings, and Jane, my best friend.

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ABBREVIATIONS

ABI – Ankle-Brachial Index

ALT – Alanine Aminotransferase

AST – Aspartate Aminotransferase

BMI – Body Mass Index

CFU/g – Colony Forming Units Per Gram

CLED – Cysteine-, Lactose- And Electrolyte-Deficient

CLSI – Clinical and Laboratory Standards Institute

CONS – Coagulase-Negative Staphylococci

D. Bil – Direct Bilirubin

DFI – Diabetic Foot Infection

DFU – Diabetic Foot Ulcer

DM – Diabetes Mellitus

DNA – Deoxyribonucleic Acid

ERC – Ethics and Review Committee

HbA1c – Glycated Hemoglobin

HDL-C – High-Density Lipoprotein Cholesterol

Hz – Hertz

IDF – International Diabetes Federation

IFCC – International Federation of Clinical Chemistry Principles

IQR – Interquartile Range

KAVI-ICR – Kenya AIDS Vaccine Initiative Institute of Clinical Research

KNH - Kenyatta National Hospital

LDL-C – Low-Density Lipoprotein Cholesterol

LFTs – Liver Function Tests

MDROs – Multidrug-Resistant Organisms

MIC – Minimum Inhibitory Concentration

MRSA – Methicillin Resistant *Staphylococcus Aureus*

NCDs – Non-communicable Diseases

NDS – Neurological Disability Score

NFGNB – Non-Lactose Fermenting Gram-Negative Bacteria

OHA – Oral Anti-Hyperglycaemic Agents

PCR – Polymerase Chain Reaction

RBS – Random Blood Sugar

RNA – Ribonucleic Acid

rRNA – ribosomal RNA

RT-PCR – Real Time PCR

SD – Standard Deviation

sp. – species

T. Bil – Total Bilirubin

T. Chol – Total Cholesterol

T1DM – Type 1 Diabetes Mellitus

T2DM – Type 2 Diabetes Mellitus

TGs – Triglycerides

TMPSMX – Trimethoprim-Sulphamethoxazole (Co-trimoxazole)

UECs – Urea, electrolytes and creatinine

UNITID- University of Nairobi Institute of Tropical and Infectious Diseases

UoN – University of Nairobi

USD - United States Dollar

DEFINITION OF TERMS

Aerobe - a microbe that requires the presence of oxygen for life and growth

Anaerobe - a microbe that is able to live and grow in the absence of free oxygen

Antibiotic - a substance produced by or derived from a living microorganism or artificially synthesized that destroys or inhibits the growth of other microorganism

Bacteria - microorganisms that lack distinct nuclear membrane and have a cell wall of unique composition

Clean wound – A sterile wound, with no bacteria and no inflammation

Colonized wound – A wound where bacteria are present, attached to the surface and replicating.

Contaminated wound – A wound with bacteria or foreign material but the bacteria are not attached or replicating.

Cross-sectional design – Cross- sectional studies are simple in design and are aimed at finding out the prevalence of a phenomenon, problem, attitude or issue by taking a snap-shot or cross-section of the population**.**

Culture - population of microorganism usually bacteria grown in solid or liquid laboratory medium

Diabetes mellitus - a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both

Diabetic foot infection - an infection occurring below the ankle in diabetic patients

Diabetic foot ulcer - breach on the normal skin occurring as induration, ulceration or change of colour on the foot for duration equal to or more than two weeks. It is associated with neuropathy and/or peripheral arterial disease of the lower limb in a patient with diabetes

Etiology - the cause of a specific disease

Gram-negative organism - bacteria lose the crystal violet stain (and take the color of the red counterstain) in *Gram's* method of staining. This is characteristic of bacteria that have a cell wall composed of a thin layer of a particular substance called peptidoglycan.

Gram-positive organism - retain the color of the crystal violet stain in the *Gram* stain. This is characteristic of bacteria that have a cell wall composed of a thick layer of peptidologlycan.

Incidence - is a measurement of the number of new individuals who contract a disease during a particular period of time.

Infected wound – In an infected wound, the bacteria are invasive and they interfere with healing. The body also mounts an immune response.

Pathogen - a microorganism such as bacterium that infects an animal or man and produces disease

Prevalence - a measurement of all individuals affected by the disease at a particular time

Sample - a representative portion of the target population

Sensitivity test disc – a disc infiltrated with antibiotic which is placed in a plate inoculated with bacteria to test the zone of inhibition of growth

Target population - collection of all subjects of interest

Type 1 diabetes mellitus - the cause is an absolute deficiency of insulin secretion

Type 2 diabetes mellitus - the cause is a combination of resistance to insulin action and an inadequate compensatory insulin secretory response.

ABSTRACT

BACKGROUND: The burden of diabetes mellitus is increasing in resource-poor settings and this is associated with a rise in diabetic complications. Diabetic foot ulcers (DFUs) often lead to hospital admissions, amputations and deaths among diabetic patients. In Kenya, there is a lack of up-to-date information on microbial isolates from diabetic foot ulcers and no mention of utilization of molecular techniques.

Significance: Identifying bacteria and their sensitivity patterns in infected diabetic foot ulcers could lead to appropriate antibiotic prescriptions, curb antimicrobial drug resistance, and better clinical outcomes thereby reducing health expenditure.

OBJECTIVE: To isolate bacteria and determine their antibiotic sensitivity patterns in patients with infected diabetic foot ulcers in a clinical setting using culture-based and molecular techniques while also investigating their risk factors and clinical outcomes.

METHODS

The study had a cross-sectional design and recruited 84 adult inpatients and outpatients at Kenyatta National Hospital over 12 months with any type of diabetes mellitus and having active foot ulcers. Consecutive sampling was used to enroll participants and informed consent taken. History and physical examinations findings were recorded through a structured questionnaire. The most recent blood tests were also recorded. Samples were collected from the centre of the wound and taken to the laboratory. One part of the sample was used for microbiological tests, and the other for RT-PCR was stored at −80 °C. On Day 1, specimens were inoculated on culture media and incubated in aerobic conditions. On Day 2, the most predominant colony was isolated and identified using standard biochemical tests and then VITEK ® 2 machine was utilized for further identification and antibiotic sensitivity. For 51 samples, DNA extraction was performed using kits from Qiagen Hilden, Germany. *Staphylococcus aureus* DNA, Methicillin-resistant *S. aureus* (MRSA) DNA and positive control were identified through real-time PCR. Thereafter, clinical outcomes for patients with foot ulcers were retrieved from the patients' medical records.

RESULTS: Majority (68%) were inpatients. The mean age was 60.30 $\pm$ 12.88 years with 68% living in urban areas and 60% having minimal or no formal education. Eight percent (8%) were newly-diagnosed with diabetes mellitus (DM). The median (IQR) duration of DM was 6.5 (1.25, 12.5) years. A majority (96%) had type II DM. Forty-five percent (45%) were on insulin only,

18% on oral drugs only and 32% on a combination of both. The median (IQR) random blood sugar was 9.60 (5.32, 15.45) mmol/L and glycated haemoglobin was 8.80 (7.17 to 9.92)%. A majority of the patients had good lipid profile, 85% with desirable total cholesterol and 70% having ideal low-density lipoproteins. Eighty-three percent (83%) had one or multiple comorbidities. The median duration of the DFU was 8 (4, 16) weeks. Eighty swabs (94%) were culture-positive; 29% were Gram-positive and 65% were Gram-negative. The organisms isolated were *Staphylococcus aureus* (16%), *Escherichia coli* (15%), *Proteus mirabilis* (11%), *Klebsiella pneumonia* (7%) and *Pseudomonas aeruginosa* (7%). The bacterial isolates showed resistance to commonly used antibiotics such as ampicillin, amoxicillin, cefazolin, cefepime, ceftazidime, cefotaxime, cefuroxime, clindamycin, co-trimoxazole (TMPSMX), erythromycin, piperacillin-tazobactam, and tetracycline. More than half (62%) of the *S. aureus* isolated and 40% of the Gram-negative bacteria were MDROs. MRSA were not identified using culture methods but were identified using PCR. PCR was also more sensitive than culture-based methods to identify *S. aureus.* There was a high mortality rate (11%) among patients with DFUs.

CONCLUSION: There are poor outcomes for patients with DFUs in this setting such as poor wound healing, high recurrence rates, increased lower limb amputations and mortality compared to previous studies. Almost all DFUs were infected, with Gram-negative bacteria being the most common. In order of frequency, *S. aureus, E. coli, P. mirabilis, K. pneumonia, P. aeruginosa* were the most common isolated species. There was a high prevalence of nosocomial bacteria. In this study, the bacteria isolated showed high resistance to commonly used antibiotics with Gram-negative bacteria showing higher resistance patterns.

Recommendations: Patients with DFUs should have microbial swabs for culture and sensitivity performed routinely for correct management and antibiotic stewardship. PCR is an effective way of species identification, and in particular, RT-PCR for *S. aureus* and MRSA should be considered for patients with adequate healthcare resources. The high prevalence of poor outcomes for patients with DFUs warrants the need to investigate bio-psychosocial risk factors.

1. Chapter One: Introduction

1.1. Background

The global prevalence of diabetes mellitus (DM) is 8.8% among people aged 20 - 79 years and is expected to rise to 10.4% by 2030 [1]. By 2030, diabetes will have affected 188 million adults in their fourth and fifth decades and four-fifths of these patients will come from resource-poor countries [2]. Within the African region, it is estimated that 3.2% of the adult population (14.2 million) have diabetes [1,2]. This is expected to rise to 3.7% adults (34.2 million) by 2040 [1,2] . In addition, at least 1 to 3 out of every 20 deaths globally of adults between 20 and 79 years results from diabetes in the years 2010 and 2015 respectively [1,2].

Like most resource-poor countries, Kenya is in the transition from communicable to non-communicable diseases (NCDs) or what you might call a 'double burden of disease' where both infectious and NCDs are prevalent [3–5]. This arises from globalization and urbanization. Currently, the prevalence of diabetes in Kenya is 2.8% within the 20 - 79 years' age group [2]. The Ministry of Public Health in Kenya reports that over 50% of all hospital admissions and over 55% of all deaths are due to NCDs [6]. In Isiolo, northern Kenya, the prevalence of diabetes was 16% over a two-day free medical screening [6]. Therefore, the burden of diabetes in resource-poor countries is increasing and it is often associated with diabetic complications such as heart disease, stroke, nephropathy, retinopathy, peripheral neuropathy and foot complications.

Foot complications result in 25 - 50% of all hospital admissions in patients with diabetes [7–10]. Foot ulcers are one of the most common diabetic complications and often lead to amputations [11]. In the United Kingdom, a fifth of all admissions of patients with DM were due to diabetic foot ulcers [12,13]. Despite significant improvements in healthcare among patients with diabetes mellitus in the UK, there are more pronounced emergency admission rates among poorer communities in the UK [14]. In a recent hospital-based study in the UK, 14 out of 98 admissions were diabetes-related but only one was due to infected foot ulcer [15].

According to Alexidou and Doupis, Diabetic Foot Ulcer (DFU) is any foot with ulceration and linked to neuropathy and/or peripheral vascular disease in a patient with DM [16]. Other publications define a DFU as an induration, ulceration or change of colour on the skin on a diabetic patients' foot for two weeks or more [17,18]. There are three clinical forms of DFUs: neuropathic, neuro-ischaemic and ischaemic ulcers. Foot ulcers are considered ischaemic when there is peripheral arterial disease without neuropathy, neuropathic when there is neurological disability

but no obvious peripheral vascular disease and neuro-ischaemic if both neuropathy and peripheral vascular disease are present [17,18]. Wagner and colleagues classified foot ulcers into six stages [19] (Refer to Table 1 below).

Wagner Stage	Definition
0	foot at risk; intact skin
1	superficial ulcer
2	deep ulcer with no bone involvement or abscess
3	deep ulcer with abscess or osteomyelitis (bone involvement visible on Xray)
4	localised gangrene e.g. toe, heel
5	gangrene of the entire foot

Table 1: Wagner staging. *Staging based on physical examination of foot ulcer and other investigations*

Diabetic Foot Infection (DFI) refers to infection occurring below the ankle in diabetic patients [20]. Patients with DFU are often under frequent antibiotic treatment without any investigations to confirm infection. This practice can lead to development of resistance. Treating an uninfected wound with antibiotics has no value in infection prevention or improved wound healing [21]. There are limited antibiotic options for treating diabetic foot ulcers and thus, antibiotics ought to be administered judiciously and only when necessary. This is because antibiotic resistance is a key public health threat of the twenty-first century [22,23].

Patients with advanced foot ulcers often require minor or major surgery. The risk of amputation is 15 – 46% higher in patients with DM compared to those without DM [24–26]. In fact, diabetic foot ulcer is the leading cause of lower limb amputations [26,27]. More recently, reports state that DFUs precede 84% of all lower limb amputations [28].

Global healthcare expenditure to treat and prevent diabetes and its complications is estimated to add up to at least US Dollar (USD) 376 billion in 2010 and 673 billion in 2015 [1,2]. Some studies estimate that diabetes takes up 11% of world's total health expenditure [29]. The estimated costs of treating a DFU, whether a patient requires an amputation or not, range from USD 18,000 to USD 34,000 in resource-rich countries [30]. Recent estimates are much higher, with the mean global health care cost being twice the cost of any other ulcer at USD 44,200 [31]. In Tanzania, the costs are quite low and stand at USD 3,060 [32]. In Nigeria, the average costs of successfully treating patients with DFU is USD 1,808 for Stage 4 ulcers, USD 1,104 for Stage 3

ulcers and 556 for Stage 2 ulcers [33]. As noted from these costs, resource-poor settings that have an increasing burden of diabetes partly due to the high number of cases and low resources available for healthcare [18]. Patients with diabetic foot ulcers require prolonged treatment, hospitalization and home-based nursing care and they spend a significant amount of the family resources. Indirect costs also arise from loss of work, loss of income, and premature death [7]. This economic burden is enormous considering the fact that type 2 diabetes (T2DM) affects the age group that is most productive (34 to 64 years) [34]. In Sudan, the total annual medical expenditure for people with diabetes was USD 579 almost 4 times that of non-diabetic patients [29]. In this study, diabetic patients had more adverse social effects such as being away from work or school.

Understanding the profile of microbes in DFUs in Kenya will be helpful in prescribing the right antibiotics, curbing antibiotic resistance, reducing hospital stay, preventing major surgical interventions, and thereby saving resources.

2. Chapter Two: Literature Review

2.1. Epidemiology

The annual prevalence of diabetic foot problems among diabetic patients is approximately 2.5% [35]. It is estimated that 10% - 15% of diabetic patients will develop diabetic foot ulcer at some point in their life [10,36]. The prevalence of foot ulcers varies from 4% - 10% in patients with diabetes mellitus in the United States and this translates to an annual population-based incidence of 1.0% - 4.1%, and a lifetime incidence of 24.3% - 25% [30,37]. The prevalence of active foot ulceration therefore ranges from approximately 1% in Western countries to more than 11% in African countries [36]. In the United Kingdom, the prevalence of DFU ranges from 7.0% - 7.4% [13,38]. In Denmark, the estimated prevalence of foot ulcers in the region of Southern Denmark is 7% [39]. In Netherlands, the prevalence of DFU is much higher at 20.4% [40] while in Iran the prevalence is 20% [41].

In a recent, meta-analysis of 55 studies from 19 African countries, the overall prevalence of DFU was 13% [42]. In Africa's most populous country, Nigeria, the prevalence of DFU is 11.7% - 19.1% [43,44]. In South Africa, the prevalence of DFU is 5.4% while in Malawi it is 4.5% [45,46]. Within East Africa, the prevalence of DFUs among diabetic patients in Tanzania is 3.2% - 15% whilst in Ethiopia, the prevalence is 13.6% – 14.8% [18,47–49]. In Sudan, the prevalence of DFI is 12.7% [50]. At Kikuyu Mission Hospital in Kenya, 29.2% of diabetic patients had foot complications [51]. Nyamu and colleagues found the overall prevalence of DFUs among diabetic patients to be 4.6% at Kenyatta National Hospital (KNH) [17]. The prevalence of DFUs among inpatients in this study was higher (11.4%).

2.2. Risk factors for developing DFUs and DFIs

The risk factors of developing DFUs in Kenya are well known and have already been reported. In Kenya, Karugu listed the following to be risk factors for developing DFUs: limited awareness among patients and clinicians on foot care, few podiatrists in the public sector, walking bare footed, wrong footwear, poor foot care and alternative medicine practices [34]. In a randomized control study in Nyeri, Central Kenya, the risk factors for diabetic foot ulcers included poor glycemic control, blood pressure higher than 130/80 mmHg, poor drug adherence, poor nail care and calluses [52]. From this study, protective factors included wearing appropriate shoes, examining feet regularly and following a prescribed diet and exercise plan. In males, no fungal infections were protective while in females, having appropriate foot care education was helpful. In a cross-

sectional study at KNH, poor glucose control, elevated diastolic blood pressure, poor lipid profile, infection and poor self-care were identified as risk factors for DFUs [17].

In Ethiopia, DFU was associated with rural residence, T2DM, increased weight, poor foot self-care, and neuropathy [49]. In Sudan, longer duration of DM and urban residence were significant risk factors [50]. In another study using a new screening tool, 35.5% of participants had at least one risk factor for developing DFU [53]. In Tanzania, polyneuropathy and insulin treatment were risk factors for DFU[48]. In the US, kidney disease has been associated with more incidences of DFU. In a cohort study comparing patients on dialysis and those with just DFU, there were more foot-related admissions in patients with renal failure compared to just having foot ulcers alone [37]. In addition, previous DFU, previous amputation and poor vision are high risk factors for DFU [30].

2.3. Clinical features and diagnosis of DFUs

DFU is often undiagnosed because patients fail to examine their feet. Secondly, patients may have already developed peripheral neuropathy and even if they notice the ulcer, they do not feel any pain and do not tell their doctors about this [54]. Lastly, clinicians may omit to ask or examine for the diabetic foot due to the pressure of the large number of patients they see in the clinic. Patients therefore present late with DFUs. For instance, in Chad, there was a one-month delay in presentation after onset of the ulcer [55]. In Western Sudan, 74% of patients presented with Wagner stage 4 ulcers [56]. In Libya, Wagner's stage 3 ulcers were the most prevalent (31%), followed by stage 2 (25%) [57]. In a recent study in Tanzania, the average duration of ulcer at presentation was 18.34 weeks [18]. For those patients with DFUs, the average duration of DM was 8.2 years but a sizeable proportion (27.9%) were newly-diagnosed with DM. In this Tanzanian study, 10.3% had a previous ulcer while 4.4% had a previous amputation. According to Chalya and colleagues, the forefoot was the most affected anatomical site (60.3%) [18]. In Nigeria, over 50% of patients presented with DFUs after 3 weeks of onset [58].

In Kenya, the major type of DFUs are neuropathic ulcers (47.5%) followed by neuro-ischaemic (30.5%) and then ischaemic ulcers (18%) [17]. Neuropathic ulcers were common in those with poorer glycaemic control while ischaemic ulcers were common in those with elevated total cholesterol and elevated diastolic blood pressures. In this study, Wagner stage 2 was the most common presentation (49.4%) while Wagner stage 4 had the highest neuropathic score (7.8/10) and the longest duration (23.3 weeks). The mean duration of DM in this study was 7.98 years and the duration of ulcers was 16.36 weeks at presentation [17]. In this study, 8.5% of patients with DFUs

were newly diagnosed diabetic patients. In another Kenyan study, the duration of diabetes in patients with foot ulcers was slightly lower at 5 years [59]. In another study of diabetic patients without foot ulcers at KNH, neuropathy was present in 42% of the participants [60]. It is clear from the above studies that there are country-to-country variations in the presentation of DFUs.

Full assessment of diabetic foot includes the skin, blood vessels, nerves, and musculoskeletal system. The examination of the skin involves visual inspection of the legs and feet and each toenail [16]. Skin temperature should be cool and not elevated. Peripheral neuropathy is assessed clinically using four tests: sensation to pain, vibration, pressure and deep tendon reflex [17]. Vibration is tested by examining for vibration sense at the medial and lateral malleoli using a 128Hz tuning fork. Pressure sensation is tested using a monofilament that buckles at 10g on several areas of the foot. Deep tendon reflexes are examined using a patella hammer on the Achilles tendon and noted whether present, absent or present only after enhancement. Pain sensation is tested using a pinprick on various sites of the foot. A neurological disability score (NDS) is then used to grade the range of neuropathy by summing up the scores of the above tests. The prevalence of diabetic polyneuropathy was 29.4% in Mulago Hospital, Uganda and was associated with a history of DFU [61]. In a screening study in Nigeria, 37% of patients with diabetes had symptoms for diabetic neuropathy [43]. In Tanzania, 44% had peripheral neuropathy [48].

It is necessary to assess pulsation in the following vessels to rule out peripheral vascular disease: dorsalis pedis, posterior tibial, popliteal, and superficial femoral arteries [16]. Poor circulation may result in poor wound healing and infection. The ankle–brachial index (ABI) is a ratio of systolic blood pressures in the brachial arteries and posterior tibial or dorsalis pedis arteries using a Doppler probe [16]. Normal ABI values range from 1.0 to 1.3, since the pressure is higher in the ankle than in the arm [16]. In a screening study, 40% of patients with diabetes had symptoms for peripheral arterial disease in Nigeria [43]. In a study of patients with peripheral arterial disease in South Africa, diabetics presented with more occlusive arterial disease in the tibioperineal segment [62].

At presentation, about half of DFUs are clinically infected [63]. DFIs are sometimes difficult to define. Some specialists define infection in wounds as $\geq 10^5$ colony forming units per gram (CFU/g) [21,63,64]. This threshold differentiates colonization from infection. Other authorities recommend the use of clinical signs and symptoms of inflammation such redness, swelling, pain, and warmth [21,63]. However, due to the presence of peripheral neuropathy and ischaemia, these

cardinal signs of inflammation may be delayed or absent. In certain cases, infection in wounds may be detected from 'secondary' findings such as a friable, poorly granulated, foul smelling or slow healing wound [21,63]. Waiting for systemic signs such as fever, chills, low blood pressure, tachycardia, confusion, leukocytosis, elevated erythrocyte sedimentation rate, elevated C-reactive protein or positive blood cultures may be too late [21,63]. It is thought that in the diabetic patient, inflammatory responses do not appear in time [65]. Therefore, neither local nor systemic inflammatory signs or symptoms, or even biological markers should be regarded as reliable for diagnosing foot infection in diabetic individuals [63]. Although not the least invasive, performing a culture and sensitive test using a wound swab is the most reliable test to confirm infection in a DFU. Imaging by X-rays is useful for deep ulcers to confirm osteomyelitis. Wound biopsies should also be performed on non-healing chronic ulcers. Kaposi Sarcoma was once missed out in a HIV-infected patient who was on treatment for diabetic foot ulcer [66].

2.4. Causative bacteria in DFIs

Staphylococcus aureus and beta-hemolytic *Streptococci*, both Gram-positive bacteria, are the most common causes of skin infections [20,21,64,65,67–69]. Some studies however show, that in resource-poor countries, Gram-negative bacteria like *Pseudomonas aeruginosa* to be more prevalent than *S. aureus* [21,65]. In Brazil, *Staphylococcus aureus*, *Staphylococcus saprophyticus*, *Proteus sp.*, *Staphylococcus epidermidis*, *Streptococcus agalactiae*, *Pseudomonas sp.*, *Escherichia coli*, *Streptococcus pneumoniae* and *Citrobacter sp.* were the most common aerobic bacteria in decreasing order [70]. Other aerobic bacteria isolated from DFIs include *Enterococcus sp.*, *Coliform bacteria* and *Acinetobacter baumannii* [71]. In India, *S. aureus*, *E.coli*, *P. aeruginosa*, coagulase-negative *Staphylococcus* (CoNS), No growth and *Klebsiella sp* were the most common organisms isolated in decreasing order [72]. In another study in India, *P. aeruginosa*, *Citrobacter sp.*, *K. oxytoca* and *Proteus sp.* were the most common species isolated after *S. aureus* [69]. In patients with chronic wounds and previous antibiotic treatment, DFIs are usually polymicrobial and include both aerobic Gram-negative bacilli and obligate anaerobic bacteria [65].

A study in Tanzania revealed that *S. aureus* was the most frequently isolated organism in DFUs [18]. In Nigeria, *Staphylococcus* and *Pseudomonas* species were found in diabetic foot ulcers [10]. In one of the few Kenyan studies, *S. aureus* and *E. coli* was found to be the most common organisms in 88% of the DFIs [59]. Taken together, there is a gap in the current level of knowledge on the types of microbes associated with DFIs in Kenya with the last published study on microbial

isolates in DFUs in Kenya, published more than 15 years ago. In this study, 73.2% of the DFUs were infected while 26.8% were culture-negative under aerobic conditions [17]. Although unconfirmed, culture-negative ulcers in this study that were also in advanced Wagner stages could possibly have had anaerobic pathogens. In Morocco, Gram-negative bacilli were isolated from 43% DFIs [73]. The most commonly isolated bacteria in this recent study were *Enterobacteriaceae, S. aureus, Streptococcus sp.*, non-fermenting gram-negative bacilli (NFGNB), and *Enterococcus sp*. Fungal infection have been implicated as a cause of DFIs in Kenya [74].

Biofilms, present in chronic wounds, are a defensive mechanism for bacteria against the effects of antibiotics. Biofilms have been fronted to be one of the evidences of evolution in our present times. The biofilm has many layers of bacteria surrounded by an envelope of proteins, DNA, and polysaccharides [69]. The bacteria communicate and work together to hinder entry of antibiotics into this environment [65]. It is thought that there is direct transfer of plasmids with resistant genes from one cell to another. Bacteria in biofilm are slow-growing, encased around a polysaccharide matrix and have altered extracellular milieu (pH and osmosis) preventing drug action and adequate immune response (phagocytosis, complement system and antibody reaction). Using Congo-red agar, biofilms were isolated in 46.3% DFUs which was almost half the prevalence from previous studies [69]. The bacteria that form biofilms in patients with DFUs are the same bacteria that infect DFUs without biofilms. In order of decreasing frequency, *S. aureus*, *P. aeruginosa*, *Citrobacter sp*., *E. coli, Proteus sp.* and *K. oxytoca* have been isolated in biofilms in patients with DFUs [69]. Management of biofilms include sharp debridement and application of anti-biofilm substances such as xylitol, Ethylenediaminetetraacetic acid (EDTA), surfactants, and Cadexomer Iodine (CI) [31].

Anaerobes rarely cause DFUs on their own but are present in deep-seated and chronic infections [64]. Samples for anaerobic culture must be collected in special conditions where a syringe devoid of air and the sample and the specimen inoculated on culture medium and placed in the anaerobic Gas cylinder immediately while at the bedside.

2.5. Antimicrobial Management and Resistance

DFUs are often treated with broad-spectrum oral antibiotics while severe infections require parenteral treatment. Antibiotic resistance is an emerging problem globally. In Tanzania, antibiotic sensitivity tests of bacterial isolates from DFUs revealed a high resistance of commonly used

antibiotics such as ampicillin, Augmentin, co-trimoxazole, tetracycline, penicillin, gentamycin, erythromycin and oxacillin [18].

	Moxifloxacin resistance	B-lactamases producing	Carbapenemase producing	Piperacillin-tazobactam resistance	Imipenem resistance	Ciprofloxacin resistance
Enterobacteriaceae		14.1	3.8	7.5	4.7	25.5
NFGNB		5.1	38.5	35.9	30.7	35.9
Streptococcus	4.9	4.9				

Table 2: Prevalence of resistant bacteria. *Proportion (%) isolated from DFUs and their antibiotic resistance patterns (Adapted from literature)* [73]**.**

Previously, *S. aureus* was sensitive to Augmentin, amikacin, clindamycin and novobiocin while Gram-negative bacilli were sensitive to amikacin, Augmentin, cefotaxime and piperacillin [59]. In this earlier study, anaerobes were partly resistant to chloramphenicol, clindamycin and lincomycin. Repeat bacterial cultures and sensitivity tests after a month of treatment were largely positive; *S. aureus* persisted in 63 ulcers despite therapy, while *E. coli* persisted in 35 DFUs [59]. Although no new organisms were isolated from repeat cultures, no ulcer was completely sterile [59].

In Nigeria, *Staphylococcus sp.* and *Pseudomonas sp.* found in diabetic foot ulcers were susceptible to quinolones [10]. A hospital-based study in Benin, Nigeria showed that fluoroquinolones such as ciprofloxacin and ofloxacin are more sensitive than chloramphenicol in treating wounds colonized by *Proteus sp.* [75]. In this study conducted among patients with burns and not patients with DM, *Proteus sp.* was more common than *Klebsiella sp*. contrary to previous literature. *Proteus sp.* and *Klebsiella sp.* have also been isolated in DFUs [18,70].

2.6. MRSA

Methicillin-resistant *S. aureus* (MRSA) was first observed in the early 1960s and has been associated with increased hospital stay, healthcare costs and mortality [76]. MRSA is often thought to colonize the nose, rectum and even wounds. As an infection control measure, nasal swabs are therefore collected from healthcare workers and inpatients in resource-rich countries for screening of MRSA [77]. Apart from hospitals, MRSA is also prevalent in the community. Once a patient is identified to be having MRSA, the patient undergoes isolation, treatment and follow-up [76]. Contacts

of the patient are also traced and tested. In Kenya, MRSA screening has been implemented as a standard procedure in some private hospitals.

MRSA is common in DFUs [78]. MRSA represented 4.7% of *S. aureus* isolated in a study in Morocco [73]. Studies have shown that MRSA are sensitive to amoxicillin-clavulanic acid, cephalexin, cefuroxime, ciprofloxacin, clindamycin, doxycycline, levofloxacin, ofloxacin and sparfloxacin [71]. Among 41 patients in Brazil, nine cases of MRSA were resistant to cefoxitin, and among 3 of these were vancomycin-resistant [70]. Other resistant bacteria include glycopeptide-intermediate *S. aureus* (GISA), vancomycin-resistant *Enterococci* (VRE), extended-spectrum β-lactamase- (ESBL) or carbapenamase–producing Gram-negative bacilli and highly resistant strains of *P. aeruginosa* [65].

2.7. Molecular Tests

Polymerase chain reaction (PCR) is a molecular method used to amplify a genomic region of interest. The 16S ribosomal RNA (rRNA) gene is a highly conserved gene present in prokaryotes and contains hypervariable regions that can be used for identifying specific bacterial species [63,65]. PCR amplifies a few pieces of DNA, of a specific sequence, into thousands or millions of copies [79]. The steps undertaken during PCR include denaturation, annealing, and extension [80]. Gel electrophoresis is performed after DNA amplification to estimate the size of the amplified PCR product. It involves separation of DNA or RNA based on their molecular sizes using an electric field. The gel is then visualised by trans-illumination under ultraviolet radiation.

Real-time PCR (RT-PCR) on the other hand, allows detection of the DNA or RNA as the PCR is ongoing through production of fluorescence light during the reaction. DNA sequencing of the 16S rRNA gene denote methods used to determine the order of nucleotides bases in DNA; adenine, guanine, cytosine and thymine [81]. Common methods of gene sequencing include Sanger sequencing, pyrosequencing, nano sequencing and sequencing by synthesis. Other molecular techniques for determining biodiversity include full ribosomal amplification, cloning and Sanger sequencing (FRACS), partial ribosomal amplification with a gel band identification and Sanger sequencing (PRADS), temperature gradient gel electrophoresis (TGGE), pyrosequencing, multi-target PCRs and density gradient gel electrophoresis (DGGE) [63,65].

Compared to molecular techniques, culture methods underestimate the presence of *S. aureus* when it is in low quantities. PCR also reveals more obligate anaerobes in wounds than standard cultures. In a study on biopsies from 128 DFIs, RT-PCR resulted in a higher detection of

S. aureus, S. pyogenes, S. agalactiae, S. dysgalactiae subspecies equisimilis, and *S. anginosus* group, compared to culture-methods [20].

In India, 4 out of 22 strains isolated were multidrug-resistant organisms (MDROs) [71]. The 4 strains identified were *S. aureus, Morganella morganii, A. baumannii* and other *Acinetobacter sp.* following PCR amplification of the 16S rRNA gene. A phylogenetic tree was later constructed to analyze the evolutionary relationship between the isolates and closely related species. In a genetic study in France, virulence in MRSA was associated with the absence of a ROSA-like gene, which was otherwise found located in CC8- and CC5-Methicillin sensitive *S. aureus* (MSSA) [68]. MRSA is coded by the staphylococcal chromosome mec (SCCmec) which contains the *mecA* gene [76]. This gene codes for the penicillin-binding protein (PBP) resulting in methicillin resistance [82].

In Africa, the only available study in literature to our knowledge, on utilization of molecular techniques in DFIs is from Algeria [83]. The study showed a high prevalence of MDROs (58.5%) among DFIs in Algeria. There was 1.51 strains isolated per sample from 183 samples in 128 patients. A majority (54.9%) were aerobic Gram-negative bacilli. MDROs included 85.9% of the *S. aureus* (MRSA), 83.8% of the *K. pneumonia* and 60% of the *E. coli*. ST239, the most common MRSA strain globally was isolated from 82.2% of MRSA while PVL+ ST80 strain , the cause of more than a third infections in Algeria, was found in 13.7% of the MRSA [83]

However, PCR technology amplifies not only the living but also the dormant or dead bacteria in a sample [63]. Another disadvantage is that the 16S rRNA primers may neglect some microbial populations such as viruses and fungi in chronic wounds [63]. Lastly, due to high costs, these techniques may not be applicable in the clinical setting. MRSA screening has mainly been performed using nasal swabs in resource-rich countries. In a laboratory assessment, out of 88 wound swabs, 93.18% had similar culture and PCR results while 6.82% had differing PCR and culture results [77]. On use of enrichment media, 2 wound samples that were culture positive and PCR negative were confirmed to be truly positive. Compared to culture-based methods using VITEK ® 2 machine, Multiplex PCR had a sensitivity of 100% and a specificity of 94% in a samples from 6 hospitals in Denmark [76].

2.8. Surgical Management

DFUs may require surgical management such as wound debridement or amputations. The prevalence of diabetic amputations in Sub Saharan Africa varies but was generally thought to be low especially in the rural areas [84,85]. However, in a study in Cameroon, 78% of diabetic patients

had amputations while 22% had surgical debridement [86]. In Ivory Coast, 46.9% below knee amputations and 11.2% below elbow amputations were due to diabetes [87]. In Nigeria, 21% of amputations were secondary to diabetes foot gangrene and diabetes complications were the leading cause of amputations in North-East of the country [88,89]. In Tanzania, the proportion that required surgery was 72.1% [18]. Lower limb amputations in this Tanzanian study occurred in 56.7% of diabetic patients with DFUs. This was similar to Pakistan where a majority of patients with DFUs (81%) required surgery [90]. In Sudan, diabetic foot is a major cause of hospitalization and lower limb amputation (19.2%) [91]. In Zimbabwe, the prevalence rate of diabetes related amputations was 9% [92]. Diabetic foot complications are the leading cause of lower limb amputation in Trinidad and Caribbean islands where diabetes is common [93]. In a recent meta-analysis of studies from 19 African countries, 15% of patients with DFU underwent major amputations [42].

Vascular amputations in Kenya range from 25% to 56% [94]. In a Kenyan provincial hospital, 25% of all lower limb amputations were due to DFUs [94].Among the elderly at KNH, diabetes accounted for 50% of all lower limb amputations [95]. Of the total vascular amputations in this centre, majority were however not related to diabetes. The prevalence of diabetic amputations was 17.5% [95]. At Kikuyu Mission Hospital, a Level 4 referral centre in Kenya, 11.4% of amputations were due to diabetic vasculopathy [96]. Among 150 patients who underwent amputations at Tenwek hospital, a rural referral health facility in Kenya, 48 (32%) had diabetes gangrene while 5.3% had other dysvascular disease [97]. Following surgery, 87.5% complicated with infection.

There are complications to surgery; in Tanzania for instance, 33.5% of diabetic foot surgery resulted in complications with 18.8% being surgical site infections [18]. In Benin, there was a mortality rate of 14.3% following surgical management of diabetic foot [58]. Even without considering post-operative complications, DFUs are still a major cause of mortality among patients with DM. In Cameroon, diabetic foot is the fifth leading cause of mortality in diabetic patients at an outpatient centre [98]. In Mombasa Kenya, the mortality rate as 13% among inpatients with DFU and 28% for those who had amputations [99]. In Sudan, mortality rate due to diabetic foot was 6.7% [91]. In comparison, the overall mortality rate of patients with T2DM in Nigeria was 30.2 per 100 000 population, with a case fatality rate of 22.0% [100]. In Libya, 34% of patients underwent amputations (10% major and 24.2% minor) and the mortality rate was 2% [57]. A pooled prevalence of mortality from a review of studies of DFU in Africa was 14.2% [42]. The mortality due to diabetic related complications in Africa occurs in a much younger age-group than in other parts of the world [42].

Moreover, patients with diabetic foot complications have a much higher mortality rate than the general population or even diabetic patients without foot complications [101].

2.9. Supportive Management and new Therapies

The following instructions should be given to the diabetic patients with or without a foot ulcer [26]:

- Stop smoking
- Regularly inspect the legs/feet for scratch marks, blisters et cetera
- Wash the feet with warm water daily followed by careful drying between the toes
- Apply emollients on dry skin
- Cut toe nails regularly; however, see your doctor for calluses and ingrown toe nails
- Inspect shoes regularly
- Wear properly fitting shoes
- See a chiropodist regularly
- Avoid sandals and pointed shoes which may lead to foot trauma
- Never walk bare footed
- See your doctor immediately if there is any injury

Other than antibiotic treatment and surgical wound debridement, the following management options for DFUs have been studied and are in current use:

- Hyperbaric Oxygen – The affected leg is covered by a disposable polythene bag through which oxygen is flowed (15Litres/min, six hours/day, four days/week). Pressure is monitored and kept at 25 – 30mmHg (3.3 – 4.0kPa), using a Y tube connected to a sphygmomanometer [26]. Interspersed periods of tissue hypoxia help to stimulate formation of granulation tissue, so that continuous oxygen therapy is not desirable.
- Offloading pressure – Total contact casts (TCCs) are the gold standard for neuropathic ulcers [102]. A cheaper option is the Samadhan System which can be made using just one foam sheet (4’ x 10’) and an adhesive applied until the foam is rolled into a cylindrical shape.
- Vacuum assisted negative pressure therapy – The technique entails placing an open cell foam into the wound, sealing the site with an adhesive drape, and applying sub-atmospheric

pressure (125mmHg (16.7kPa) below ambient) that is transmitted to the wound in a controlled manner [26]. Blood flow levels increased about fourfold when 125mmHg sub-atmospheric pressure is applied.

- Antiseptic dressings – In one study, topical antimicrobial peptide (pexiganan) and gentamicin-collagen sponge were comparable to systemic antibiotics [21]. However, antiseptic dressings were not shown to add any benefit.
- Ghee and Honey – Twenty-nine cases of diabetic foot ulcers were treated using honey with good outcomes [103]. Ulcers healed on an average of 21 days and healthy granulation tissue seen in 16 days.
- Phototherapy – Animal and human studies have shown improved healing of wounds after irradiation with light of different wavelengths [104]. A pilot study in South Africa among diabetic patients revealed positive outcomes of combining podiatry services with phototherapy in a number of DFU cases [105].
- Maggot therapy – The larvae of *Chrysomya megacephala* were used for over 40 days on an elderly patient with a DM for 30 years and an advanced foot ulcer. The treatment led to reduction in necrotic tissue, wound size and elimination of MDROs. Maggot therapy is thought to reduce necrotic tissue due to the activity of the larvae' mandibles and secretion of lytic enzymes [106].

2.10. Prevention

DFU is a major problem that is associated with illiteracy, lack of health education and dominance of wrong beliefs [107]. Interestingly, 40% - 50% of DFUs can be prevented by education and foot care [108]. The impact of DFU is heaviest in resource poor settings due to lack of efficient diagnosis, treatment and rehabilitation tools [18]. Patients usually present late for instance in Chad, Sudan, Nigeria and Tanzania as earlier mentioned. In Sudan, the awareness of diabetes management is very low among adults [107]. In this study, few patients were aware of prior screening or could explain why they had diabetic complications such as retinopathy or nephropathy. Most Kenyans are ignorant about diabetes mellitus and its prevention. In a local Kenyan study conducted in 4 provinces with the highest rate of DM, only 27.2% were aware of DM and 41% undertook preventive measures [109]. In another study, 10% of the patients did not realize they had a DFU meaning they did not regularly inspect their feet [17]. In South Africa, only 22.2% of study

participants examined their feet but only when they experienced a problem [110]. In Ghana, 51.5% of patients rightly mentioned DFU as a complication of diabetes [111]. In this study, gender, marital status, education level, duration of DM and residence were significant factors to the level of awareness. Despite a long hospital stay and a high amputation and mortality rate secondary to diabetes foot, 60% of patients at a Teaching Hospital in Enugu state, Nigeria had no knowledge of foot care despite having been educated on dietary control [112]. In a study in Tanzania, 48% of patients had received foot care education while 27.5% had ever had a foot examination by a doctor [48]. There is therefore need for comprehensive education on foot care among DM patients.

Education of diabetic patients and their healthcare workers has been shown to improve foot-care, leading to earlier detection of foot lesions and prevention of serious foot complications [113]. Technology can bridge gaps where human resource is limited. However, patient monitoring of DFUs in Denmark using telemedicine fared much worse than standard care [39]. Although outcomes such as healing and amputation did not differ in this study, there was a higher mortality rate among those who were monitored using telemedicine compared to regular care. Therefore, the best way of monitoring DFUs is by physician reviews at the outpatient department as often as is deemed necessary. In developing countries, with limited number of doctors, there is a crucial role for podiatrists and nurses specialized in foot ulcers. Community health workers can also be trained on foot care and they increased the level of diabetic foot screening in a study in South Africa [114].

3. Chapter Three: Research Definition

3.1. Study justification

DFU is a chronic issue that contributes significantly to morbidity and mortality. From local studies, DFUs last from 2 weeks to 6 years, with a mean duration of 6 months. DFUs can lead to lower limb amputation which can be prevented by proper wound care.

Patients with DFUs are commonly treated with broad-spectrum antibiotics even when there is no evidence of bacterial colonization through microbiological tests. When providers look at necrotic, non-healing ulcers, they are usually prompted to use antibiotics. The chronicity of the ulcers puts pressure on healthcare providers and patients to consume antibiotics. However, not all DFUs are infected. Furthermore, if the wounds are actually colonized and drug sensitivity tests are not performed, then the patient is subjected to inadequate or ineffective treatment.

There is a changing pattern in antibiotic use, susceptibility and resistance patterns. Prolonged use of antibiotics, appropriate or inappropriate, for several months confers the development of resistance patterns. Common pathogens are now resistant to old and new antibiotics. Unfortunately, the development of resistance is not keeping up with the discovery of new antibiotics. This calls for rational decision-making and antibiotic stewardship.

Pathogens in DFU are often poly-microbial in nature [57,70]. Understanding the current microbial profile in DFU and their sensitivity patterns will save scarce resources and prevent antimicrobial resistance. There is a gap in this setting in up-to-date information on the current antimicrobial isolates in DFUs and their sensitivities in Kenya. A previous study in KNH on bacterial isolates in DFUs and the drug sensitivities was conducted in 1991 but a lot could have changed since then [59]. KNH is Kenya's national, regional referral and teaching hospital. Based on more recent studies on DFUs conducted elsewhere like in Tanzania, a neighbouring country, drug resistance is an increasing problem [18]. It is also important to understand the current risk factors for DFUs in an effort to prevent their occurrence and for early detection.

No molecular characterisation of bacterial isolates in DFUs has been previously reported in Sub-Saharan Africa. The only other study conducted in Africa was in Algeria [83]. It showed a high number of MDROs including MRSA species. Molecular studies are useful since they have better sensitivities compared to culture-based methods. Furthermore, it is possible to detect resistant genes as a marker of drug resistance using PCR. MRSA is predominant in DFUs and could perhaps be the most likely resistant bacteria isolated from our Kenyan population [78].

3.2. Study Questions

1. What are the sociodemographic and clinical characteristics of patients with diabetic foot ulcers?

2. What are the microbial pathogens in diabetic foot ulcers in Kenya and what are their sensitivity patterns to commonly used antibiotics?

3. What is the molecular characterisation of drug-resistant *Staphylococcus aureus* species found in diabetic foot ulcers in Kenya?

4. What is the outcome of diabetic foot ulcers; complete healing, non-healing, surgery (minor or amputation), death or loss to follow-up?

3.3. Objectives

3.3.1. Broad Objectives

The aim of this study is to describe the microbial profile of pathogens in diabetic foot ulcers and their antibiotic sensitivity patterns in patients with diabetes in KNH and to describe their predisposing risk factors and clinical outcomes.

3.3.2. Specific Objectives

1. To describe the sociodemographic characteristics of diabetic patients with diabetic foot ulcers at KNH.

2. To determine the microbial pathogens isolated from diabetic foot ulcers and the bacterial drug sensitivity patterns in patients with diabetes at KNH using culture-based methods

3. To determine the presence of *Staphylococcus aureus* DNA and drug-resistant *S. aureus* (MRSA) DNA in diabetic foot ulcers in patients with diabetes at KNH using RT-PCR.

4. To determine the clinical outcomes of diabetic foot ulcers of patients at KNH and associate this with the antimicrobial sensitivity patterns.

3.4. Research Methodology

3.4.1. Study site

The study was conducted at KNH within the following departments:

- Medical Wards (7A, 7B, 7D, 8A, 8B, 8D)
- Diabetes and Endocrinology Outpatient Clinic

KNH is located in Nairobi, the politico-administrative and economic capital of Kenya, approximately 2 km to the west of the city Centre. KNH is one of two Kenya's national referral hospitals and is also a teaching hospital for the University of Nairobi's College of Health Sciences. The Hospital has a bed capacity of 1800 and has 4800 members of staff. It caters for over 80,000 in-patients and over 500,000 out-patients annually. About 75% of the patients treated as outpatients and inpatients are residents of Nairobi through self-referral or referral from the public and private health facilities. KNH runs specialized diabetes clinics managed by a team of specialist, endocrinologists, physicians, graduate resident doctors, nutritionists, diabetic educators, medical assistants, and nurses. Approximately 400 clients with DM are seen per week at the clinics which run daily excluding weekends [115].

3.4.2 Study design

Cross-sectional study

3.4.3 Sampling method

Consecutive sampling

3.4.4 Study population

Adult diabetic patients with diabetic foot ulcers from medical inpatient departments and diabetes outpatient clinic.

3.4.5 Inclusion criteria

i. Diabetic patients older than 18 years.
ii. Diabetic patients with active diabetic foot ulcers.
iii. Diabetes patients willing to provide written consent.

3.4.6 Exclusion criteria

i. Patients less than 18 years.
ii. Patients with HIV/AIDS, cancer patients and other known immunosuppressive states such as steroid medication.
iii. Patients with superficial ulcers (to eliminate the possibility of isolating colonizing bacteria).
iv. Patients not willing to give consent.

3.4.7 Sample size determination

Sample size was calculated using the (Daniel, 1999) formula;

$$n = \frac{Z^2 x\ P(1-P)}{d^2}$$

Where

n = Desired sample size

Z = value from standard normal distribution corresponding to desired confidence level (Z=1.96 for 95% CI)

P = expected true proportion (estimated at 0.046 according to a study at KNH [17]..

d = desired precision (0.05)

$$n = \frac{1.96^2 x\ 0.046(1-0.0.046)}{0.05^2} = 67$$

Adjusting for a 10% non-response or drop-out rate,

$$n = \frac{67}{1-0.1} = 74$$

Although the target minimum sample size was 74 patients, we however enrolled 84 participants with DFUs.

3.4.8 Enrollment

Subjects were enrolled consecutively for a period of 12 months (5th September, 2017 to 15th August, 2018). Patient enrollment took place on Monday to Thursday and no enrollment took place during weekends or Public Holidays.

3.4.9 Patient characteristics

Basic patient and clinical characteristics were collected as part of the routine history taking and usual physical examinations. Patients' basic characteristics included age, sex, area of residence, and level of education. Clinical characteristics including presence of comorbidities, smoking history, alcohol use, duration of diabetes, type of diabetes, duration

of DFU, diabetes medications used, previous history of healed foot ulcers, type of DFUs, Wagner's classification, and location of the lesion were recorded for each patient. Peripheral neuropathy and peripheral vascular disease was also assessed. Height, weight and blood pressure with the adult cuff standard technique was also measured. An X-ray was ordered for patients with advanced ulcers to rule out osteomyelitis.

Equipment used for Patient Characteristics

- Digital Blood pressure machine
- Digital Thermometer
- 128Hz Tuning fork
- Patella hammer
- 5.07 Semmes Weinstein Mono-filament 10g
- Glucometer
- X-ray machine

3.4.10 Clinical Outcomes

It was difficult to follow-up patients every four weeks due to the protracted nature of the study and interruptions by doctors' strikes. The outcomes of the diabetic foot ulcer were collected for patients at the end of the study by reviewing through their medical records. Patients' medicals records were reviewed to retrieve the latest doctors' notes from the diabetic clinic or the discharge notes from the medical wards. The 1st interview was conducted on the day the patient was enrolled into the study. The 2nd interview was based on the dated of the last clinical notes.

The interval between 1st enrollment and 2nd follow-up interview (in days) was calculated and outcome of the DFU [complete healing, non-healing, surgical intervention (minor or amputation) or mortality]. Permission was obtained from the KNH Medical Records department to extract data from the hospital registries.

4. Chapter Four: Materials and Methods

4.1. List of Materials and Equipment

MATERIALS	EQUIPMENT
Microbiology Tests Sterile cotton swabs Culture media (Sheep Blood Agar & CLED) Glass slides and pencil Reagents for Grams' Stains and Biochemical tests (Lactose Fermentation Catalase, Indole, Citrate test) Normal saline in dispenser Pipette and Test tubes Turbidometer/turbidity check ID cards and AST cards (P580 & GN83)	Safety cabinet VITEK ® 2 machine (bioMe´rieux, Durham, United States) Bunsen burner Wire loop Microscope
Laboratory Tests Blood collecting bottles (EDTA and serum) Reagents by Dirui ®, Changchun, China for HbA1c, LFTs (AST, ALT, T. Bil, D. Bil, Albumin), Kidney function tests (electrolyte tests, Urea) and Lipid profile (TGs, HDL-C, LDL-C) Reagents by StanBio ® Laboratory, Texas, United States for creatinine	DIRUI ® CS 4000 automated Clinical Chemistry Analyzer (Istanbul, Turkey)
DNA extraction and PCR amplification Dulbecco's phosphate buffered solution (Sigma ® - Aldrich, Steinheim, Germany) Proteinase K Wash (Buffer AW1) & Wash (Buffer AW2) DNA extraction kits (QIAamp DNA Blood Minikit (250)/QIASymphony Kit (Qiagen Hilden, Germany) Disposable powder-free gloves and laboratory coat Disposable tips with aerosol barriers (100 or 200 µl) in tube racks Disposable polypropylene microtubes for PCR or PCR-plate	Safety cabinet for DNA extraction Water bath at 56^{o}C Automatic adjustable pipettes (from 5 to 20 µl and from 20 to 200 µl). Tube racks Vortex mixer/desktop centrifuge Refrigerator for 2–8 °C. Deep Freezer (– 20°C to – 80°C) PCR box/hood Real Time PCR instrument (Rotor-Gene Q (Hilden, Qiagen)

Waste bin for used tips Spin columns (Standard 1.5 ml microcentrifuge tubes) MRSA Quant Real-TM kit (Sacace™ Biotechnologies, Como, Italy) 15µL of PCR Master Mix (PCR mix-1 FRT MRSA, PCR-mix-2 FRT, TaqF polymerase, and Internal Control) DNA standard QS1 *MRSA* DNA standard QS2 *MRSA* Negative Control (C–) Positive Control DNA *MRSA*	DNA extraction instrument (QIASymphony (Qiagen Hilden, Germany)
	Software Microsoft ® Excel IBM® SPSS® Statistical Package Version 23.0 Rotor-Gene Q-Rex Series Software 2.3.1. (Build 49)

Table 4.1 Table of list of materials and equipment used for laboratory tests

4.2. Wound site preparation and sample collection

After rinsing the wound area with normal saline, pus samples were collected from the centre of the diabetic wound. For patients undergoing wound debridement, tissue samples were collected after debridement. Samples were also collected from patients with deep-seated wounds while undergoing dressing or minor surgical procedures from the bedside. Specimens were collected using sterile cotton swabs and taken to the KNH Microbiology Laboratory within 30min. On arrival at the laboratory, samples were presented to the laboratory technicians assisting in the research for recording. Samples were processed for immediate culture and identification by using standard clinical laboratory procedures.

4.3. Standards of care clinical tests

On the same day, as part of standard of care, 2mls of blood from the cubital vein was collected into an EDTA bottle for glycated heamoglobin (HbA1c) and another 5mls into a serum tube for urea, electrolytes and creatinine (UECs), Lipid profile [(Total cholesterol (T. Chol), Triglycerides (TGs), High Density Cholesterol (HDL-C) and Low Density Cholesterol (LDL-C)],

and Liver function tests [(Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Total Bilirubin (T. Bil), Direct Bilirubin (D. Bil) and Albumin (Alb)]. All these parameters were analysed using a DIRUI ® CS 4000 automated Clinical Chemistry Analyzer (Istanbul, Turkey).

Laboratory Test	**Method**	**Reference Range**
HbA1c [a]	Latex agglutination method (antigen-antibody reaction)	Normal: < 7.0% Elevated: >7.0%
Kidney Function Tests		
Sodium	ion selective method	135 – 145mmol/L
Potassium		3.5 – 5.1mmol/L
Chloride		96 – 106mmol/L
Urea	enzyme Method	2.5 – 10.7mmol/L
Creatinine [b]	modification of earlier method [116]	62 – 106mmol/L
Lipid profile [c]		
T. Chol	enzyme method	Desirable: < 5.2mmol/L Borderline high: 5.2 – 6.2mmol/L High ≥ 6.2mmol/L
TGs	oxidase method	Desirable: <1.7mmol/L Borderline High: 1.7 – 2.2 High: 2.3 – 5.6 Very High: >5.6
HDL-C	direct method	Poor: <1mmol/L Better :1-1.5mmol/L Best: > 1.5mmol/L
LDL-C		Best for patients DM <1.8mmol/L High 1.8 – 4.9mmol/L Very high > 4.9mmol/L
HDL/LDL Ratio	calculation	Bad >5 Not bad 3.5 – 5 Ideal < 3

Liver Function Tests		
AST (U/L) ALT (U/L)	IFCC [d] method	7 – 40U/L 5 – 35U/L
T. Bil D. Bil	Diazo Salt Method [e]	3 – 22μmol/L 0 – 5μmol/L
Albumin (g/L)	dye-binding lysine (DBL) with Bromocresol Green Method	35 – 50g/L

Table 4.2: Summary of principles and methods of Laboratory tests according to manufacturer's instructions. *a, c, d, e=Reagents by Dirui ®, Changchun, China; a=Sample collected in anti-coagulation bottle; b=Reagents by StanBio ® Laboratory, Texas, United States; c= The product measured is quinone imine pigments; d= International Federation of Clinical Chemistry Principles; e= The product measured is azo-bilirubin pigment.*

The patients' primary physician received a copy of the patients' results for further management. Results for these tests within the last 6 months were also considered relevant.

4.4 Microbiological Procedures

On Day 1, specimens were inoculated on Sheep Blood Agar and CLED Media under aerobic conditions for the isolation of aerobic bacteria. Inoculation on culture media was performed through the streak method after Cheesbrough [117]. The plates were streaked aseptically with a sterile wire-loop to form discrete colonies. The media plates were then incubated at 35°C – 37°C for 24- 48 hours under aerobic conditions. On Day 2, positive growth was noted as colonies on the culture media. The most predominant colony was isolated using a sterile swab and specific tests such as Gram stain, motility, oxidase test, catalase test, carbohydrate utilization tests, indole formation and citrate tests were performed for preliminary identification. The VITEK ® 2 machine (bioMe´rieux, Durham, United States) was then utilized for further identification (ID) and antibiotic sensitivity tests (AST). The patients' primary physician also received a copy of the patients' results for further management. The specimen swab was then stored at –20ºC to −80 °C for subsequent DNA isolation and PCR analysis.

4.5 Identification and Antimicrobial Sensitivity Tests using the VITEK ® 2 Machine

On Day 2, the most predominant colony from the culture plate was again isolated for further tests. Pairs of ID and AST cards were set on the VITEK ® 2 cassete and scanned on the barcode reader so that the patient details (study ID number, name, hospital number and medical department) could be entered into the computer. The straws on the ID and AST cards were kept facing away from the technician to avoid contamination.

For the ID cards, 3ml of normal saline was drawn from the dispenser onto plastic test tubes. The most predominant colony was again picked from the culture plate and diluted with the saline. Using a pipette, the solution was mixed up by draining in and out water. The test tube was then placed onto a turbidometer/turbidity check and the machine turned on. The test tube was rotated 360^{o} and the reading taken. The target MacFarland's turbidity was 0.5 to 1.0 standards. If the solution was of lower turbidity than 0.5 standards, more bacterial isolates were picked from the culture plate and added into the solution. On the other hand, if the turbidity was higher than 1.0 standards, more saline was added to further dilute the solution while taking care not to contaminate the dispenser.

For the AST cards, 3ml of normal saline was drawn from the dispenser. One millimetre (1 ml) of dissolved bacteria was transferred from the ID test tubes and added to the 3ml of the AST test tubes to further dilute the mixture. The straws of the ID and AST cards were then dipped into their respective test tubes and the cassette placed into the filling chamber of the VITEK ® 2 machine and the machine turned on. Once the filling of the cards was complete, the cassette was moved from filling chamber VITEK ® 2 machine to the incubation chamber. The cards took roughly 8hrs in the incubation chamber at a temperature of 35 - 37^{o}C. Quality control was assured through the use of positive controls of previous specimens stored in the laboratory.

Antimicrobial sensitivity tests were performed using a set 20 different types of antibiotics for each sample. The types of antibiotics included beta-lactamase, cefoxitin screen, oxacillin, benzylpenicillin, gentamicin, tobramycin, levofloxacin, moxifloxacin, inducible clindamycin resistance, erythromycin, clindamycin, linezolid, teicoplanin, vancomycin, tetracycline, trimethoprim/sulfamethoxazole, ampicillin, amoxicillin/clavulanic acid, ampicillin/sulbactam, piperacillin/tazobactam, cefazolin, cefuroxime, cefuroxime axetil, cefotaxime, ceftazidime, ceftriaxone, cefepime, aztreonam, meropenem, amikacin, ciprofoxacin, nitrofurantoin, cefoxitin, tigecycline, fosfomycin, fusidic acid, mupirocin, and rifampicin.

The ID and AST results were delivered to the patients' primary physician for further management and another copy for the Research records. MDROs are bacteria that are resistant to more than one or all classes of antibiotics. To be classified as MDROs, *S. aureus* was resistant to methicillin, oxacillin or other penicillin and Gram-negative bacilli resistant to third generation cephalosporins [83]. All samples positive for were stored for further molecular testing.

Figure 1: VITEK ® 2 *(a) Image of machine (b) Dilution of cultured pathogens (c) ID and AST cassettes*

4.6 Molecular tests

4.6.1 Procedure of DNA extraction & RT-PCR Amplification

Wound swabs were stored at – 70^{o}C and transported to KAVI-ICR Molecular Laboratory (located within KNH) at the end of the study. Dulbecco's phosphate buffered solution (Sigma ® - Aldrich, Steinheim, Germany) in quantities of 200µL to 500µL was added to the wound swabs to dissolve the specimen. Both manual and automated methods were employed for DNA extraction. Manual DNA extraction was performed using QIAMP DNA Blood Minikit (250), Qiagen Hilden, Germany according to manufacturer's instructions. Automated extraction was performed using QIASymphony Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Identification of resistant *Staphylococccus sp* was confirmed by screening for presence of *mecA* gene using real-time PCR as previously described [118]. PCR-amplification and real-time hybridization was carried out using the MRSA Quant Real-TM kit (Sacace™ Biotechnologies, Como, Italy). *S. aureus* DNA was detected in the FAM/Green channel, MRSA DNA (amplification

of *mecA* fragment) was detected in the JOE/HEX/Yellow channel, and Internal Control (IC) DNA was detected in the ROX/Orange channel using RotorGene Q (Hilden, Qiagen). Amplification was set up in a 1.0µl PCR tubes containing 15µL of PCR Master Mix (PCR mix-1 FRT MRSA, PCR-mix-2 FRT, TaqF polymerase, and Internal Control). The sequences for actual primers and probes used from the commercial from Sacace™ Biotechnologies, Como, Italy were proprietary information and not revealed to the general public.and not revealed. However, the table below shows a sample sequence of probles and primers for S. aureus used in a previous study [119].

S. aureus	
FORWARD PRIMER	5'-ACGA CTARATAA ACGCTCAT TCG-3'
PROBE	5'-HEX-TGAAAT CTCATTACGT TGCATCGGA- BHQ1-3'
REVERSE PRIMER	5'-GACGGC TTTTACAT ACAGAA CACA-3'

Table 4.3: Nucleotide sequence of primers and probes for S. aureus from a Universal kit. *Adapted from Hopman and colleagues* [119].

Quality control of the RT-PCR tests was further assured by running 4 additional samples alongside the 51 specimen: Positive control, Negative control, DNA Quality Standard (QS) 1 MRSA and DNA QS2 MRSA. The reaction tubes were subjected to Thermal cycling reactions on a Rotor-Gene Q machine (Qiagen, Hilden, Germany) comprising of 15 min at 95°C followed by 5 cycles of 15sec at 95°C, 30 sec at 60°C, and 15 sec at 72°C, and finally 40 cycles of 15 sec at 95°C, 30 sec at 55°C and 15 sec at 72°C for measuring fluorescent signal.

Figure 2: DNA extraction and PCR amplification Equipment. *(a) QIASymphony for automatic DNA extraction (b) Rotor-Gene Q (Qiagen, Germany)*

4.6.2 Interpreting the RT-PCR results

The PCR reports were generated using Rotor-Gene Q-Rex Series Software 2.3.1. (Build 49). The RT-PCR reports included sigmoid curves of the 3 channels: Green (*S. aureus* DNA), Yellow (MRSA DNA) and Orange (Internal Control). The PCR cycle threshold (C_t) used was 20 since that was where the exponential curve of the Internal Control (Orange channel) begun. Samples $\geq$ 20 were considered positive for the DNA tested. However, comparisons between the DNA concentrations in copies/ml of the Green and Yellow channel were used to definitely determine if the RT-PCR test was truly positive. The C_t values and the Calculated concentration (copies) reported by the software were used to calculate the concentration of DNA in copies/ml using formulas in the manufacturer's kit (Refer to Table 4.4 below). Finding the values of the DNA concentrations of the QS1 and QS2 standards to be within the expected ranges 100,000 copies/ml and 100 copies/ml respectively verified that the calculations of the DNA copies was accurate.

FAM (S.aureus)	***JOE (mec A gene)***	***ROX (IC)***	***Result (copies/ml)***
+	-	+/-	*MSSA (= (A/C)*IC coefficient*N)*
-	+	+/-	*MRCoNS (= (B/C)* IC coefficient*N)*
+	+	+/-	*MRSA (= (B/C)* IC coefficient *N)*
A – concentration in FAM/Green channel *B – concentration in JOE/HEX/Yellow channel* *C – concentration in ROX/Orange channel* *N=100 / extraction volume, ml* *IC coefficient is specific as provided in the kits manual*			

Table 4.4: Formula for the calculation of DNA concentration in copies/ml using manufacturer's instructions

4.7 Data collection procedures

Data was collected using a structured questionnaire designed by the investigators. The questionnaire was pretested to a small sample of 10 diabetic patients to determine whether respondents had any difficulty in understanding the questions or if any questions were ambiguous or biased. A data collection form was used to collect results of the laboratory procedures. A UoN Research Notebook was also used to record data collection processes, laboratory record keeping and meetings with supervisors in line with good laboratory practices and university protocols.

4.8 Data management and analysis

Data was entered into a template Microsoft ® Excel spreadsheet designed by the study statistician. Scalar variables were explored to check for normality. A null hypothesis that data was not normally distributed was tested using Shapiro-Wilk normality test. When the $p>0.05$ the null hypothesis for normality was rejected and the data shown to be normally distributed. Mean and Standard deviation (SD) were used as measures for central tendencies for normally distributed data while median and interquartile range (IQR) for data not normally distributed. Variables such as age, time and, blood tests were classified using cut-off points by transforming the data and recoding it into a nominal values. Nominal variables were analyzed as frequencies and percentages. Data was also presented in the form of diagrams, tables, cross-tabulations, stem and leaf plots, box plots, pie charts, bar charts and histograms where appropriate. Independent 2-tailed t-test was used to compare means of continuous variables across gender with the Levene's test being used to determine equality of variances. Chi-square test was used to verify association of categorical variables across gender with Pearson's Chi-square test being used when 0 cells had expected count less than 5 and Fischer's Exact test when at least one cell had expected count less than 5. Comparison across multiple groups (e.g. Clinical Outcomes) was performed using one-way ANOVA. The results were considered statistically significant at $p < 0.05$. All analyses were performed using IBM® SPSS® Statistical Package Version 23.0.

4.9 Ethical consideration

Approval was sought from the KNH-UoN Ethics and Research Committee; Approval Number P769/10/2016. Permission to carry out the study was also sought from the Head of Medical Department, KNH. For confidentiality the patient's, laboratory records and medical files were confined to the KNH Wards, Laboratories and the Hospital's Registries department. The patients' full name was not included in the data collection form. Instead, the patients' initials and a unique identifier number were recorded. Only the investigator had access to both the laboratory records and medical files for the purposes of the study. Raw data in form of filled forms was filed in a secure cabinet only accessible to the investigator. Soft copy data was stored in a password protected computer.

4.10 Study limitations

We only enrolled patients in the medical wards and diabetic clinic into the study. This was a limitation to the study because patients with diabetes are also routinely treated and admitted to other departments of the hospital. However, patients with DFU are mostly found in the medical units.

We recruited fewer patients per day than expected. This was due to the seasonality of cases in the clinic. Similar patients are usually seen at the clinic for a couple of months before they are discharged following wound healing or closure and then another batch of new patients is recruited to the clinic. There was also a strike of healthcare workers at KNH and other hospitals leading to fewer patients seeking care at the study site. KNH is mainly a referral hospital and patient flow was affected by this disruption. The stud lasted 12 months; requiring the investigators to seek an extension of the Ethical approval from KNH-UoN ERC.

4.11 Dissemination plans

The results of the study were disseminated to the Medical department of KNH, KNH Microbiology Laboratory and will be shared with the UoN library and UNITID library. Since the study was partly funded by public resources, systematic reviews and original articles will be submitted to high-quality, peer-reviewed openly accessible journals and online repositories including the UoN Library Digital Repository.

4.12 Study Risks

We performed an observational cross-sectional study design. The procedures performed were part of standard care and present minimal risks. Outpatients were interviewed while awaiting to see their primary physician. Inpatients were interviewed at a convenient time during the day. The interview lasted 10 minutes and was restricted to questions on DM, and DFUs. There was minimum discomfort during wound site preparation and drawing of blood samples. A qualified clinician performed these procedures ensuring that the patient did not undergo unnecessary stress and pain. Although no monetary compensation was given, patients benefitted by receiving the culture and sensitivity results of their DFUs within 3-4 days at no fee. We were also not testing for any therapeutic intervention and so the patients were at no risk of developing side effects or adverse effects. Any new diagnosis made during the study was reported to the patients' primary physician.

5. Chapter Five: Results

5.1 Flow Chart

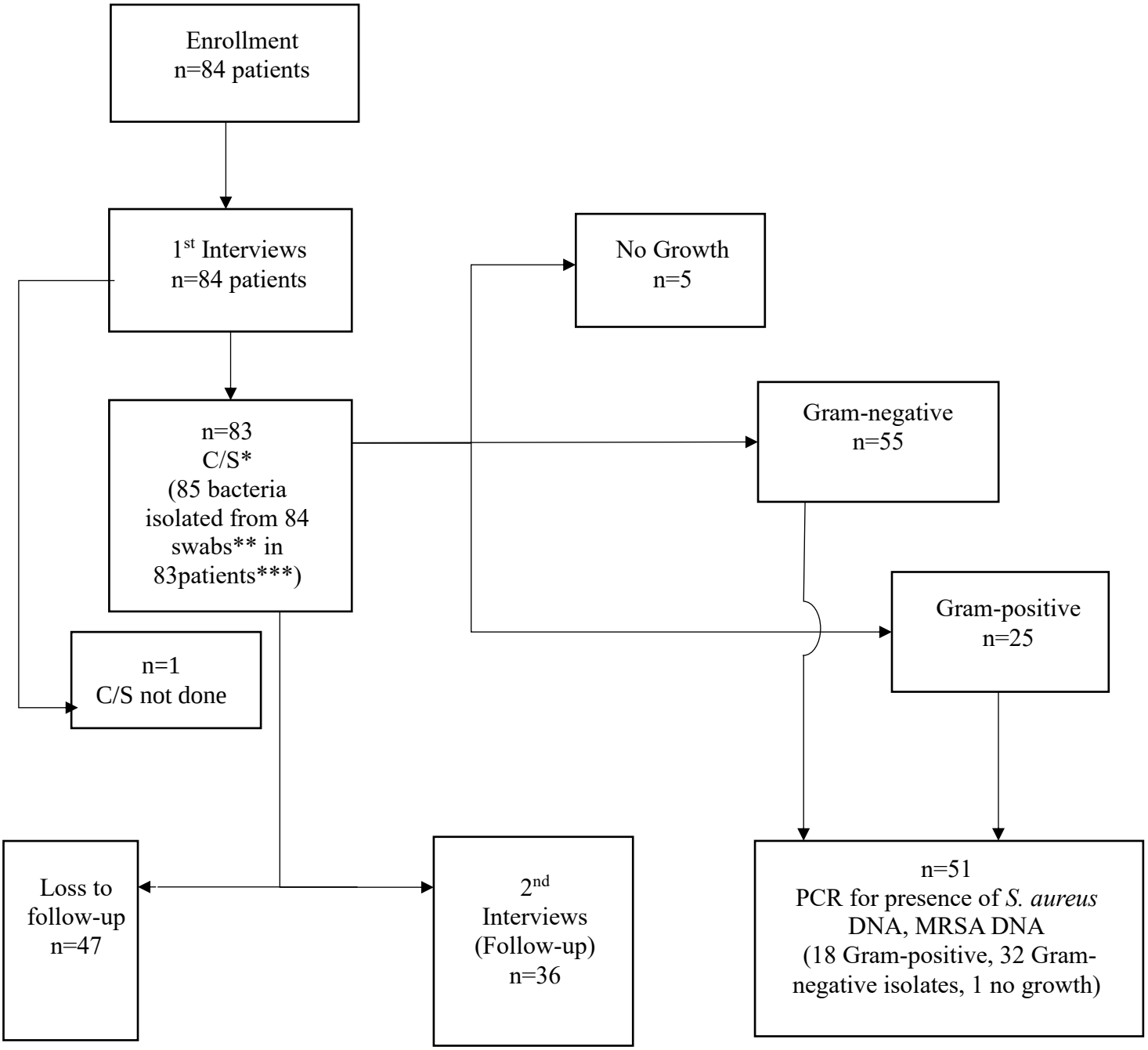

Chart 1: Flow chart of the study. *It depicts recruitment of patients into the study and study directed activities; *C/S = Culture and Sensitivity; **One patient had 2 swabs collected from 2 separate ulcers from both feet; *** One other patient had 2 different organisms isolated from the same ulcer. For all other patients, 1 organism, the most predominant and more likely pathogenic was isolated.*

5.2 Patients' socio-demographic characteristics

	Population	*n (%)*
Sex	Male	39 (45.9)
	Female	46 (54.1)
Age Group	≤ 30	1 (1.2)
	31-50	15 (18.1)
	51-65	42 (50.6)
	66-80	20 (24.1)
	≥ 81	5 (6.0)

		Population	*Male*	*Female*	*p value*
Age (years) Mean (SD)		60.30 (12.88)	58.62 (11.38)	61.65 (13.95)	0.29
Education level n (%)	None	12 (15.6)	2 (5.9)	10 (23.3)	**0.001**
	Primary	34 (44.2)	12 (35.3)	22(51.2)	
	Secondary	24 (31.2)	13 (38.2)	11(25.6)	
	Tertiary	7 (9.1)	7 (20.6)	0 (0.0)	
Residence n (%)	Urban	56 (68.3)	29 (78.4)	27 (60.0)	0.097
	Rural	26(31.7)	8 (21.6)	18 (40.0)	

Table 5.1: Sociodemographic characteristics of study. *The table shows sociodemographic characteristics of the study patients. Ages were classified into 5 groups. The lower part of the table shows differences in variables across gender that were analysed using Chi-square test and considered statistically significant at $p < 0.005$*

We enrolled 84 patients into the study. Most of the study patients were inpatients (68.4%). The patients underwent a questionnaire-based interview at the start of the study to determine sociodemographic and clinical findings. The mean age was 60.30 ± 12.88 years. Most patients were in their fifth and sixth decades; 50.6% were 51-65 years while 24.1% were 66-80 years. The youngest patient was 25 years while the oldest was108 years. The females were 54.1%. Most of the patients (68.3%) lived in urban areas while 31.7% lived in rural areas. A majority (44.2%) had primary education and 15.6% did not have any formal education. The difference in education levels across gender was statistically significant ($p = 0.001$) with males being more educated.

5.3 Clinico-Laboratory Findings

	Population	*n (%)*
Type of Diabetes Mellitus n (%)	Type 1 DM	2 (2.4)
	Type 2 DM	81 (96.4)
	Gestational DM	1 (1.2)
History of Diabetes Mellitus n (%)	Yes (Previously diagnosed)	75 (91.5)
	No (Newly diagnosed)	7 (8.5)
Duration of DM (years) median (IQR)	**6.5 (11.25*)**	
Duration of DM (years) n (%)	<5	36 (46.2)
	6 - 10	18 (23.1)
	10 - 20	19 (24.4)
	>21	5 (6.4)
Type of Treatment n (%)	None	2 (2.4)
	Diet	1 (1.2)
	OHA	15 (17.6)
	Insulin	38 (44.7)
	Both Diet and OHA	2 (2.4)
	Both OHA and Insulin	27 (31.8)

Risk factors		Total n (%)	Male n (%)	Female n (%)	p value
Smoking habits	Yes	13 (17.3)	11 (31.4)	2 (5.0)	**0.004**
	No	62 (82.7)	24 (68.6)	38 (95.0)	
Alcohol intake	Yes	25 (33.3)	18 (51.4)	7 (17.5)	**0.003**
	No	50 (66.7)	17 (48.6)	33 (82.5)	
Number of Comorbidities	None	14 (16.5)	7 (17.9)	7 (15.2)	0.509
	1	33 (38.8)	15 (38.5)	18 (39.1)	
	2	19 (22.4)	7 (17.9)	12 (26.1)	
	3	13 (15.3)	5 (12.8)	8 (17.4)	
	4	5 (5.9)	4 (10.3)	1 (2.2)	
	5	1 (1.2)	1 (2.6)	0 (0.0)	

Clinical Parameters	Total Mean (SD)	Male	Female	p value
Systolic Blood Pressure (mmHg)	136.05(34.51)	138.25 (34.72)	138.39 (24.77)	0.985
Diastolic Blood Pressure (mmHg)	80.08 (18.74)	80.56 (19.47)	80.61 (12.41)	0.991

Laboratory Parameters	n	Total Mean (SD)	Total Median (IQR)	Male	Female	p value
RBS (mmol/L)	62	14.12 (11.03)	9.60 (8.95)	10.08 (4.96)	11.57 (7.28)	0.373
HbA1c (%)	31	8.40 (2.29)	8.80 (2.80)	9.59 (3.75)	8.51(1.74)	0.358
Urea (mmol/L)	61	12.05 (10.58)	7.1 (11.85)	19.36 (20.90)	9.67 (16.68)	**0.049**
Creat (mmol/L)	56	147.53 (74.06)	114.55 (111.2)	228.19 (156.38)	133.12 (100.72)	**0.015**
LDL-C (mmol/L)	27	1.40 (0.34)	1.51 (0.81)	1.65 (0.90)	1.82 (0.79)	0.627
Albumin (g/L)	44	29.97 (6.81)		28.5 (7.53)	30.66 (6.46)	0.333

Table 5.2: Clinical characteristics and laboratory parameters of study patients. *Table represents the frequency, percentages, means (SD) and median (IQR) of various clinico-laboratory parameters. Mean (SD) was reported when data was normally distributed while median (IQR) was reported when data was not normally distributed following normality tests. Where both mean and median are reported, the data was not normally distributed but mean was calculated for comparison with the difference in means across gender. Duration of DM classified into 4 groups. Chi-square used to determine difference in proportions across gender. Statistically significant* $p < 0.005$. *n=sample size*

The median (IQR) duration of DM was 6.5 (1.25, 12.5). A small proportion, (8.5%) of patients were newly-diagnosed with diabetes, 17.3% had ever smoked cigarettes while 33.3% had ever taken alcohol. Males were more likely to be smokers ($p = 0.004$). Fourteen patients (16.5%) had no comorbidities, while the rest of the patients (83.5%) had one or more co-morbidities: fifty-one patients (61.44%) had hypertension, 18 patients (21.69%) had kidney disease, 10 (12.05%) had heart disease, 8 (9.64%) had anaemia, 7 (8.43%) had brain disease, 5 (6.02%) had cellulitis, 4 (4.82%) had sepsis and 1 (1.20%) had high cholesterol. Most of the study patients had T2DM and were currently on medication; 44.7% were on insulin, 17.6% oral anti-hyperglycemic agents and

17.6% on a combination of both insulin and oral drugs. The maximum duration of DM was 30 years.

The mean blood pressures (BP) were within normal ranges but 39.7% had hypertension and severe hypertension based on systolic BP whereas 23.3% had high blood pressure and severe hypertension based on diastolic BP. The median RBS (IQR) was normal at 9.60 (5.32, 15.45) mmol/L while the median (IQR) HbA1c was elevated at 8.80 (7.17, 9.92)%. Refer to Table 4.2 for normal reference values (*Chapter 4*). Glycaemic control was poor in 31.8% of based on RBS and 83.9% of patients had elevated HbA1c levels. The median (IQR) urea levels were normal, 7.1 (11.85) mmol/L, while the mean and median (IQR) creatinine levels were markedly elevated, 114.55 (111.2) mmol/L. Females had better urea ($p = 0.049$) and creatinine levels ($p= 0.015$). Thirty-nine percent (39.3%) of patients had high urea levels and 58.9% had elevated creatinine levels. A majority of the patients had desirable T. Chol levels (84.6%), TGs levels (63%), LDL-C levels (70%), while a small proportion had the best HDL-C levels (7.7%), and HDL/LDL ratio (12.5%). Seventy percent (70.5%) of the patients had low albumin levels. A large number of patients did not have recent laboratory results; 63.53% lacked HbA1c levels, 70% lacked lipid profile tests, 47% lacked liver function tests and 30% lacked kidney function tests.

The median (IQR) duration of the DFU was 8 (4, 16) weeks with 37.5% having lasted for less than 6 weeks and 52.8% between 7 and 26 weeks. The longest duration of DFU was 312 weeks. Half of the patients had a previous history of DFU and a third had a prior history amputation. At the time of the study, 77.6% had recently used antibiotics to treat the ulcer. A majority (88.10%) of the patients had ulcers on only one foot. We analysed results for 92 out of 94 ulcers in total. Forty-nine ulcers (53.26%) were located on the right foot and 43 ulcers (46.74%) on the left. On the right side, DFUs were on the forefoot (51%) and hindfoot (22.4%).and were mainly neuropathic (51%) or ischaemic (40.8%). On the left foot, DFUs were mainly located on the forefoot (55.8%) and hindfoot (37.2%) of ischaemic (51.2%) or neuropathic (41.9%) type. The majority of the ulcers on both feet were Wagner Stage 1 and 2.

	Category	*n (%)*
Duration of DFU (weeks) 8 (12*)		
Duration of DFU (weeks) n (%)	>6	27 (37.5)
	7 to 26	38 (52.8)
	27-52	7 (9.7)
	>52	0 (0.0)
History of previous DFU n (%)	Yes	40 (50)
	No	40 (50)
History of Previous Amputation n (%)	Yes	26 (33.3)
	No	52 (66.7)
History of any recent antibiotic use n (%)	Yes	45 (77.6)
	No	13 (22.4)

		RIGHT n (%)	LEFT n (%)	TOTAL n (%)
Anatomic Site of foot ulcer	Forefoot	25 (51.0)	24 (55.8)	49 (53.3)
	Midfoot	7 (14.3)	2 (4.7)	9 (9.8)
	Hindfoot	11 (22.4)	16 (37.2)	27 (29.4)
	Forefoot & Midfoot	4 (8.2)	0 (0.0)	4 (4.4)
	Midfoot & Hindfoot	1 (2.0	0 (0.0)	1 (1.1)
	Forefoot, Midfoot & Hindfoot	1 (2.0)	1 (2.3)	2 (2.2)
Type of Ulcer	Ischaemic	20 (40.8)	22 (51.2)	42 (45.6)
	Neuropathic	25 (51.0)	18 (41.9)	43 (46.7)
	Neuro-Ischaemic	4 (8.2)	3 (7.0)	7 (7.6)
Wagner Stage	0	1 (2.0)	0 (0.0)	1 (1.1)
	1	11 (22.4)	16 (37.2)	27 (29.4)
	2	16 (32.7)	12 (27.9)	28 (30.4)
	3	10 (20.4)	9 (20.9)	19 (20.7)
	4	10 (20.4)	4 (9.3)	14 (15.2)
	5	1 (2.0)	2 (4.7)	3 (3.3)

Table 5.3 Clinical characteristics of diabetic foot ulcers of the study patients*. Table represents duration, types, location and other clinical characteristics of DFUs among the study patients. Proportions and median (IQR) have been calculated and differences across gender determined using Chi-square. *The interquartile range of Duration of DFU was from 4 to 16.*

5.4 Antimicrobial tests

The results below describe the identification of the isolates and their sensitivity patterns to commonly used antibiotics.

5.4.1 Distribution of bacterial pathogens isolated from DFUs

We isolated the most predominant growth on culture plate per specimen. Out of 85 culture and sensitivity tests performed from 83 patients, 78 swabs had mono-microbial growth, 1 had poly-microbial growth (2 isolates) and 5 had no growth. Most organisms, 55 (64.71%) were Gram-negative and 25 (29.41%) organisms were Gram-positive. The most common organisms isolated were *S. aureus (*16.47%), *E. coli* (15.29, *P. mirabilis* (10.59%), *K. pneumonia* (7.06%) and *P. aeruginosa* (7.06%). Some of the other rare bacteria isolated from patients included *Staphylococcus lentus, Staphylococcus simulans, Staphylococcus xylosus, Acinetobacter baumanni, Burholderia cepacia, Kocuria kristanae, Leuconostoc mesenteroides, Pantoea agglomerans, Providencia stuartii* and *Raoultella ornithinolytica*.

5.4.2 Antibiotic Resistance Patterns

5.4.2.1 Resistance patterns for Gram-positive bacteria

S. aureus was highly resistant to benzylpenicillin and TMPSMX but sensitive to cefoxitin, oxacillin, nitrofurantoin, levofloxacin, linezolid, and vancomycin. There was no MRSA identified using microbiology tests. Isolates of *S. epidermidis, S. intermidius* and *S. simulans* were either 100% resistant, 50% resistant or 100% sensitive to the antibiotics tested (Refer to Table 5.4).

5.4.2.2 Resistance patterns for Gram-negative bacteria

E. coli were highly resistant to ampicillin, aztreonam, cefuroxime and TMPSMX but sensitive to amikacin and nitrofurantoin. *P. mirabilis* and *K. pneumonia* showed a similar resistance to ampicillin but sensitive to amikacin. *P. aeruginosa* was sensitive to nitrofurantoin, TMPSMX, ampicillin, amoxicillin-clavulanic acid, ciprofloxacin, ceftazidime and aztreonam. *S. fonticola* species showed resistance to TMPSMX, ampicillin, amoxicillin, piperacillin-tazobactam, cefazolin, ceftazidime, and cefepime. Refer to Table 6.3 (*Appendix*).

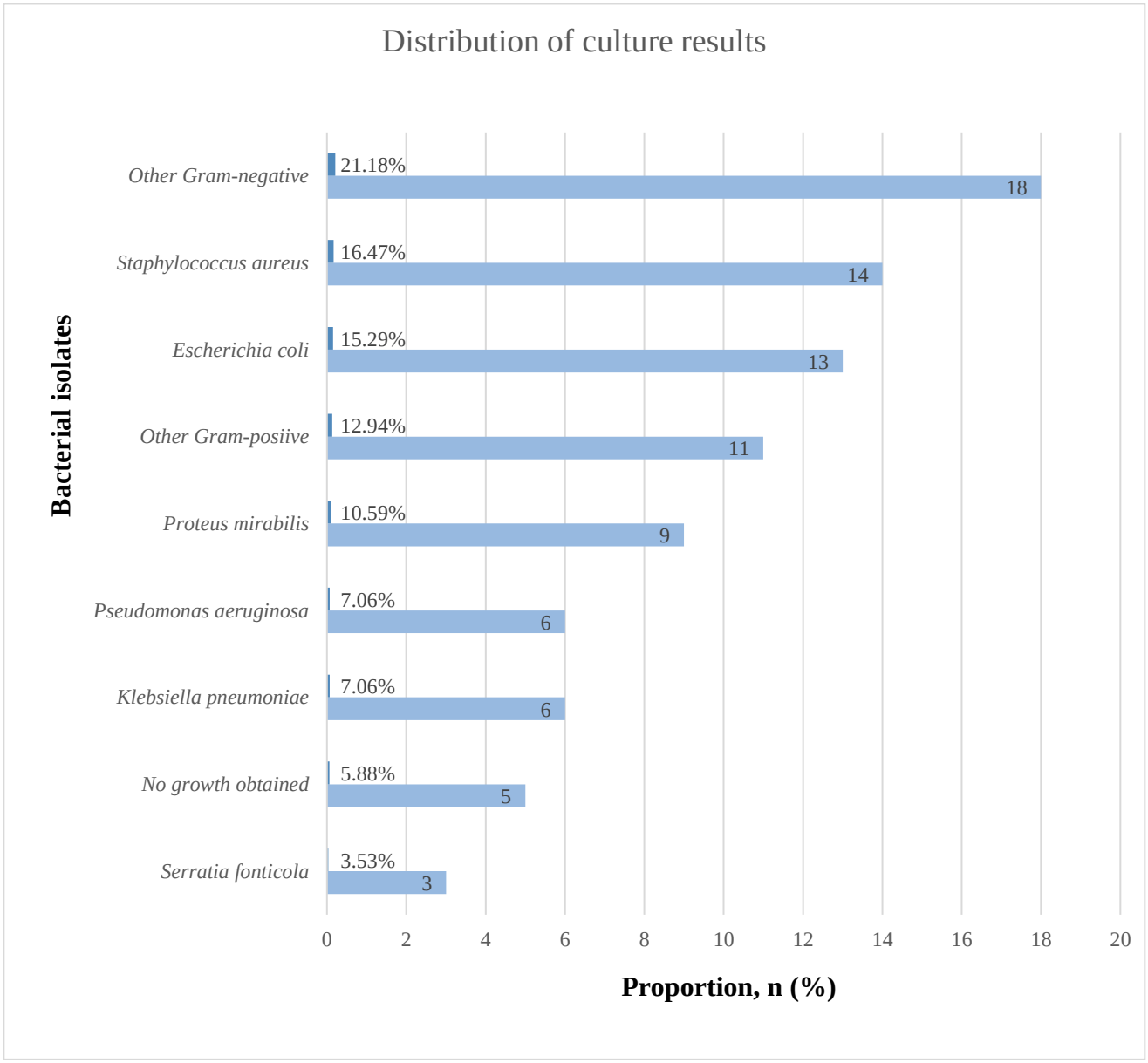

Chart 2: Distribution of Gram-positive and Gram-negative isolated*. It shows the organisms identified from the study patients and the distribution in counts and percentage in descending order. Refer to Table 6.1 (Appendix) for the full list of organisms*

	n (%)			
	Staphylococcus aureus n=14	*Staphylococcus epidermidis n=2*	*Staphylococcus intermedius n=2*	*Staphylococcus simulans n=1*
Benzylpenicillin	12 (85.7)	2 (100.0)	2 (100.0)	1 (100.0)
Beta Lactamase	0 (0.0)	ND	2 (100.0)	ND
Cefoxitin Screen	0 (0.0)	1 (50.0)	ND	ND
Clindamycin	2 (14.3)	0 (0.0)	1 (50.0)	0 (0.0)
Erythromycin*	3 (21.4)	0 (0.0)	1 (50.0)	0 (0.0)
Gentamicin*	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Inducible Clindamycin* Resistance	1 (7.1)	0 (0.0)	0 (0.0)	0 (0.0)
Levofloxacin*	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Linezolid*	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Nitrofurantoin*	0 (0.0)	0 (0.0)	ND	ND
Oxacillin	0 (0.0)	1 (50.0)	ND	0 (0.0)
Rifampicin	0 (0.0)	0 (0.0)	ND	0 (0.0)
Teicoplanin*	0 (0.0)	1 (50.0)	1 (50.0)	0 (0.0)
Tetracycline	2 (14.3)	1 (50.0)	1 (50.0)	1 (100.0)
Tigecycline*	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Tobramycin	1 (7.1)	0 (0.0)	0 (0.0)	0 (0.0)
Trimethoprim-Sulfamethoxazole	7 (50.0)	1 (50.0)	1 (50.0)	0 (0.0)
Vancomycin*	0 (0.0)	0 (0.0)	1 (50.0)	0 (0.0)

Table 5.4 Resistance patterns for Gram-positive organisms*. The table depicts the number of organisms resistant to antibiotics and their proportions (%) based on the total organisms isolated per species. n = Total number of organisms per species; ND = test for a particular antibiotic was not determined; * Commonly used antibiotics at KNH* [120].

	n (%)			
	Escherichia coli n=13	*Proteus mirabilis n=9*	*Klebsiella pneumonia n=6*	*Pseudomonas aeruginosa n=6*
Amikacin*	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)
Amoxicillin ClavulanicAcid*	7 (53.8)	0 (0.0)	4 (66.7)	ND
Ampicillin*	13 (100.0)	6 (66.7)	5 (83.3)	ND
Ampicillin-Sulbactam	8 (61.5)	0 (0.0)	3 (50.0)	ND
Aztreonam	11 (84.6)	2 (22.2)	2 (33.3)	ND
Cefazolin	12 (92.3)	3 (33.3)	3 (50.0)	3 (50.0)
Cefepime*	9 (69.2)	3 (33.3)	2 (33.3)	1 (16.7)
Cefotaxime	9 (69.2)	3 (33.3)	2 (33.3)	6 (100.0)
Cefoxitin	4 (30.8)	0 (0.0)	0 (0.0)	ND
Ceftazidime*	9 (69.2)	3 (33.3)	2 (33.3)	0 (0.0)
Ceftriaxone*	9 (69.2)	3 (33.3)	2 (33.3)	2 (33.3)
Cefuroxime*	12 (92.3)	3 (33.3)	5 (83.3)	0 (0.0)
Cefuroxime Axetil	9 (69.2)	2 (22.2)	2 (33.3)	1 (16.7)
Ciprofoxacin*	5 (38.5)	1 (11.1)	2 (33.3)	0 (0.0)
Gentamicin*	2 (15.4)	0 (0.0)	3 (50.0)	1 (16.7)
Meropenem*	3 (23.1)	0 (0.0)	1 (16.7)	0 (0.00)
Nitrofurantoin*	0 (0.0)	3 (33.3)	1 (16.7)	ND
Piperacillin-Tazobactam*	9 (69.2)	0 (0. 0)	2 (33.3)	1 (16.7)
Trimethoprim-Sulfamethoxazole	10 (76.9)	5 (55.6)	4 (66.67)	0 (0.00)

Table 5.5 Resistance patterns for Gram-negative organisms. *Table depicts the number of Gram-negative organisms resistant to antibiotics and their proportions (%) based on the total organisms isolated per species. n = Total number of organisms per species; ND = test for a particular antibiotic was not determined; * Commonly used antibiotics at KNH* [120].

5.4.2.3 Distribution of Multiple-Drug Resistant organisms isolated

MDROs were classified as those resistant to different classes of antibiotics. *S. aureus* had to be at least resistant to oxacillin or benzylpenicillin while Gram-negative bacilli had to be at least resistant to a third generation cephalosporins (ceftazidime or ceftriaxone) to be considered as a MDROs. More than half (61.54%) of the *S. aureus* were considered as MDROs while 40.38% Gram-negative bacilli were MDROs. The difference in means in Age, Glycemic Control and

Kidney functions between patients who had MDROs and those who did not was not statistically significant. Refer to Table 6.4 (*Appendix*).

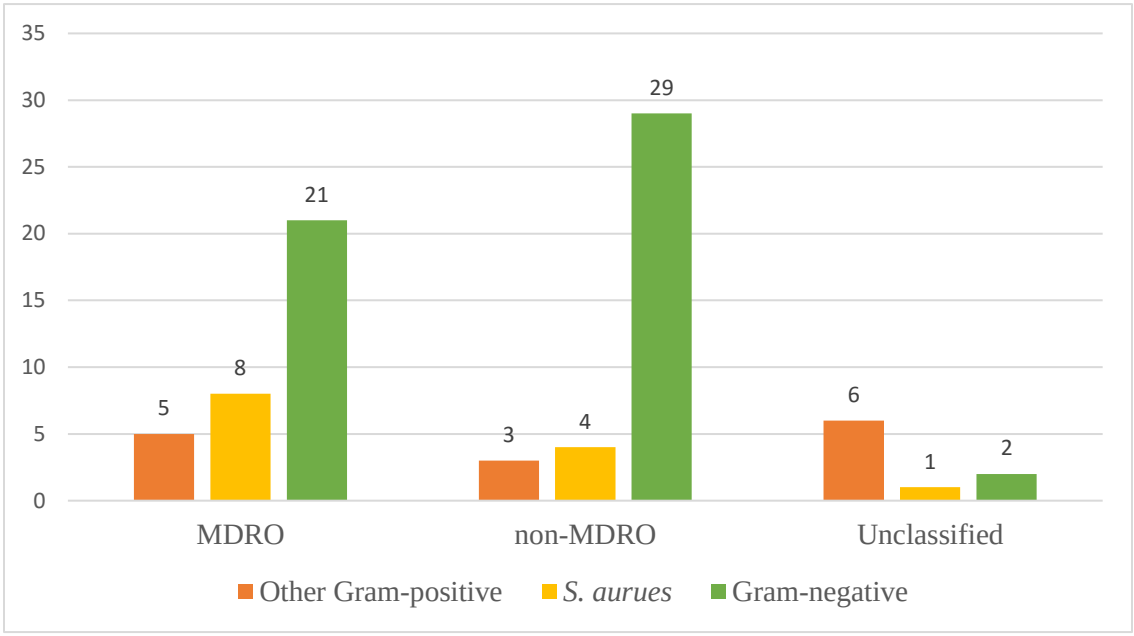

Chart 3: Distribution of MDROs among Gram-positive and Gram-negative Organisms. *The table illustrates the distribution of MDROs among Gram-positive and Gram-negative bacteria. S. aureus and other Gram-positive organisms are displayed on different rows. Antibiotic sensitivity was not determined for tests that showed no growth, Kocuria kristinae, Leuconostoc mesenteroides. MDROs = Multiple Drug Resistant Organisms; Non-MDRO=not resistant to multiple classes of antibiotics; Unclassified= MDRO could not be defined because the organism did not undergo test with definitive antimicrobials. According to Djahmi and colleagues, S. aureus had to resistant to methicillin, oxacillin or other penicillin while Gram-negative bacilli resistant to third generation cephalosporins* [83].

5.5 Molecular Tests

Molecular tests were performed on 51 out of the total 85 samples tested. MRSA For the Gram-positive pathogens, 11 were positive for *S. aureus* while 7 yielded other *Staphylococcus sp.* PCR was considered to be the gold standard for species identification. RT-PCR on the 11 *S. aureus* confirmed 9 of the culture results to be true-positives and 2 to be false-positives. Since no MRSA were detected by culture-based methods, there were therefore 5 false-negatives on the culture results for the 9 *S. aureus species* and 2 false negatives from the other *Staphylococcus sp.*

One sample with other *Staphylococcus sp*. and suspected to have skin contaminants based on microbiological tests was subjected to RT-PCR. The culture result was confirmed to be a false-negative after *S. aureus* DNA was detected by RT-PCR. No antibiotic sensitivity tests were

performed on this sample to rule out cefoxitin resistance. One sample without any growth was also tested for *S. aureus* and MRSA DNA. PCR confirmed the presence of *S. aureus* DNA but not that of MRSA DNA. Of note is that MRSA were not identified using culture methods but were identified using PCR.

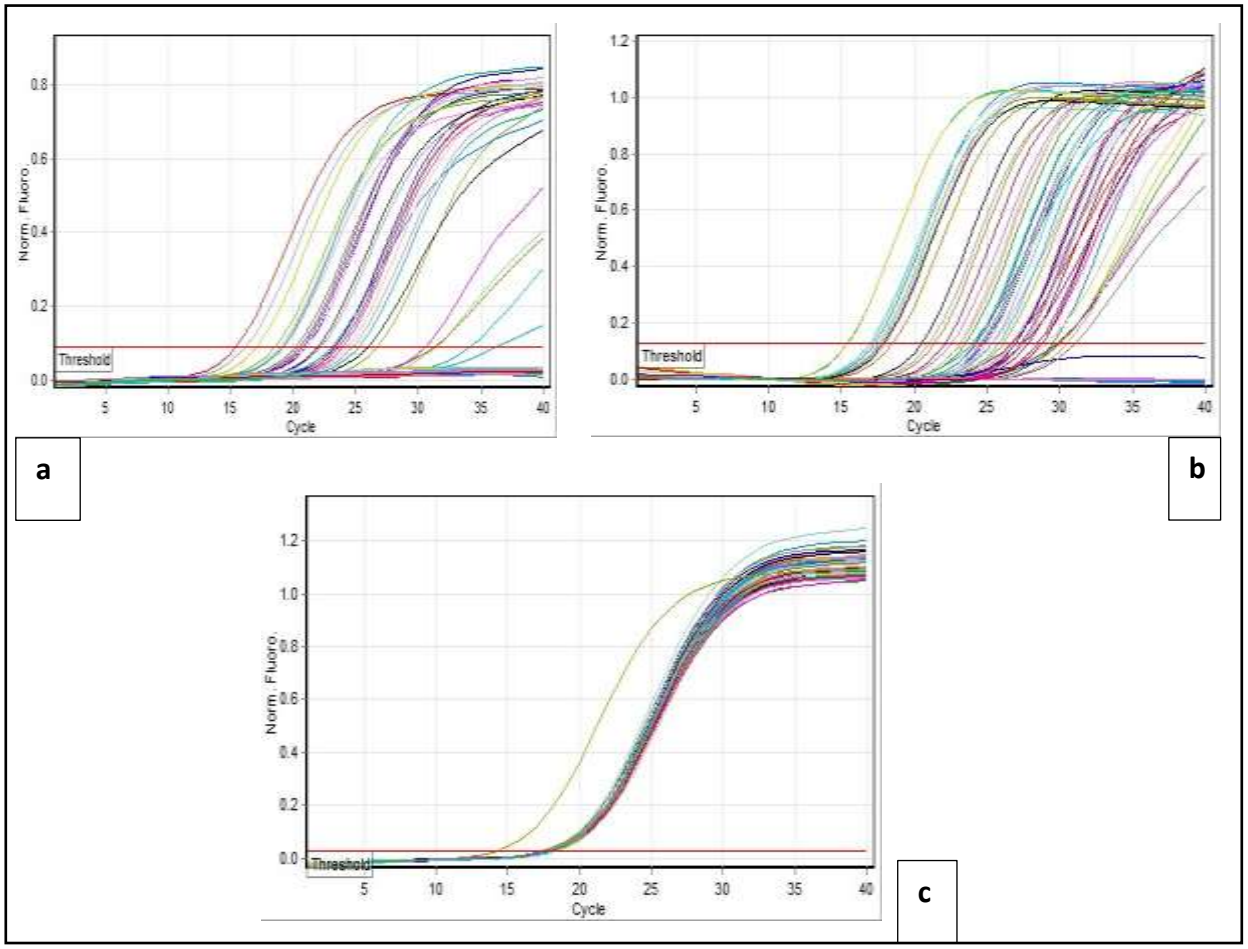

Chart 4: Quantitation data for Cycling A *for a) Green Channel (DNA for Staphylococcus aureus) (b) Yellow Channel (DNA for MRSA) (c) Orange Channel (Internal Control). The C_t used was $\geq$ 20 since the exponential of the sigmoid curve of the Internal Control (Orange Channel) begins at this point.*

	Gram-positive	RT-PCR	
	Culture-positive	*S. aureus*	MRSA
1	*Staphylococcus aureus*	Positive	Positive
2	*Staphylococcus aureus*	Positive	Positive
3	*Staphylococcus aureus*	Positive	Negative
4	*Staphylococcus aureus*	Positive	Negative
5	*Staphylococcus aureus*	Positive	Negative
6	*Staphylococcus aureus*	Negative	Negative
7	*Staphylococcus aureus*	Positive	Positive
8	*Staphylococcus aureus*	Negative	Positive
9	*Staphylococcus aureus*	Positive	Negative
10	*Staphylococcus aureus*	Positive	Positive
11	*Staphylococcus aureus*	Positive	Negative
12	*Staphylococcus epidermidis*	Negative	Negative
13	*Staphylococcus intermedius*	Positive	Positive
14	*Staphylococcus lentus*	Positive	Positive
15	*Staphylococcus pseudintermedius*	Positive	Negative
16	*Staphylococcus simulans*	Negative	Negative
17	*Staphylococcus xylosus*	Negative	Negative
18	*Suspected contaminants isolated*	Positive	Positive

Table 5.6: RT-PCR results for S. aureus and MRSA DNA compared with positive culture results for Gram-positive bacteria.

	Culture-negative	RT-PCR	
		S. aureus	MRSA
1	*No growth obtained*	Positive	Negative

Table 5.7: RT-PCR results for S. aureus and MRSA DNA compared with culture-negative results.

	Gram-negative	RT-PCR	
	Culture results	*S. aureus*	MRSA
1	*Acinetobacter baumannii*	Negative	Negative
2	*Acinetobacter baumannii*	Negative	Negative
3	*Citrobacter freundii*	Positive	Positive
4	*Enterobacter aerogenes*	Negative	Negative
5	*Escherichia coli*	Negative	Negative
6	*Escherichia coli*	Negative	Negative
7	*Escherichia coli*	Negative	Negative
8	*Escherichia coli*	Positive	Negative
9	*Escherichia coli*	Negative	Positive
10	*Escherichia coli*	Negative	Negative
11	*Escherichia coli*	Positive	Positive
12	*Escherichia coli*	Negative	Negative
13	*Klebsiella oxytoca*	Negative	Positive
14	*Klebsiella pneumoniae*	Negative	Negative
15	*Klebsiella pneumoniae*	Negative	Negative
16	*Klebsiella pneumoniae*	Negative	Negative
17	*Pantoea agglomerans*	Negative	Negative
18	*Proteus mirabilis*	Negative	Negative
19	*Proteus mirabilis*	Negative	Negative
20	*Proteus mirabilis*	Negative	Negative
21	*Proteus mirabilis*	Negative	Negative
22	*Proteus mirabilis*	Negative	Negative
23	*Providencia stuartii*	Negative	Negative
24	*Pseudomonas aeruginosa*	Positive	Negative
25	*Pseudomonas aeruginosa*	Negative	Negative
26	*Pseudomonas aeruginosa*	Negative	Negative
27	*Pseudomonas aeruginosa*	Negative	Negative
28	*Raoultella ornithinolytica*	Negative	Positive
29	*Serratia fonticola*	Negative	Negative
30	*Serratia fonticola*	Negative	Negative
31	*Serratia fonticola*	Negative	Negative
32	*Serratia marcescens*	Negative	Positive

Table 5.8: RT-PCR results for S. aureus and MRSA DNA compared with positive culture results for Gram-negative bacteria.

Only 28 samples (87.5%) from the specimens that had Gram-negative bacteria were true-negatives for *S. aureus* while, there were 6 false-negatives for MRSA from these samples. Table 5.9 and Table 5.10 below show the distribution of the organisms. Eighty organisms were subjected to Culture-methods while only 51 underwent RT-PCR.

	S. aureus Detection			
	Gram-positive	Gram-negative	No growth	**Total**
Culture-positive	14 (56.0)	0 (0.0)	0 (0.0)	**14 (17.5)**
Culture-negative	11 (44.0)	55 (100.0)	5 (100.00	**66 (82.5)**
RT-PCR-positive	13 (72.2)	4 (12.5)	1 (100.0)	**18 (35.3)**
RT-PCR-negative	5 (27.8)	28 (87.5)	0(0.0)	**33 (64.7)**

Table 5.9: Frequency and proportion of organisms based on culture and RT-PCR results for S. aureus. *There were 80 culture-positive tests conducted and only 51 RT-PCR tests*

	MRSA Detection			
	Gram-positive	Gram-negative	No growth	**Total**
Culture-positive	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**
Culture-negative	25 (100.0)	55 (100.0)	5 (100.0)	**80 (100.0)**
PCR-positive	8 (44.4)	6 (18.8)	0 (0.0)	**14 (27.5)**
PCR-negative	10 (55.6)	26 981.3)	1 (100.0)	**37 (72.5)**

Table 5.10: Frequency and proportion of organisms based on culture and RT-PCR results for MRSA. *There were 80 culture-positive tests conducted and only 51 RT-PCR tests*

We calculated the sensitivity and specificity of the culture tests for the 51 samples compared to RT-PCR (gold standard). The sensitivity of the VITEK ® 2 machine to detect *S. aureus* was 90.9% while the specificity was 82.5%. The positive predictive value (PPV) was 58.8% while the negative predictive value (NPV) was 2.9%. The sensitivity of the culture tests to detect MRSA could not be calculated due to missing culture-positive results. However, its specificity was 72.7%.

S. aureus			
	RT-PCR- positive	RT-PCR- negative	**Total**
Culture-positive	10	1	**11**
Culture-negative	7	33	**40**
Total	**17**	**34**	**51**

Table 5.11 2 X2 Table for comparison of culture versus RT-PCR results for S. aureus

MRSA			
	RT-PCR positive	RT-PCR negative	**Total**
Culture-positive	0	0	**0**
Culture-negative	3	8	**11**
Total	**3**	**8**	**11**

Table 5.12 2 X2 Table for comparison of culture versus RT-PCR results for MRSA

5.6 Clinical Outcomes of DFUs

The interval between these two dates was calculated and its median (IQR) was 98 (147) days. A majority of the patients were reviewed within 1-3 months after the first interview (28.9%), 3-6 months (28.9%) and 6-12 months (21.1%).

Days	*n (%)*
<30	7 (18.4)
31-90	11 (28.9)
91-180	11 (28.9)
181-365	8 (21.1)
>366	1 (2.6)

Table 5.13: Intervals between 1st interview and 2nd interview. *Table indicates the distribution of the intervals between the first interviews and the second interview.*

We reviewed 36 patients (43.4%) while 47 patients (56.6%) were lost to follow-up. A majority of the patients had good progress; 30.6% had ongoing healing whereas 19.4% had complete healing. However, 25% of the patients on follow-up had non-healing DFUs. The mortality rate was equally high at 11.1%.

Outcome Measure	*n*
Complete Healing	7
Ongoing Healing	11
Non-Healing	9
Major Surgery	1
Minor Surgery	4
Mortality	4

Table 5.14: Distribution of clinical outcomes of DFUs for the study patients*: The table displays the frequency of study patients reviewed with a particular outcome of interest.*

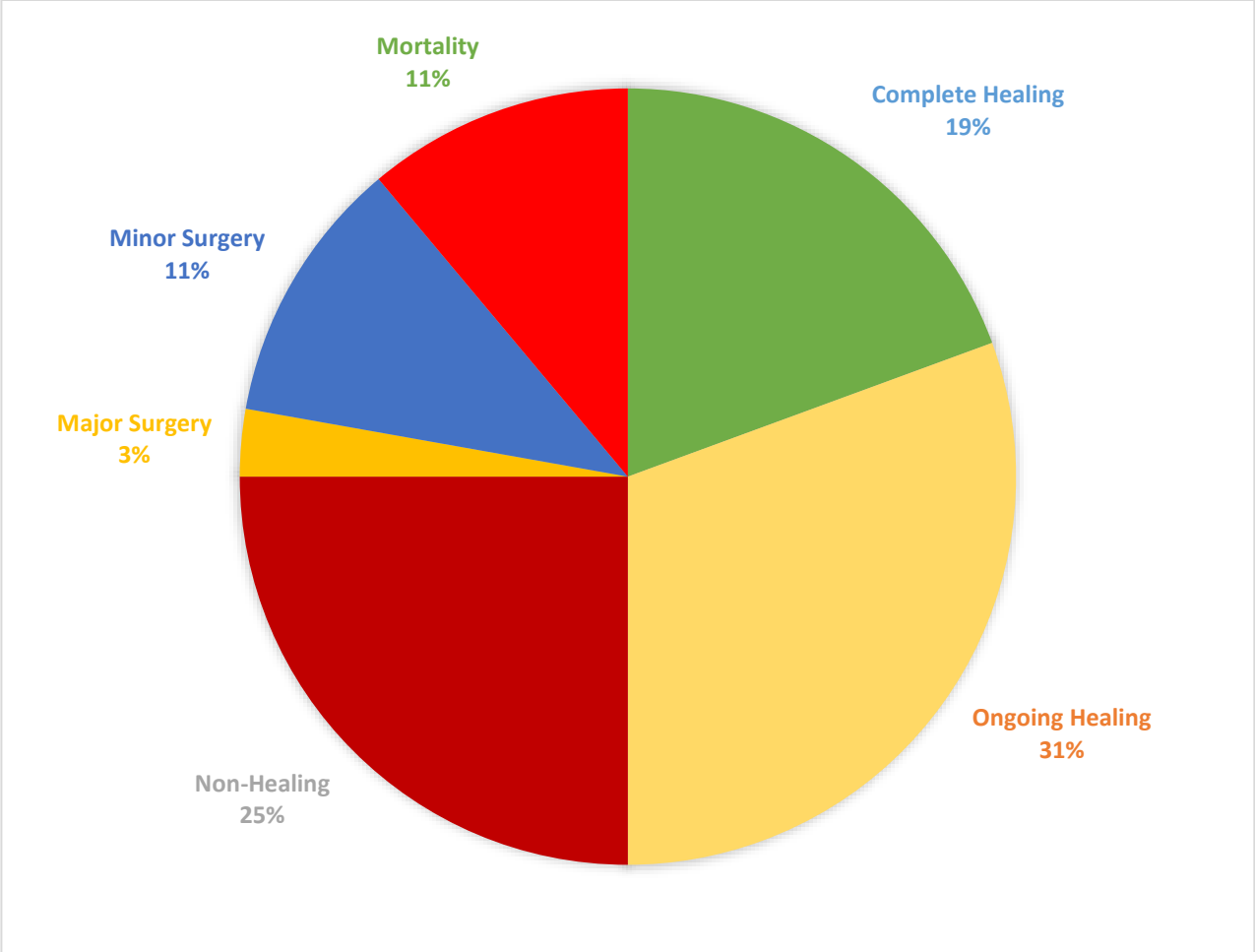

Chart 5: Distribution of clinical outcomes of DFUs for the study patients. *The chart depicts the proportion of study patients reviewed with a particular outcome of interest.*

6. Chapter Six: Discussion

6.1 Prevalence and socio-demographic factors

There is a common notion that the burden of DFUs is much higher in resource-poor countries [18]. The prevalence rate of DFUs among patients with DM varies widely, the highest rate being about 30% in Kenya [17,51]. This wide variation in prevalence is also reported in other African countries, with some studies reporting a prevalence of 20% [18,42–44,47,49,50]. In resource-rich countries, there are country-country variations in the prevalence of DFUs, and to the best of our knowledge, the highest rate is 20% [13,30,36,38–40]. In a meta-analysis the prevalence of DFUs in decreasing order was North America, Africa, Asia and then Europe [121].

In this current study, the prevalence of DFUs was not determined since all the 84 patients enrolled into the study had active DFUs. Based on the rate of previous history of DFU among the study patients, the prevalence of DFUs was 50%, which is higher than previous studies from South Africa, Malawi, Tanzania, Sudan, Ethiopia and Kenya [18,42,45–47,49,50]. This high rate can be explained by the high recurrence rate of DFUs (70%) reported even in facilities that follow the best practices [64]. The high recurrence is disturbing and warrants further investigation to see if patients who have had prior DFUs adhere to proper foot care. Depression, which is often associated with DM, and worsening peripheral arterial disease could also be linked to this poor outcome.

Previous studies in Kenya have reviewed DFUs among both inpatients and outpatients [17,51,52]. Similarly, study patients in this present study were enrolled from both the medical wards and diabetic clinic, inpatients being the majority. This distribution can be accounted for by the fact that DFUs are a major cause of hospitalization for patients with DM [7,8]. The mean age of patients in this study was slightly higher (60.30 years) than in earlier studies [17,18,52]. Most of the study patients were however in their fifth and sixth decades just like in other studies in East Africa [18,47,49,51,52]. In this current study, we had 6% of the patients being older than 80 years. There were more females than males enrolled in this present study similar to an earlier study in Kenya [51]. This was a contrast to a previous study in Kenya where the distribution of males and females was equal and other studies in Sudan, Tanzania, and Ethiopia where males were more [17,18,47,107].

According to Karugu, lack of education on foot care is a significant risk factor for developing DFUs in Kenya [34]. In this present study, about 60% had minimal or no formal education similar to studies in Sudan and Tanzania [18,107]. In this present study, males were significantly more educated than females. A majority of patients in this study were from urban areas similar to

publications from Sudan and Ethiopia [49,107]. From a recent study in Sudan, urban residence was a significant risk factor in the development of DFUs [50]. In contrast, rural residence was a significant factor to developing DFUs in Ethiopia [49]. A study in Tanzania also enrolled patients mainly from rural areas [18]. According to Kibachio and colleagues, patients from rural areas, who are mainly farmers, are more likely to walk bare-feet and have poor foot care habits [52]. This could confer increased risk of developing DFUs and infection by resistant Gram-negative bacteria. Obimbo and colleagues conducted a study in a rural area within the outskirts of Nairobi and confirmed most patients had not been educated on foot care and therefore had poor foot care habits [51]. In this current study, more patients were from urban areas possibly due to the location of KNH being in Nairobi.

6.2 Clinical characteristics and Patient Outcomes

Mariam and colleagues reported that having T2DM is a significant risk factor for developing a DFU [49]. In this present study, almost all of the patients had T2DM. A small proportion of the study patients were newly-diagnosed with DM comparable to an earlier study at the same hospital [17] . The average duration of DM was 8 years, similar to earlier studies in Kenya [17,52] . The median duration of DM, the measure of central tendency in this study, was however 6.5 years, revealing that DFUs are developing much earlier than before. This could be an indicator of poor foot care or inadequate foot care. In this present study, a smaller portion of patients had DM for less than a year compared to previous findings [18]. Having a longer duration of DM was a significant risk factor for developing DFU in Sudan [50].

In this present study, only a small proportion of patients were not on medication. A majority (77%) were on insulin therapy whether alone or in combination with oral drugs. The high number of patients on insulin could result from the fact that inpatients often have deranged glucose levels and require insulin for strict glucose control. According to Nyamu and colleagues, insulin therapy among T2DM is associated with pancreatic beta cell failure and is also associated with sepsis secondary to DFUs [17]. However, less than half of the current study patients were on insulin only which is comparable to earlier studies in Kenya and Ethiopia [17,49]. Although poor drug adherence and poor glycaemic control are known risk factors for DFUs in Kenya, insulin treatment is in itself a significant risk factor for developing DFUs in Tanzania [17,48,52]. The glycemic control based on HbA1c level for patients in this present study was similar to an earlier study in KNH but much worse than in a recent study in rural Kenya [17,52].

The prevalence of uncontrolled hypertension in this study was much lower than an earlier study in Kenya where 60% of the patients had BPs higher than 130/80mmHg [52]. In this present study, although 61% of patients had co-morbid hypertension, on recording of BPs during physical examination, only about 40% had systolic hypertension while 23% had diastolic hypertension. Hypertension is a known risk factor for DFUs [17,47,52]. In the current study, 22% had kidney disease similar to a study in Ethiopia, while 12% had heart disease, which was higher than a study in Sudan [47,107]. Males in this present study had significantly higher levels of urea and creatinine. The poor kidney function may be attributable to lower health seeking behavior among men [122]. In this present study, the lipid profile was much better than that reported by Nyamu and colleagues and could be a result of improved patient awareness or better management of dyslipidaemia by the clinicians [17]. Hypertension and dyslipidaemia have been previously associated with DFUs in Kenya [17,52].

A small proportion of patients reported a positive history of smoking cigarettes and drinking alcohol, which could possibly reflect under-reporting as noted by Nyamu and colleagues [17]. Smoking habits and alcohol use were much higher among surgical patients with DFUs in Tanzania [18]. Males were also more likely to smoke and take alcohol in this present study as expected, a reflection of cultural norms across gender in resource-poor countries. Based on studies comparing participants with different smoking rates, smoking cessation has been recommended as an effective preventive measure to prevent DFUs and to avoid amputations [121]. In Tanzania, drinking alcohol was a protective factor against DFU but this association was not present after multivariate analysis [48]. Kibachio and colleagues similarly found different risk factors for DFUs depending on gender [52].

The median duration of DFU was 8 weeks in this current study. This was shorter than in earlier studies in Kenya and Tanzania and could perhaps indicate increased patient awareness, increased foot care, and increased foot care assessment by the clinicians [17,18]. Similar to this study, the forefoot and the right leg were the most affected anatomical sites similar to studies in KNH, Tanzania and Brazil [17,18,70]. The causal pathway for DFUs among patients from two outpatient settings in resource-rich countries was peripheral neuropathy, foot deformity and minor trauma [7,30]. In Kenya, prior history of trauma was present in 50% of the patients with DFUs at KNH and in 75% at Kikuyu Hospital [17,51]. However, mild continuous trauma is the most likely cause of DFU [64].

Neuropathy is a significant risk factor for developing DFUs [48,49]. Peripheral neuropathy usually begins with autonomic dysfunction, and then sensory and motor nerves are finally affected [64]. Although the prevalence of neuropathy in this present study is much lower than in earlier studies [17,51], diabetic polyneuropathy among the study patients was much higher than in Uganda and Nigeria [43,61]. This confirms a higher rate of neuropathy among Kenyan patients. A majority of the DFUs in this present study were in Wagner Stage 1 and 2 in contrast to studies in Tanzania, Sudan and Libya [18,56,57]. This could indicate early presentation of the patients to this hospital. In Brazil, 54% of DFUs were superficial ulcers similar to this present study [70]. Neuropathy among patients in Nigeria was associated with increasing age, male sex, longer duration of DM, poor glycemic control and dyslipidaemia [43]. In Kenya, neuropathy has also been associated with poor glycemic control [17].

In this present study, a third of the study patients had a prior amputation, which was thrice the rate of previous amputation in Tanzania [18]. However, prospectively, only 14% of patients in this study had a surgical procedure compared to 90% in Tanzania, mainly because the current study was among medical patients while the other was among surgical patients [18]. Half of the patients followed-up in this present study had good clinical outcomes. The mortality rate in this present study was also similar to the study in Tanzania and a literature review from 19 African countries [18,42]. In Sudan, Adam and colleagues reported a much lower mortality rate (6.7%) following diabetic foot [91], possibly because of a shorter duration of DM and less chronic complications [107]. Mortality in Tanzania was associated with diabetes complications and advanced DFUs [18].

6.3 Microbiology results

Wounds can be classified as clean, contaminated, infected or colonised [72]. Infection is determined by performing culture and sensitivity tests on wound swabs and a high CFU/g [21,63,64]. In this present study, over 90% of the DFUs were infected. This was higher than in an earlier study in Kenya, Tanzania, and Libya where about 70% of DFUs had positive cultures [17,18,57]. There is therefore need to manage diabetes mellitus effectively which was identified as a risk factor for infectious wounds in India [72]. In this current study, Gram-negative bacteria were more predominant than Gram-positive organisms similar to studies in Morocco and Brazil [70,73]. The most common species isolated in this study in order of frequency was *S. aureus, E. coli, P. mirabilis, K. pneumonia, P. aeruginosa* and *S. fonticola.* Many other previous studies have also detected *S. aureus* as the most predominant species in DFUs [10,18,20,21,57,59,65,67,68,70]. Similar to the present

results, *E. coli* and *P. aureginosa* are common Gram-negative bacteria isolated from DFUs [21,57,59,65,70].

Although not all bacteria infecting a DFU were identified, in one patient, there were two different bacteria colonizing one wound (*E. coli* and *Raoultella ornithinolytica*) and in another patient, *S. aureus* and *E. coli* were identified from ulcers on different feet. Therefore, in this study, most of the DFUs had mono-microbial growth. The identification of bacteria was limited to the most predominant species and so poly-microbial infections were lower than expected. This mono-microbial pattern could also be associated with the fact that most of the DFUs in this present study were in early Wagner stages and most patients were already on antibiotic treatment [69,123]. In this present study, 77.6% of the patients were taking antibiotics at the time of the sample collection. Poly-microbial growth, associated with advanced DFUs, was reported in Libya, Tanzania and Brazil [18,57,70]. However, in study at a tertiary referral facility in India, mono-microbial growth was present in all 100 patients with chronic DFUs [69].

Citrobacter sp. isolated from DFUs in this current study has also been previously isolated from DFUs in Brazil [70]. Majority of the rare Gram-negative species isolated in this study such as *Acinectobacter baumanni, Burholderia cepacia* and *Providencia stuartii* and cause nosocomial infections. *L. mesenteroides* is utilized in milk processing while *P. agglomerans* is considered an opportunistic infection [124].

Unjustified use of antibiotics is not only a precursor to antibiotic resistance, but leads to inefficient use of scarce health resources and further burdens patients and their families [7,21,34]. In this present study, there was high resistance to antibiotics among the Gram-negative organisms compared to the Gram-positive bacteria. *S. aureus* was resistant to benzyl-penicillin and TMPSMX in this present study. MRSA is predominant in DFUs and sensitive to sparfloxacin, levofloxacin, ofloxacin, ciprofloxacin, amoxicillin- clavulanic acid, cephalexin, cefuroxime, doxycycline and clindamycin .[71,78]. In Brazil, 22% of DFUs were identified as having MRSA following cefoxitin screen, and 33% of these were also resistant to vancomycin [70]. In this present study, no MRSA was identified by culture methods since cefoxitin screen for *S. aureus* was negative for all. *S. aureus* was also sensitive to oxacillin, nitrofurantoin, gentamicin, levofloxacin, linezolid, teicoplanin, tigecycline and vancomycin in this study.

S. aureus and *E. coli* isolated from DFUs by Perim and colleagues were resistant to multiple classes of antibiotics [70]. In this present study, antimicrobial sensitivity tests revealed that most *E.*

coli and *K. pneumonia* were resistant to ampicillin and cefuroxime. *P. aeruginosa* was resistant to cefotaxime while *S. fonticola* was resistant to most antibiotics. From previous studies, antibiotics that used to work before are now showing increasing resistance [18,59]. For example, in Tanzania, most pathogens isolated from DFUs had high resistance to commonly used antibiotics [18]. Common practice in Kenya is to empirically administer one of the following 6 antibiotics to treat chronic wounds before a culture and sensitivity test is performed: Augmentin, ciprofloxacin, cefuroxime, ceftriaxone, clindamycin, flagyl or floxapen.

Our results therefore offer guidance on recommendations for treating DFUs. From this present study, amikacin is effective against most Gram-negative bacteria in this setting. The high resistance to ampicillin should warrant careful consideration of this drug during empirical therapy. It is also worth noting that although some *E. coli* isolates were resistant to meropenem (a third-line antibiotic); all were sensitive to nitrofurantoin (a first-line antibiotic). On the other hand, in this present study, *C. freundii* isolated had resistance to piperacillin-tazobactum and most cephalosporins. From literature, *C. freundii* is associated with over-use of broad-spectrum antibiotics and is also known to be resistant to piperacillin, piperacillin-tazobactam, vancomycin and cephalosporins [124]. There is therefore need to use antibiotics judiciously and be guided by routine culture and sensitivity results which should be the standard practice. More sensitive procedures should be employed to detect infection-causing bacteria. A study by Stappers and colleagues reported high number of false-negatives from culture-based methods [20].

6.4 Molecular

Molecular methods include amplification of the hypervariable region in the 16S rRNA [67]. In the present study, molecular tests were more sensitive than culture-based methods. PCR revealed pathogens that were not previously recognized by culture methods which included MRSA species. The sensitivity of the culture tests was 90.9% while the specificity was 82.5% for detection of *S. aureus*. The PPV and NPV were 58.8% and 2.9% respectively. Compared to RT-PCR, culture tests had a specificity of 99%, sensitivity of 31%, PPV of 59% and NPV of 98% also found significant discrepancies between culture and sequencing methods [20,67]. Stappers and colleagues reported that 81% of samples were RT-PCR-positive for *S. aureus* while only 41% were culture positive for the same species [20]. In the present study, 72% of the samples were RT-PCR-positive for *S. aureus* while 56% were culture-positive. Weighing costs, PCR should be considered as a microbiological procedure for diagnosing pathogens in DFUs.

6.5 Wound Healing

In this present study, there was a non-healing rate of 25% among the study patients who were followed-up. Healing of wounds mainly occurs in three main stages: inflammation, proliferation (re-epithelization, granulation, neo-angiogenesis) and extracellular matrix remodeling [54,64]. Mendes and Neves describe the process of wound healing [64]. First, platelets degranulate at the wound site and release transforming growth factor (TGF-β1), platelet-derived growth factor (PDGF) and fibroblast growth factor (β-FGF) leading to blood vasoconstriction. A clot then forms, producing vascular endothelial growth factor (VEGF) which attracts fibroblasts around the wound area and later leukocytes (neutrophils) and monocytes infiltrate. Cells (keratinocytes, fibroblasts, endothelial progenitor cells, macrophages and platelets), and normal quantities of chemokines and cytokines are all important for proper wound healing. At the molecular level, there are many pathological causes for poor wound healing. Delayed healing of DFUs among patients with DM has been attributed to on one hand by reduced TGF, nerve growth factor (NGF), insulin-like growth factor (IGF), epidermal growth factor receptor (EGFR), stromal cell-derived factor 1 (SDF-1α), endothelial nitric oxide synthase (eNOS) leading to elevated NO, imbalance of matrix metalloproteinases (MMPs); and on the other hand by increased c-myc, β-catenin, cathepsin-D, inflammatory markers (IL-6, IL-8, IL-19, IL-15, neurotrophin-3, substance P, CGRP) and glucocorticoid pathways activity [28,54]. Immunological and collagen cross-linking disturbances have also been associated with poor healing of DFUs [30].

6.6 STUDY LIMITATIONS

1. The present study was a hospital-based study in a referral centre and the results cannot be generalized to the entire population. There was a prolonged doctors and lecturers strike in the beginning of the year, which slowed down patient enrollment. However, we surpassed the minimal sample size expected based on previous prevalence studies in Kenya.
2. Although some of the samples showed mixed growth from preliminary tests, due to funding limitations, this present study did not identify all the bacteria using both culture-based. The study did also test for anaerobic bacteria which are commonly suspected in deep seated infections. Identification of anaerobic bacteria requires immediate collection and streaking of sample in the culture media and placing it into an anaerobic jar with packs at the bedside of the patient. The ideal scenario requires a laboratory technician to collect the sample himself.

However, from personal experiences of technicians at the KNH Microbiology Laboratory, the results from aerobic cultures are often similar with results from anaerobic procedures; mostly because aerobic and facultative, which are the majority, can do well in presence of oxygen. However, further research needs to confirm this notion.

3. Antimicrobial sensitivity did not test for all antibiotics. It would have been interesting to note sensitivity of Gram-positive bacteria to Augmentin, ciprofloxacin, cephalosporins, and flucloxacillin, antibiotics which are commonly used by clinicians. However, sensitivity of the gram-positive organisms to clindamycin, another commonly used drug, was tested. Resistance to metronidazole among gram-negative organisms was not tested.
4. Retrieval of records from patients' files was not efficient enough to obtain full laboratory results and patient outcomes on follow-up. With the necessary resources, it would have been prudent to collect blood samples for relevant laboratory tests as we performed the wound culture and sensitivity tests and to have a prospective arm for the follow-up results.
5. PCR was used to identify and quantify DNA of the target *S. aureus* and resistant *mecA* gene for MRSA. Limited funding and time prevented the use of PCR to fully sequence part or the entire genome with Universal primers. This would have not only helped to compare molecular versus culture-based tests and also detect resistant strains. PCR tests are thought to have better sensitivity and would be ideal in identifying bacteria in low populations. However, genotyping is quite expensive, takes time and is beyond the scope of this study. It is also worth noting that molecular tests may detect dead bacteria which could include contaminants.

6.7 CONCLUSION

There are poor outcomes for patients with DFUs in this setting such as poor wound healing, high recurrence rates, increased lower limb amputations and mortality compared to previous studies. The study patients were slightly older, more likely female, less educated but urbanised. The study patients had better control of hypertension and dyslipidaemia, lower levels of neuropathy and earlier presentation of DFUs reflecting increased patient awareness and better management by clinicians. Majority of the patients in this study were on insulin and antibiotics. Almost all DFUs were infected, with Gram-negative bacteria being the most common. In order of frequency, *S. aureus, E. coli, P. mirabilis, K. pneumonia, P. aeruginosa* were the most common isolated species. There was a high prevalence of bacteria which are nosocomial in nature. In this study, the bacteria isolated showed high resistance to commonly used antibiotics with Gram-

negative bacteria showing higher resistance patterns. MRSA were not identified using culture methods but were identified using PCR. PCR was also more sensitive than culture-based methods to identify *S. aureus.*

6.8 RECOMMENDATIONS

1. Patients with DFUs should have microbial swabs for culture and sensitivity performed routinely for correct management and antibiotic stewardship.
2. The presence of nosocomial infections should encourage infection control measures among patients with DM.
3. Further studies should be performed to identify bacteria from mixed infections in DFUs and anaerobic organisms.
4. PCR is an effective way of species identification, and in particular *S. aureus* and MRSA and should be considered as an ideal test for patients who have resources.
5. The high prevalence of poor outcomes for patients with DFUs warrants the need to investigate bio-psychosocial risk factors.
6. Screening and foot assessment should be encouraged during each clinical visit. Examination focuses on peripheral neuropathy, peripheral arterial disease and plantar pressure [64].Certain professional organisations recommend different prevention measures based ion the patients' risk levels [30]. Patients with DM should be educated to smoking cessation and foot-care.
7. Patients with DM should have laboratory tests such as UECs, LFTs, Lipid profile, and HbA1c every 6 months to rule out and properly manage diabetic complications.

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8. Chapter Eight: Appendix

8.1 Informed Consent Form (English Version)

PATIENT STUDY NUMBER _____ PATIENT INITIALS_____

Introduction

My name is Dr. Daniel Munyambu Mutonga, a finalist student in Masters of Science in Tropical and Infectious Diseases. I am conducting a study on:

Isolation, sensitivity patterns and molecular characterisation of bacterial isolates from infected diabetic foot ulcers in patients at Kenyatta National Hospital.

I would like to invite you to participate in this study.

Type of Research Intervention

This study entails looking at the type of bacteria that infect diabetic foot ulcers in patients at Kenyatta National Hospital and their sensitivity to commonly used antibiotics.

After enrollment of this study, information about your condition including age, gender, residence, type of diabetes, current medications, and about the diabetic foot ulcers will be noted and filled up in this questionnaire. Your height, weight and blood pressure readings will also be recorded.

With your permission, we will clean your diabetic wound and collect samples from the wound which we shall send to the laboratory for identification of bacteria and antibiotic sensitivity tests. We will also collect blood samples to check your blood sugar level, cholesterol levels and kidney function. These tests will help your doctor manage your diabetic condition better.

Participation in this study

Participation in this study is voluntary and you can withdraw your participation at any time even after signing the consent. Refusal to participate in this study will not result in any penalty or loss of rights to good medical care. I assure you that the information collected will remain confidential. You can ask any other questions appertaining to assessment and treatment and this will be availed to you at any time.

Purpose of Study

We want to find out the types of bacteria that infect diabetic foot ulcers and their sensitivity to commonly used antibiotics. The study will help us manage diabetic foot infections better. From this study, we will also be able to make recommendations for better treatment of the diabetic foot infection.

The results of this study will be published as a book (thesis) and in a Medical Journal. The results will also serve as information for teaching purposes. Results will be made available to the community for better understanding of this illness.

We will inform you on the results and make these available to the healthcare giver at Medical Ward and the Diabetes Outpatient Clinic for better follow up. We assure you that we will NOT use your name anywhere in the presentation of these results.

Cost

No added costs will be incurred to the patient other than those of routine laboratory tests.

Duration of participation

Every participant will be enrolled only once but the study will take place over a 4-month period at the Diabetic Out-patient Clinic or in the Medical or Surgical Wards.

Risks and Benefits

While participating in this study, you will not be exposed to any risks and you will not incur any losses. About 5mls of blood will be drawn from you for blood sugar levels, blood cholesterol levels, kidney function and liver function tests only. A mildly unpleasant sensation may be felt during collection of a blood sample (for a few seconds). Your wound will also be cleaned during the collection of a wound sample and you may feel an unpleasant sensation.

Participants Declaration

Just as an indication that you have agreed to participate in this study, kindly sign below,

I, _____ hereby agree to participate in this study being carried out by DR DANIEL MUNYAMBU MUTONGA, the nature of which has been explained to me. I have understood the purpose of this study and my questions have been answered satisfactorily by Dr Daniel Munyambu Mutonga.

Signed (Patient): _____

Signed (PI): _____

Date: _____

Whom to Contact:

If you have any queries about this study, please feel free to contact the persons underlisted now or at any time.

Dr. Daniel Munyambu Mutonga 0711 899684

Dr. Marianne Mureithi 0703 704711

Prof. C. F. Otieno 0722 752558

Dr. Nancy Ngugi 0722 788533

The Secretary

KNH-UoN Ethics and Research Committee

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Email: uonknh_erc@uonbi.ac.ke

8.2 Informed Consent form (Swahili version)

PATIENT STUDY NUMBER _____ PATIENT INITIALS_____

KUHUSU IDHINI

Mimi ni Daktari Danieli Munyambu Mutonga na mimi ni mkufunzi katika Chuo Kikuu cha Nairobi. Ninatekeleza utafiti kuhusu:

Viambukizi katika vidonda vya nyayo kwa wale wenye ugongjwa wa kisukari (diabetes) katika hospitali kuu ya Kenyatta

Ningependa kukusajilisha katika utafiti huu.

Sababu za Kufanya Utafiti

Utafiti huu utatusaidia kujua viambukizi vinavyopatikana katika vidonda vya nyayo za wale wenye ugonjwa wa kisukari na madawa yanayofanya kazi kuua viini hivi.

Matokeo ya utafiti huu yatachapishwa katika kitabu ili kuwaelimisha wakufunzi wengine na wale wanaopatiana huduma katika Kiliniki ya Wagonjwa wa Sukari. Tutakueleza matokeo ya utafiti huu na pia kumueleza muuguzi wako ili akuhudumie vilivyo. Tunakuhakikishia ya kuwa hatutatchapisha jina lako popote katika dodoso letu.

Mambo tutakayofanya

Baada ya kujisajilisha katika utafiti huu, tutachukua historia yako kuhusu umri, jinsia, kimo, urefu, makao, aina ya ugonjwa wa kisukari, madawa unayotumia, na kuhusu kidonda cha nyayo. Tutaaandika majibu yako katika dodoso hii. Kwa idhini yako, tutasafisha kidonda chako na kuchukua sampuli ambayo tutapeleka kwenye maabara yetu ili kufanya vipimo. Tungependa pia kuchukua sampuli ya damu ili kupima kiwango cha sukari na mafuta kweye damu, na pia hali ya figo. Vipimo hivi vitamwezesha dakatari wako kukutibu vilivyo.

Manufaa Ya Kuhusika

Manufaa ya utafiti huu ni kuwaelezea madaktari kuhusu viambukizi vya vidonda katika nyayo za wagognjwa wa kisukari na dawa zenye nguvu zaidi. Mapendekezo ya utafiti huu yatasaidia kuboresha huduma wanazozipata wagonjwa wa kisukari.

Mbali na hayo, tutachukua kipimo cha damu, itakayotuonyesha kiwango cha sukari kwa miezi mitatu iliopita, kipimo cha mafuta kwenye damu, na pia hali ya figo

Madhara Ya Kuhusika

Hakuna madhara yoyote yatakayotokana na kuhusika katika utafiti huo. Unaeza kuhisi uchungu kidogo kutokana na sindano wakati wa kutolewa damu. Uchungu huu ni sawa na ule unaosikika wakati unapotolewa damu kwa vipimo vingine. Kiwango cha damu ni mililita tatu pekee.

Gharama

Hakuna gharama yoyote ya ziada kwa wewe ambaye umekubali kupatiana idhini isipokua kwa vipimo vya kawaida ambavyo daktari wako atakavyoagiza.

Idhini ya kuhusika

Kuhusika kwako katika utafiti huu ni kwa hiari yako, na unaeza kujiondoa wakati wowote hata baada ya kupatiana idhini yako. Matibabu yanayostahili yatapewa kwa watu wote wahusika na wanaokataa kujihusisha na utafiti huu hawatabaguliwa kwa njia yoyote ile.

Sahihi (Mhusika)_____ **Sahihi (Mtafiti)_____**

Tarehe _____ **Tarehe _____**

Mawasiliano:

Ijapo uko na maswali ama mapendekezo, kuwa huru na utuelezee kwa namba hizo zilizoandikwa.

Dr. Daniel Munyambu Mutonga 0711 899684

Dr. Marianne Mureithi 0703 704711

Prof. C. F. Otieno 0722 752558

Dr. Nancy Ngugi 0722 788533

The Secretary

KNH-UoN Ethics and Research Committee

Tel: 2726300, Ext 44102

Barua pepe: uonknh_erc@uonbi.ac.ke

8.3 Data Collection Form

1. BASIC CHARACTERISTICS

1. Fill in all the blanks or check the correct response.

ID. # ☐☐☐ AGE ☐☐ SEX M ☐ F ☐

Area of Residence ☐ Urban ☐ Rural

Level of Education ☐ None ☐ Primary ☐ Secondary ☐ Tertiary

Diabetes History ☐ Y ☐ N

Smoking History ☐ Y ☐ N

Alcohol History ☐ Y ☐ N

COMORBIDITIES ☐ Hypertension ☐ HIV ☐ Cancer ☐ Kidney disease ☐ Heart disease ☐ High cholesterol

Others ☐

2.5. Check for pulses in the following arteries and measure the highest systolic pressure on the posterior tibial artery or dorsalis pedis artery using a Doppler probe.

R		Y	N
	Dorsalis pedis	☐	☐
	Post. tibial	☐	☐
	Popliteal artery	☐	☐
	Sup. femoral	☐	☐

L		Y	N
	Dorsalis pedis	☐	☐
	Post. tibial	☐	☐
	Popliteal artery	☐	☐
	Sup. femoral	☐	☐

BP (mmHg) Systolic ☐☐☐

WAGNER CLASSIFICATION OF DIABETIC FOOT ULCERS

stage 0 = foot at risk; intact skin

stage 1 = superficial ulcer

stage 2 = deep ulcer without bone involvement or abscess

stage 3 = deep ulcer with abscess or osteomyeltis (bone involvement visible on X-ray)

stage 4 = localised gangrene e.g. toe, heel

stage 5 = gangrene of the whole foot

2. CLINICAL CHARACTERISTICS

2.1 Record vital measurements

BP Systolic ☐☐☐ Diastolic ☐☐☐

Weight (kg) ☐☐☐

Height (cm) ☐☐☐

Temp (°C) ☐☐.☐

2.2. Take further history on diabetes and the diabetic foot ulcer

Type of DM: ☐ Type 1 ☐ Type 2 ☐ Gestational ☐ Other

Type of DM Control: ☐ None ☐ Diet ☐ OHA ☐ Insulin ☐ Herbal

Duration of DM (yrs) ☐☐

Duration of DFU (weeks) ☐☐

Previous History of DFU ☐ Y ☐ N

Previous History of amputation ☐ Y ☐ N

Any antibiotic use in last 2 weeks ☐ Y ☐ N

If yes, Specify which drugs:

2.3 Record the site of the DFU and classify it using the 2 classification systems listed. Consult the diabetes foot specialist nurse whenever necessary. Turn to page 3 for Wagner classification.

R

Anatomical site: ☐ Forefoot ☐ Midfoot ☐ Hindfoot

Type of ulcer: ☐ Ischaemic ☐ Neuropathic ☐ Neuro-ischaemic

Wagner Stage ☐

L

Anatomical site: ☐ Forefoot ☐ Midfoot ☐ Hindfoot

Type of ulcer: ☐ Ischaemic ☐ Neuropathic ☐ Neuro-ischaemic

Wagner Stage ☐

2.4 Score for sensation pain, vibration and pressure as follows 0 = absent , 1 = present; score for Achilles tendon reflex as follows; 0 = present, 1=absent, 2 = absent without stimulation. Sum up the scores to get a NDS

R

Sensation

	0	1	2
Pain	☐	☐	
Vibration	☐	☐	
Pressure	☐	☐	
Achilles reflex	☐	☐	☐

NDS score ☐

L

Sensation

	0	1	2
Pain	☐	☐	
Vibration	☐	☐	
Pressure	☐	☐	
Achilles reflex	☐	☐	☐

NDS score ☐

3. BIOCHEMISTRY

3.1 Collect 5ml of blood into a red and purple vacutainer and send the samples to the laboratory.

RBS (mmol/L) ☐☐ HbA1c (%) ☐☐

UECs (mmol/L)		Lipid levels		LFTs	
Na^+	☐☐☐	T. Chol (mmol/L)	☐☐	AST	☐☐☐
K^+	☐☐☐	TG (mmol/L)	☐☐	ALT	☐☐☐
Cl^-	☐☐☐	HDL-C (mmol/L)	☐☐	T. Bil	☐☐☐
Urea	☐☐☐	LDL-C (mmol/L)	☐☐	D. Bil	☐☐☐
				Indirect Bil	☐☐☐
				Albumin	☐☐☐

4. MICROBIOLOGY PROCEDURES

4.1 Clean the wound with normal saline and collect a sample of specimen from the centre of the wound using a sterile swab. Send the sample to the microbiology laboratory. Following culture and biochemical tests, what species have been isolated?

S. aureus	☐	*Proteus spp*	☐
CONS	☐	*Str. agalactiae*	☐
Str. pyogenes	☐	*Str. pneumoniae*	☐
E. coli	☐	*S. saprophyticus*	☐
K. pneumoiae	☐	*Enterococcus*	☐
Pseudomonas spp	☐	*Acinectobacter*	☐
Other	☐	*S. epidemidis*	☐

4.2 Test from antibiotic sensitivity using the disk diffusion method

	S	R		S	R
Augmentin	☐	☐	*Erythromycin*	☐	☐
Ampicillin	☐	☐	*Metronidazole*	☐	☐
Azithromicin	☐	☐	*Gentamicin*	☐	☐
Chloramphenicol	☐	☐	*Imepinem*	☐	☐
Cefotaxime	☐	☐	*Meropenem*	☐	☐
Ciprofloxacin	☐	☐	*Norfloxacin*	☐	☐
Clindamycin	☐	☐	*Penicillin*	☐	☐
Cloxacilin	☐	☐	*Polymixin B*	☐	☐
Cotrimoxazole	☐	☐	*Streptomycin*	☐	☐
Tetracyclin	☐	☐	*Vancomycin*	☐	☐

Others:

5. PCR & DNA ANALYSIS

5.1. Is *Staphylococcus aureus* species present in the DNA sample?

☐ Y

☐ N

5.2 Determined by presence of *mecA gene*, is MRSA species present in this DNA sample?

☐ Y

☐ N

CLINICAL OUTCOMES

Date of Follow-up ☐☐ ☐☐ ☐☐☐☐

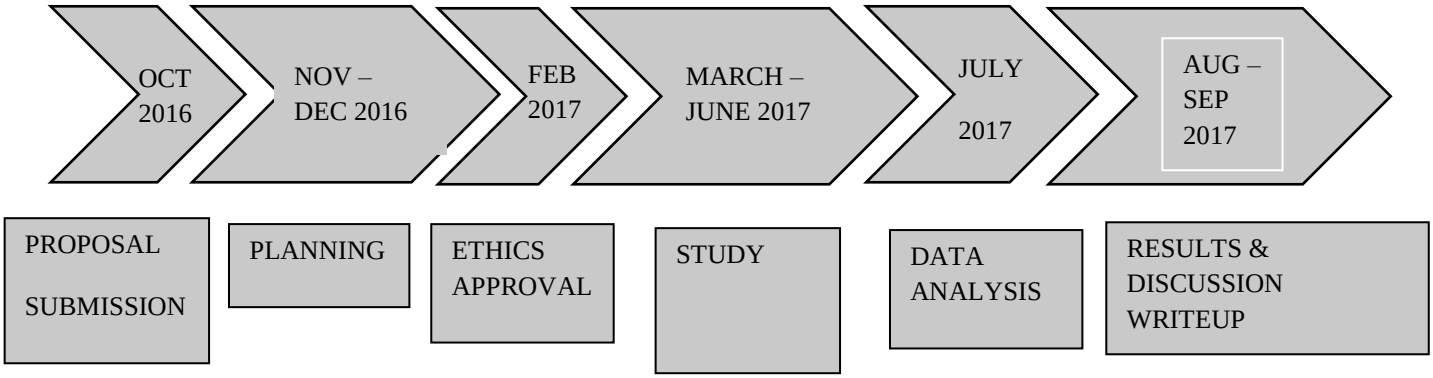
DD / MM / YYYY

Length of hospital stay (days) ☐☐☐

What were the outcomes of the DFUs on follow-up?

- ☐ ***Complete Healing***
- ☐ ***Ongoing healing***
- ☐ ***Non-healing***
- ☐ ***Major Surgery***
- ☐ ***Minor Surgery***
- ☐ ***Mortality***

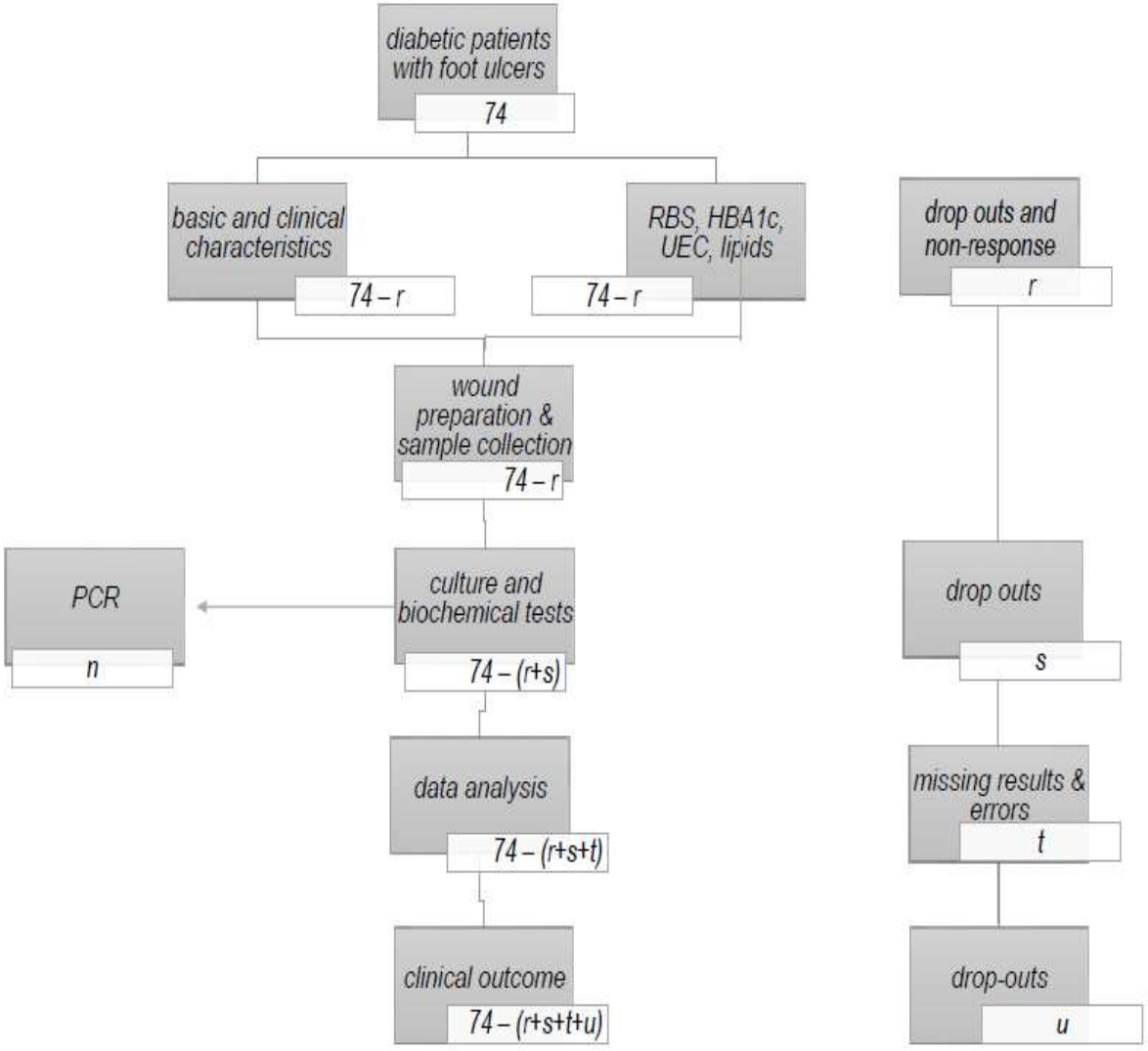
8.4 Time Plan



****Ethical Approval was obtained as planned in February, 2018. The Study started in September, 2017 as planned. However, due to ongoing strikes at the hospital and other disruptions, the study was concluded in August, 2018. Data analysis, write-up of Results, Discussions and editing the Thesis took place between August and September, 2018.*

8.5 Flow Chart

This is the flow-chart that was anticipated at the beginning of the study. We surpassed the sample size. The actual Flow-chart can be reviewed on page.



8.6 Budget

8.6.1 BUDGET FORM

COMPONENTS	UNIT OF MEASURE	DURATION/ NUMBER	COST (KSHS)	TOTAL (KSHS)
Personnel				
Research Assistant	1	13	1,500.00	19,500.00
Statistician				30,000.00
Transcribing Fees				-
Subtotal				***49,500.00***
Printing				
Consent Form	1	4	10.00	40.00
Assent Form				-
Questionnaires	1	6	10.00	60.00
Interview Guide				-
Final Report	1	100	10.00	1,000.00
Subtotal				***1,100.00***
Photocopying				
Consent Form	85	4	3.00	1,020.00
Assent Form				-
Questionnaires	85	6	3.00	1,530.00
Interview Guide				
Final Report	5	100	3.00	1,500.00
Final Report Binding	6	1	500.00	3,000.00
Subtotal				***7,050.00***
Laboratory				
Wound Swab Culture & Sensitivity	74	1	1,200.00	88,800.00
PCR	50	1	3,000.00	150,000.00
Subtotal				***238,800.00***
				-
Other Costs				
ERC Fees				2,000.00

Records Access Fees				1,500.00
Box File	2	1	250.00	500.00
Pens	5	1	25.00	125.00
Subtotal				***4,125.00***
TOTAL				**300,575.00**

8.6.2 BUDGET JUSTIFICATION

We anticipated to enrol six participants per day. To attain the sample size of 74 participants, we expected to therefore take 13 days. Daily allowance for the assistants was Ksh. 1500.00. However, do to the prolonged nature of the study, we worked with the assistant for an additional 21 days at the same rate and then the Principal investigator proceeded on his own.

We printed consent forms, questionnaires and final reports once and made photocopies as required. We will made extra copies of the questionnaires and consent forms to take care of any damage or loss to the forms.

Laboratory Wound swabs culture and sensitivity was conducted at KNH Microbiology laboratory at the quoted price. Real-time PCR test for *S.aureus* DNA, and MRSA DNA (genes *mecA)* was not available at the KNH Microbiology laboratory. Therefore, we procured services from Bio-zeq Kenya Ltd., which runs the molecular laboratory at Kenya AIDS Vaccine Initiative-Institute for Clinical Research (KAVI-ICR), University of Nairobi.

We obtained medical records of patients to obtain data on the progress of their wounds following treatment and give back results of the microbiology tests done.

The principal investigator and research assistant used procured stationery for data entry and storage of paper records.

8.7 Additional Antimicrobial Sensitivity Results

Table 6.1: Frequency of Bacteria Isolated from DFUs

	n (%)
Gram-positive	
Staphylococcus aureus	14 (16.47)
Staphylococcus epidermidis	2 (2.35)
Staphylococcus intermedius	2 (2.35)
Enterococcus faecalis	2 (2.35)
Staphylococcus haemolyticus	1 (1.18)
Staphylococcus lentus	1 (1.18)
Staphylococcus simulans	1 (1.18)
Staphylococcus xylosus	1 (1.18)
Contaminants isolated	1 (1.18)
Subtotal	*25 (29.41)*
Gram-negative	
Escherichia coli	13 (15.29)
Proteus mirabilis	9 (10.59)
Klebsiella pneumoniae	6 (7.06)
Pseudomonas aeruginosa	6 (7.06)
Acinetobacter baumannii	2 (2.35)
Serratia fonticola	3 (3.53)
Serratia marcescens	2 (2.35)
Proteus hauseri	2 (2.35)
Klebsiella oxytoca	2 (2.35)
Burholderia cepacia	1 (1.18)
Citrobacter amalonaticus	1 (1.18)
Citrobacter freundii	1 (1.18)
Enterobacter aerogenes	1 (1.18)
Kocuria kristinae	1 (1.18)
Leuconostoc mesenteroides	1 (1.18)
Pantoea agglomerans	1 (1.18)
Proteus vulgaris	1 (1.18)
Providencia stuartii	1 (1.18)
Raoultella ornithinolytica	1 (1.18)
Subtotal	*55 (64.71)*
No growth obtained	5 (5.88)
TOTAL	**85 100)**

Table 6.2: Gram-positive Antibiotic Sensitivity Results

	Enterococcus faecalis		Staphylococcus aureus					Staphylococcus epidermidis					Staphylococcus intermedius				Staphylococcus simulans			
	1	2	1	2	3	4	5	1	2	3	4	5	1	2	3	4	1	2	3	5
Benzylpenicillin			0	12	0	0	0	0	2	0	0	0	0	2	0	0	0	1	0	0
BetaLactamase			0	0	1	0	0						0	0	0	1	0	0	0	0
Cefoxitin			0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CefoxitinScreen			0	0	6	0	0	0	0	0	1	0					0	0	0	0
Clindamycin	0	1	9	2	0	0	1	2	0	0	0	0	1	1	0	0	1	0	0	0
Erythromycin	0	1	9	3	0	0	0	2	0	0	0	0	1	1	0	0	1	0	0	0
Fosfomycin			5	0	0	0	0	1	0	0	0	0								
Fusidicacid			6	0	0	0	0	1	0	0	0	0					1	0	0	0
Gentamicin			12	0	0	0	0	2	0	0	0	0	2	0	0	0	1	0	0	0
InducibleClindamycinResistance			0	0	11	1	0	0	0	1	0	0	0	0	2	0	0	0	1	0
Levofloxacin	1	0	11	0	0	0	0	1	0	0	0	1	2	0	0	0	1	0	0	0
Linezolid	1	0	12	0	0	0	0	2	0	0	0	0	1	0	0	0	1	0	0	0
Moxifloxacin			10	0	0	0	0	1	0	0	0	0	1	0	0	0	1	0	0	0
Mupirocin			5	0	0	0	0	1	0	0	0	0								
Nitrofurantoin			6	0	0	0	0	1	0	0	0	0								
Oxacillin			6	0	0	0	0	0	1	0	0	0					1	0	0	0
Rifampicin			3	0	0	0	3	0	0	0	0	1					0	0	0	1
Teicoplanin	1	0	12	0	0	0	0	2	0	0	0	0	1	1	0	0	1	0	0	0
Tetracycline	0	1	8	2	0	0	0	1	1	0	0	0	1	1	0	0	0	1	0	0
Tigecycline	1	0	7	0	0	0	0	1	0	0	0	0	2	0	0	0	1	0	0	0
Tobramycin			10	1	0	0	0	1	0	0	0	0	1	0	0	0	1	0	0	0
TrimethoprimSulfamethoxazole	0	1	3	7	0	0	0	0	1	0	0	0	1	1	0	0	1	0	0	0
Vancomycin	1	0	12	0	0	0	0	2	0	0	0	0	1	1	0	0	1	0	0	0

Table 6.3: Gram-negative Antibiotic Sensitivity Results

	Acinetobacter baumannii	Burholderia cepacia		Citrobacter amalonaticus		Citrobacter freundii			Enterobacter aerogenes		
	2	1	2	1	2	1	2	5	1	2	
Amikacin	0	0	1	1	0	0	0	1	1	0	1
AmoxicillinClavulanicAcid	0	0	0	1	0	0	1	0	0	1	5
Ampicillin	0	0	0	0	0	0	1	0	0	0	0
AmpicillinSulbactam	1	0	0	0	0	0	0	0	0	0	3
Aztreonam	0	0	0	0	0	0	0	0	0	1	2
Cefazolin	1	0	0	0	0	0	1	0	0	1	0
Cefepime	1	0	1	1	0	0	1	0	0	1	2
Cefotaxime	1	0	1	1	0	0	1	0	0	1	2
Cefoxitin	0	0	0	0	0	0	0	0	0	0	3
Ceftazidime	1	0	1	1	0	0	0	1	0	1	2
Ceftriaxone	1	0	1	1	0	0	1	0	0	1	2
Cefuroxime	0	0	0	0	1	0	1	0	0	1	1
CefuroximeAxetil	0	0	0	0	0	0	0	0	0	1	0
Ciprofoxacin	1	1	0	1	0	0	1	0	1	0	8
Gentamicin	1	1	0	1	0	0	1	0	1	0	1
Meropenem	1	0	1	1	0	1	0	0	1	0	8
Nitrofurantoin	0	0	0	0	0	0	0	0	0	0	7
PiperacillinTazobactam	1	0	1	1	0	0	1	0	1	0	4
TrimethoprimSulfamethoxazole	1	0	0	1	0	0	1	0	0	1	0

Table 6.4: Comparing means for MDROs and none-MDROs

		MDROs mean (SD)	None MDROs mean (SD)	p Value
Age (years)	60.30 (12.88)	61.25 (10.65)	59.22 (12.98)	0.481 NS
RBS (mmol/L)	14.12 (11.03)	10.65 (6.30)	11.06 (5.84)	0.813 NS
HbA1c (%)	8.40 (2.29)	8.69 (2.68)	9.97 (3.86)	0.322 NS
Urea (mmol/L)	12.05 (10.58)	18.425 (26.00)	9.355 (9.15)	0.101 NS
Creatinine (mmol/L)	147.53 (74.06)	172.76 (97.61)	155.12 (136.50)	0.623 NS

Table 6.5: Comparing means within and between Outcome Groups

	p Value
Age (years)	0.105
RBS (mmol/L)	0.414
HbA1c (%)	0.757
Urea (mmol/L)	0.867
Creatinine (mmol/L)	0.613

Table 6.6: Comparing proportions across Outcome Groups

		Total	Complete Healing	Ongoing healing	Non-healing	Major Surgery	Minor Surgery	Mortality	p value
Sex	Male	16 (44.44)	4 (57.1)	5 (45.5)	5 (55.6)	0 (0.0)	2 (50.0)	0 (0.0)	0.453
	Female	20 (55.56)	3 (42.9)	6 (54.5)	4 (44.4)	1 (100.0)	2 (50.0)	4 (100.0)	
Residence	Urban	19 (55.88)	4 (57.1)	7 (77.8)	3 (33.3)	0 (0.0)	3 (75.00	2 (50.0)	0.397
	Rural	15 (44.12)	3 (42.9)	2 (22.2)	6 (66.7)	1 (100.0)	1 (25.0)	2 (50.0)	
Smoking habits	Yes	9 (29.03)	1 (16.7)	5 (50.0)	1 (16.7)	0 (0.0)	1 (25.0)	1 (25.0)	0.446
	No	22 (70.97)	5 (83.3)	5 (50.0)	5 (83.3)	1 (100.0)	3 (75.0)	3 (75.0)	
Alcohol Intake	Yes	13 (41.94)	2 (33.3)	6 (60.0)	3 (50.0)	0 (0.0)	1 (25.0)	1 (25.0)	0.384
	No	18 (58.06)	4 (66.7)	4 (40.0)	3 (50.0)	1 (100.0)	3 (75.0)	3 (75.0)	

Table 6.7: Wagner Staging Type RIGHT FOOT and selected parameters

Wagner Stage	0	1	2	3	4	5
SBP (mmHg)	128.00	148.83 (37.12)	129.36 (28.19)	133.00 (23.152)	133.875 (24.76)	119.00
DBP (mmHg)	89.00	89.17 (23.25)	76.29 (16.14)	79.5 (11.40)	71.75 (15.30)	69.00
RBS (mmol/L)	11.1000	12.96 (8.67)	9 (2.93)	9.97 (4.35)	13.31(8.27)	
HbA1c (%)		8.8000	9.33 (3.58)	8.55 (1.91)	8.71 (0.96)	
Urea (mmol/L)		10.22 (7.88)	14.52 (12.14)	19.27 (35.57)	16.5 (29.54)	6.4000
Creat (mmol/L)		153.5 (79.54)	221.13 (159.08)	210.12 (196.49)	130.25 (51.95)	67.0000
T. Chol (mmol/L)		4.57 (1.19)	3.06 (0.81)	3.66(1.77)	4.60 (0.76)	
TGs (mmol/L)		1.58 (0.71)	1.74 (0.80)	1.19 (0.31)	1.65 (0.13)	
HDL-C (mmol/L)		1.4 (0.84)	0.85 (0.47)	0.87 (0.25)	1.10(0.16)	

LDL-C (mmol/L)		2.48 (0.89)	1.43 (0.36)	1.34 (0.90)	1.96 (0.85)	

Table 6.8: Wagner Staging Type LEFT FOOT and selected parameters

Wagner Stage	1	2	3	4	5
SBP (mmHg)	163.46 (36.41)	133.2 (24.09)	118.14 (18.00)	131 (32.91)	139.5 (28.99)
DBP (mmHg)	89.69 (17.85)	83.1 (9.68)	80.14 (14.42)	75.33 (26.50)	85.50
RBS (mmol/L)	10.63 (8.65)	9.12 (4.67)	14.33 (8.78)	8.9 (4.81)	14.4000
HbA1c (%)	8.47 (0.85)	10.58 (6.58)		8.5 (1.76)	
Urea (mmol/L)	12.24 (10.03)	15.22 (14.71)	14.16 (18.51)	9.55 (5.89)	6.75 (0.50)
Creat (mmol/L)	204.92 (117.10)	235.63 (241.97)	125.72 (57.65)	157.53 (52.29)	79 (16.97)
T. Chol (mmol/L)	4.12 (1.03)	4.18 (1.66)	3.045 (1.62)	2.5600	
TGs (mmol/L)	1.788 (0.79)	1.84 (0.83)	1.45 (0.37)	2.5600	
HDL-C (mmol/L)	1.17 (0.19)	1.01 (0.37)	1.03 (0.318)	.6200	
LDL-C (mmol/L)	2.13 (1.02)	1.77 (1.00)	1.54 (0.049)	0.9	

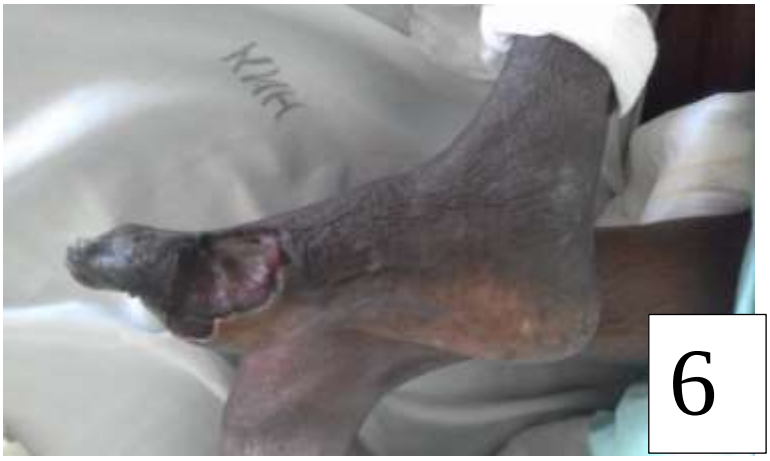
Table 6.9: RIGHT FOOT – Location of Foot Ulcer

Site	**Male**	**Female**
Forefoot	10 (52.6)	15 (50.0)
Midfoot	0 (0.0)	7 (23.3)
Hindfoot	8 (42.1)	3 (10.0)
Forefoot and Midfoot	1 (5.3)	3 (10.0)
Forefoot and Hindfoot	0 (0.0)	0 (0.0)
Midfoot and Hindfoot	0 (0.0)	1 (3.3)
Whole Foot	0 (0.0)	1 (3.3)

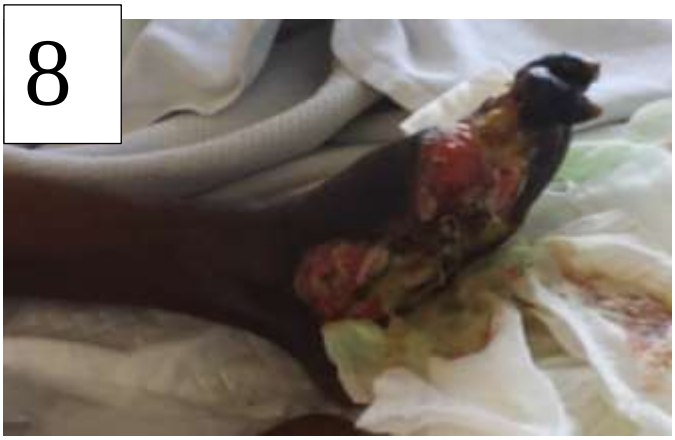
Table 6.10: LEFT FOOT – Location of Foot Ulcer

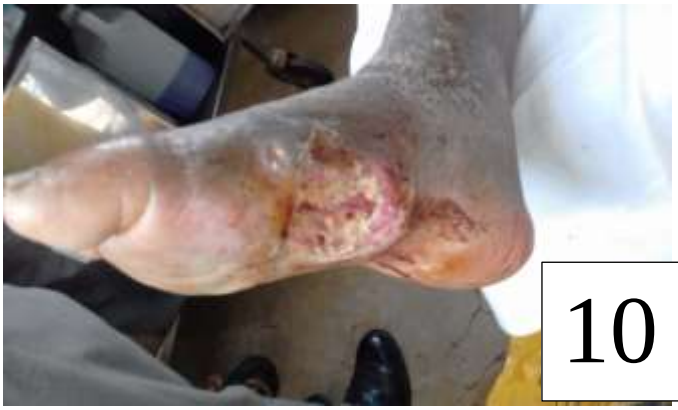
Site	Male	Female
Forefoot	11 (50.0)	13 (61.9)
Midfoot	0 (0.0)	2 (9.5)
Hindfoot	11 (50.0)	5 (23.8)
Forefoot and Midfoot	0 (0.0)	0 (0.0)
Forefoot + Hindfoot	0 (0.0)	0 (0.0)
Midfoot +Hindfoot	0 (0.0)	0 (0.0)
Whole Foot	0 (0.0)	1 (4.8)

8.8 Images of Diabetic Foot



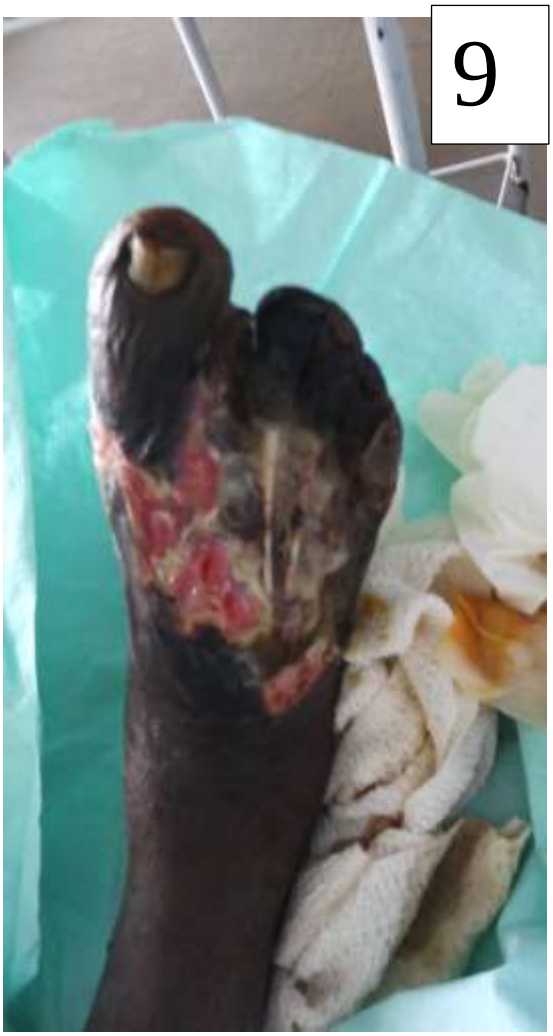
8





10

9



KEY:

3 – Neuropathic ulcer, midfoot Wagner Stage 1

4 – Forefoot ulcer Wagner Stage 0

5 – Healing Hindfoot Ulcer Stage 1

6 – Wagner Stage 5

7 - Ischaemic ulcer Wagner Stage 0

8 – Wagner Stage 4

9 – Midfoot ulcer Wagner Stage 2

10 – Midfoot ulcer Wagner Stage 2

8.9 Ethical Approvals

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Ref. No.KNH/ERC/R/29

12th February 2018

Dr. Daniel M. Mutonga
P O BOX 11692-00100
NAIROBI

Dear Dr. Mutonga

Re: Approval of Annual Renewal – Isolation, sensitivity patterns and molecular characterization of Bacterial isolates from infected diabetic foot ulcers in patients at Kenyatta National Hospital (P769/10/2016)

Refer to your communication dated January 29, 2018.

This is to acknowledge receipt of the study progress report and hereby grant annual extension of approval for ethical research protocol P769/10/2016.

The approval dates are 17th March 2018 – 16th March 2019.

This approval is subject to compliance with the following requirements:

a) Only approved documents (informed consents, study instruments, advertising materials etc) will be used.
b) All changes (amendments, deviations, violations etc.) are submitted for review and approval by KNH- UoN ERC before implementation.
c) Death and life threatening problems and severe adverse events (SAEs) or unexpected adverse events whether related or unrelated to the study must be reported to the KNH- UoN ERC within 72 hours of notification.
d) Any changes, anticipated or otherwise that may increase the risks or affect safety or welfare of study participants and others or affect the integrity of the research must be reported to KNH- UoN ERC within 72 hours.
e) Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. (*Attach a comprehensive progress report to support the renewal*).
f) Clearance for export of biological specimens must be obtained from KNH- UoN-Ethics & Research Committee for each batch of shipment.
g) Submission of an *executive summary* report within 90 days upon completion of the study
This information will form part of the data base that will be consulted in future when processing related research studies so as to minimize chances of study duplication and/or plagiarism.

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17 MAR 2017

Ref: KNH-ERC/A/92

17th March 2017

Dr. Daniel Munyambu Mutonga
Reg. No. W64/81850/2015
Institute of Tropical and Infectious Disease (UNITID)
College of Health Sciences
University of Nairobi

Dear Dr. Mutonga

Revised research proposal: Isolation, sensitivity patterns and molecular characterization of Bacterial Isolates from infected Diabetic Foot ulcers in patients at Kenyatta National Hospital (P769/10/2016)

This is to inform you that the KNH- UoN Ethics & Research Committee (KNH- UoN ERC) has reviewed and approved your above revised proposal. The approval period is from 17th March 2017 – 16th March 2018.

This approval is subject to compliance with the following requirements:

a) Only approved documents (informed consents, study instruments, advertising materials etc) will be used.
b) All changes (amendments, deviations, violations etc) are submitted for review and approval by KNH-UoN ERC before implementation.
c) Death and life threatening problems and serious adverse events (SAEs) or unexpected adverse events whether related or unrelated to the study must be reported to the KNH-UoN ERC within 72 hours of notification.
d) Any changes, anticipated or otherwise that may increase the risks or affect safety or welfare of study participants and others or affect the integrity of the research must be reported to KNH- UoN ERC within 72 hours.
e) Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. (*Attach a comprehensive progress report to support the renewal*).
f) Clearance for export of biological specimens must be obtained from KNH- UoN ERC for each batch of shipment.
g) Submission of an *executive summary* report within 90 days upon completion of the study. This information will form part of the data base that will be consulted in future when processing related research studies so as to minimize chances of study duplication and/ or plagiarism.

For more details consult the KNH- UoN ERC website **http://www.erc.uonbi.ac.ke**

"Protect to Discover"

Yours sincerely,

PROF M. L. CHINDIA
SECRETARY, KNH-UoN ERC

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The Director, CS, KNH
The Assistant Director, Health Information, KNH
The Chair, KNH-UoN ERC
The Director, UNITID, UoN
Supervisors: Dr. Marianne Mureithi, Prof. Fredrick C.F.Otieno, Dr. Nancy Ngugi