Speciation and Antifungal Susceptibility of *Candida* isolates from Diabetic Foot Ulcer Patients in Kenyatta National Hospital

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A research dissertation submitted to the Department of Medical Microbiology in partial fulfillment for the award of the Master of Science Degree in Medical Microbiology at the University of Nairobi

Declaration of originality

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Speciation and Antifungal Susceptibility of *Candida* isolates from Diabetic Foot Ulcer patients in Kenyatta National Hospital

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LIST OF ACRONYMS

ADA – American Diabetes Association

ATCC – American Type Culture Collection

CDC – Centers for Disease Control

CLSI – Clinical and Laboratory Standards Institute

DFD – Diabetic Foot Disease

DFU – Diabetic Foot Ulcer

DFUI – Diabetic Foot Ulcer Infection

DLW – Diabetic Lower Limb Wounds

DNA – Deoxyribonucleic Acid

IBM – International Business Machine

ICR – Institute of Clinical Research

IDDM – Insulin Dependent Diabetes Mellitus

IDF – International Diabetes Federation

IDSA – Infectious Disease Society of America

IWGDF – International Working Group on the Diabetic Foot

KAVI – Kenya AIDS Vaccine Initiative

KNH - Kenyatta National Hospital

KOH – Potassium hydroxide

NICE – National Institute for Health and Care Excellence

PAD – Peripheral Arterial Disease

SDA – Sabouraud Dextrose Agar

SPP - Species

SPSS – Statistical Package for the Social Sciences

UON - University of Nairobi

USA – United States of America

WHO – World Health Organization

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Abstract

Background

Diabetic foot ulcer is the leading cause of diabetic related hospital admissions, amputations and mortality among diabetic patients. Chronic wounds are a concern to public health worldwide, and the effects are a warning to the economy. Diabetic foot ulcer wounds are prone to infection with *Candida* species presenting as the principal fungal agent among other microorganisms. Identification of *Candida* isolates to species level is essential and might reduce antifungal drug resistance, cost of treatment, morbidity and mortality among diabetic patients.

Objective

To determine the prevalence, species and antifungal susceptibility of *Candida* species from diabetic foot ulcer patients receiving clinical services at Kenyatta National Hospital between June and August 2019

Methodology

This was a cross-sectional study carried out at Kenyatta National Hospital among diabetic adult patients presenting with active foot ulcers. A total of 152 swabs were consecutively collected from 152 diabetic foot ulcer patients over a three month period, from June to August 2019. We collected clinical and socio-demographic data using a structured questionnaire. Growth on Sabouraud Dextrose Agar was evaluated for colonial morphology, gram stain and germ tube. Species identification and antifungal susceptibility was determined using VITEK - 2 System according to CLSI M60 guideline. Data were retrieved and imported to WHONET through BACLINK and analysis done using WHONET version 5.6 and IBM SPSS Statistics version 21.

Results

Sixty one percent of the participants were male. The mean age was 50.7 (SD=12.9) years. Out of 152 samples, a total of 36 *Candida* species were isolated. Among these 46% were drug resistant, 11% multidrug resistant, 3% pandrug resistant and 40% susceptible to all the antifungal agents tested. *Candida albicans* was the most common species isolated with low incidence of resistance to echinocandins (26%) and triazoles (26%) but demonstrated high susceptibility to flucytosine (96%) and amphotericin B (81%). *Candida lusitaniae* and *C. dubliniensis* were the predominant non albicans *Candida* species and showed moderate resistance to voriconazole (50%) and amphotericin B (33%) respectively. Both showed 100% susceptibility to echinocandins, fluconazole and flucytosine. Eighty percent of the wounds demonstrated polymicrobial infections.

Conclusion

Candida species was isolated in a fifth of the participants and showed low resistance rates to the commonly administered antifungal agents such amphotericin B and fluconazole. However, we also noted a high number of the wounds to have mixed infection. There is need for inclusion of fungal diagnosis in diabetic foot ulcer infection, continuous antifungal resistance surveillance especially in *Candida* species and strengthening of antifungal stewardship programmes to enhance patient care and management.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Diabetes mellitus is a chronic metabolic non-communicable disorder associated with severe complications and premature death. The high morbidity and mortality occurring every year is more prevalent among patients of lower socioeconomic status due to poverty, negligence, and illiteracy (Raza and Anurshetru, 2017; Kalshetti *et al.*, 2017; Brownrigg *et al.*, 2013).

According to the International Diabetes Federation (IDF), approximately 425 million people globally have diabetes with South – East Asia and Africa recording 82 and 16 million cases respectively. In 2016, an estimated 1.6 million deaths occurred globally due to diabetes and diabetes-related complications with more than 80% occurring in low and middle-income countries. It is projected that cases of diabetes mellitus will increase to 500 and 600 million by the year 2025 and 2030 respectively. The increase is predicted to occur in the developing countries due to sedentary lifestyle, aging, unhealthy diets and population growth. The World Health Organization (WHO) and Lancet 2016 reported a global rise in the prevalence of diabetes from 4.7% to 8.5% from 1980 to 2014 (World Health Organization, 2018; Krug, 2016). Kenya is experiencing a double burden of both communicable and non-communicable diseases recording a 150% rise from 2.4% in 1980 to 6% in 2014. In addition, an estimated 190,400 Kenyans within the age group of 20-79 suffer from diabetes mellitus. It's predicted by 2030; diabetes will be the seventh cause of death (World Health Organization, 2018; International Diabetes Federation, 2017; Selva Olid *et al.*, 2015).

Resource-limited countries have reported an increasing burden of complications associated with diabetes. Among the diabetes complications, Diabetic Foot Disease (DFD) is the leading cause of hospitalization, non-traumatic amputations of lower extremities and reduction of quality of life among diabetic people. According to the Institute for Health Metrics and Evaluation 2017 report, diabetes and related complications were seventh among the health problems causing Disability-adjusted life years (DALYs) (IHME, 2017; Oostvogels *et al.*, 2015). To achieve the targets stipulated in Sustainable Development Goals (SDGs) 3 set for 2030, estimation of healthcare cost implicated in the management of diabetes and related complication is important (Mutyambizi *et al.*, 2018). Globally, healthcare expenditure in treatment, management and prevention of diabetes/diabetes-related complication is estimated to cost 400 million USD (IDF, 2015). Recent

studies on healthcare expenditure, estimate the treatment and management of diabetes to 11 – 15% of the world's total health expenditure (Elrayah-Eliadarous *et al.*, 2017). The mean annual healthcare cost (USD 44200) of DFU management is twice that of managing other chronic ulcer aetiology (Hurlow *et al.*, 2018). In Tanzania, the cost of DFU management is low (USD 3060) compared to Nigeria (USD 3468) (Kasiya *et al.*, 2017; Danmusa *et al.*, 2016). Like many African countries faced with scarcity of healthcare resources, political and economic instability, diabetes management presents as one of the major healthcare burdens to the already struggling healthcare services with the financial burden being imposed to the patient. Kenya is striving to provide affordable healthcare to its citizens through the Universal Health Care (UHC) initiative as part of the four pillars of economic development (PBO, 2018).

Annually, Diabetic Foot Ulcers (DFU) affects 1 – 4% of diabetic patients (Rice *et al.*, 2014). India as one of the WHO member states has the highest number of diabetics with approximately 15% during their lifetime developing lower extremity ulcers. Worldwide, the prevalence of DFU is 6.3% with North America and Oceania recording the highest and lowest prevalence of 13% and 3% respectively. According to a systematic review conducted in 2017, Africa has a DFU prevalence of 7.2% relatively higher than Asia (5.5%) and Europe (5.1%). In Nigeria and Cameron, the prevalence is between 9.9% - 19.1% while in Kenya, DFU prevalence is approximately 4.6% lower than Tanzania (7.3%) and Egypt (6.2%) with about 750,000 reported cases and 20,000 deaths annually (International Diabetes Federation, 2017; Zhang *et al.*, 2017; Desalu *et al.*, 2011; Nyamu *et al.*, 2003).

The lower limb amputations are preceded by the development of Diabetic Foot Ulcer and polymicrobial infections of the wound. Of the reported diabetic complications, 20% involve the feet, and the major factors contributing to the diabetic foot ulcer are the peripheral neuropathy, macro and micro angiography. It occurs frequently causing sensory impairment, weakness of intrinsic muscles and ischemia of foot tissues leading to foot deformities. This leads to the development of wounds which become infected more often with the rate of infection parallel to high blood levels of glucose (World Health Organization, 2018; Halpati *et al.*, 2014).

An estimated 60% of the amputations of the lower extremities in developed countries are associated with DFU infections. Early diagnosis and appropriate antimicrobial therapy is essential. Management of diabetic foot infection is difficult due to impaired microvascular circulation around

the lower limb. This hinders the accessibility of phagocytes and the antimicrobial agent to the infected site. In Africa, the infection rate is not known but its postulated to be similar or slightly higher to Europe at 58% (Kasiya *et al.*, 2017). Common micro-organism isolated from DFU includes aerobes of the genus *Staphylococcus, Enterococcus, Pseudomonas, Acinetobacter,* the family *Enterobacteriaceae* and some anaerobes. Among the bacteria, *Pseudomonas* species and *Enterococcus* species are isolated with fungi (Karmaker *et al.*, 2016; Sanniyasi, Balu and Narayanan, 2015). Most of the studies focus on bacteria with some reporting low cases of pathogenic yeast. In polymicrobial infections, *Candida* is the most common fungal agent isolated from diabetic foot ulcer. Common *Candida* species isolated during diabetic foot ulcer infection include *Candida albicans, Candida krusei, Candida tropicalis* and *Candida dubliniensis* (Abilah *et al.*, 2015). Fungal infection is a major health concern despite the proper surgical and antimicrobial therapy of DFU. Irrational use of antimicrobials is associated with the development of antimicrobial resistance which is a key health problem in the 21st century. Clinicians managing diabetic lower limb wounds (DLW) mostly focus on bacteria as the infecting agent without considering samples from the deep portion of the wound for fungal culture and sensitivity (Peters, 2016; Chellan *et al.*, 2010).

With the speculation of deep fungal diabetic wound infection contributing to delay wound healing consequently resulting in high cost of treatment and development of antifungal resistance; this study aimed at determining the prevalence, species and antifungal susceptibility pattern of *Candida* species isolated from diabetic lower limb wounds.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Introduction

Diabetes mellitus is an endocrine disorder resulting in high blood glucose levels. This is due to the pancreas secreting insufficient insulin or inability of the target cells to utilize properly the insulin produced. According to the American Diabetes Association there are two main types of diabetes mellitus: type I, also known as the insulin dependent diabetes mellitus (IDDM), insulin production by the body is impaired and type II in which the insulin produced is not enough for proper function or there is no response to insulin by the body cells: insulin resistance. Other forms include gestational diabetes that affects female during pregnancy. Worldwide, approximately 10% of the diabetic cases are of type I while 90% are of type II (American Diabetes Association, 2015).

Over time, elevated blood sugar (hyperglycemia) leads to complications associated with multiple organ failure. The most common reported diabetes complication include kidney, eye, heart and blood vessels, nervous system and the foot complications which leads to amputation. Other acute complications associated with diabetes are diabetic ketoacidosis and the diabetes non-ketotic coma (hyperosmolar). Diabetic foot ulcers is characterized by a classical triad of peripheral neuropathy, ischemia and infection (Lal, 2016). Risk factors associated with the infection include recurrent foot ulcers, previous amputation of the lower extremities, long duration of more than 30 days of foot ulceration, existing wounds due to trauma, walking bare foot, peripheral sensory neuropathy and renal insufficiency (Peters, 2016).

2.2 Epidemiology and aetiology

Diabetic foot ulcer is one of the most common complications of diabetes with 15% of all diabetic individuals developing it during diabetic life. Approximately 85% of the lower limb amputations are preceded by diabetic foot ulcer. Although there are numerous predisposing factors for diabetic foot ulcer, the most important is peripheral sensory neuropathy and peripheral vascular disease. Lesions in diabetic are neuropathic, neuroischemic and ischemic. Ischemic foot ulcer presents with peripheral arterial disease with no neuropathy, while neuroischemic is considered if neuropathy and peripheral vascular disease are both present and neuropathic when neurological disability is present with no clear presentation of peripheral vascular disease (International Diabetes Federation, 2017;

Desalu *et al.*, 2011). Different systems of classifying the diabetic foot ulcer are used. These systems facilitate treatment and aid in predicting the outcome. The most widely used and accepted classification system is the Wagner ulcer classification system (Danmusa *et al.*, 2016; Nyamu *et al.*, 2003).

- Stage 0 – No open lesions, foot at risk
- Stage 1- Superficial ulcer
- Stage 2 – Deep ulcer (extending to the ligament, tendon, joint capsule or deep fascia) without abscess or bone involvement
- Stage 3 – Deep ulcer with bone involvement and abscess
- Stage 4 – Localized gangrene to the portion of toes and heels
- Stage 5 – Gangrene involving the entire foot

Diabetic foot ulcers have a negative social impact and functional ability resulting in financial instability, reduced work productivity and high hospital care cost. An open wound due to foot ulceration and immunological response associated with diabetes often lead to an infection. Diabetic foot infection is the most common cause of diabetic related hospital admissions and accounts for approximately 80% of the lower limb amputations (Jneid *et al.*, 2017). Diabetic individuals are 23 times at risk of undergoing a lower extremity amputation due to diabetic foot ulcer compared to an individual without diabetes. According to the National Diabetes Audit, in England and Wales, 7 out of 10,000 people with diabetes in 2008 – 2009 underwent a major lower limb amputation with an estimated 72,000 hospital admissions recorded in 2010 – 2011 due to diabetes-related complications. Belgium records the highest prevalence of DFU at 16.6% followed by Canada (14.6%), USA (13.0%), Trinidad (12.2%) and India (11.6%). Korea, Poland and Australia record the lowest DFU prevalence (1.5% – 1.7%) (Zhang *et al.*, 2017; Brownrigg *et al.*, 2013).

Through a multifactorial and matrix interaction, the aetiology of DFU involves distal polyneuropathy (autonomic, motor and sensory), abnormal foot anatomy, peripheral arterial disease (PAD) and functional changes in the microcirculation. Painless neuropathic foot trauma leads to ulcer development which due to PAD maybe poorly perfused; hence healing takes longer. Ulceration and infection increase the oxygen demand impairing wound healing; other factors include defective humoral immunity and abnormal inflammatory responses. Micro-organisms representing the normal

flora from the surrounding skin are usually present in DFU as in all chronic wounds. Colonizing microorganisms cause no host tissue inflammation as compared to infecting micro-organisms. Basing on clinical diagnosis, signs and symptoms of host tissue inflammation in infected DFU includes pyrexia, warmth, purulent secretions and induration (Raza and Anurshetru, 2017; Brownrigg *et al.*, 2013; Fata *et al.*, 2011).

2.3 Antifungal agents and development of resistance

Fungal infection especially among the immunocompromised is a public health challenge in healthcare settings worldwide. Empirical antifungal therapy is required for successful patient management. Due to limited classes of antifungal drugs, choices of drugs for treatment are restricted. The chemical classes include those which modify the cell membrane (azoles and the polyenes), nucleic acid and protein flucytosine (5 – fluorocytosine) and those which act on the cell wall (echinocandins). The onset of antifungal drug resistance especially in immunocompromised patients is marked by the rampant use of antifungal therapy.

Antifungal resistance can be microbiological (fungal factors due to genetic alteration) which can further be classified into intrinsic and acquired or clinical (due to host or drug-related factors). Intrinsic resistance is found naturally within some fungal strains before exposure to drugs while acquired resistance occurs due to alteration of genes upon drug exposure to a previously susceptible fungal strain (Sanguinetti, Posteraro and LassFlorl, 2015). There is increasing resistance to the first line and second line antifungal drugs like fluconazole and the echinocandins among the *Candida* species. Resistance to fluconazole has been constant for the past 20 years with surveillance data from CDC indicating that an estimated 3% of the *Candida glabrata* isolates are resistant to echinocandins (Wiederhold, 2017; Perlin, Shor and Zhao, 2015). Multidrug resistant *Candida* infections pose a threat in patient management especially among very sick and immunocompromised patients, as a consequence, Amphotericin B used in treatment of such cases is known to be toxic to human tissues (Sanglard and Odds, 2002).

2.4 Management of Diabetic Foot Ulcer infections

Wound closure is the ultimate goal in the management of diabetic foot ulcer. Severity in terms of grading, vascularity and presence of an infection determine the management of the wound. Due to the multifaceted nature of the wound, a systematic and multidisciplinary approach is required for the wound management as this has shown significant improvement and reduction in major lower limb amputations (Raza and Anurshetru, 2017; Danmusa *et al.*, 2016).

For the past few years, numerous guidelines and working group recommendations have been published with a focus on improving the management and care of people with DFUs. These include (1) the UK National Institute for Health and Care Excellence (NICE), a guideline on inpatient management of diabetic foot ulcers (2) the International Working Group on the Diabetic Foot (IWGDF) that focuses on the management and prevention of the diabetic foot (3) the Infectious Disease Society of America (IDSA), a guideline for the diagnosis and treatment of diabetic foot infections (Kwon and Armstrong, 2018; Xie *et al.*, 2017).

According to NICE, a diabetic patient presenting with DFU should be evaluated clinically at three levels: the diabetic patient as a whole, the limb affected and the infected wound. Before empiric therapy, NICE recommends obtaining an appropriate sample for culture after the wound has been cleansed and debrided. In addition, the IWGDF recommends a gram stain to be performed before the culture (Nelson *et al.*, 2018). The IDSA is currently the most comprehensive guideline with a review to strengthen the recommendation and quality of the supporting evidence on the diagnosis and management of DFUs (Kwon and Armstrong, 2018; Xie *et al.*, 2017).

2.5 Rationale of the study

Globally, the prevalence of diabetes is on the rise with developing countries recording high rates compared to developed countries. Challenges in the management of diabetes have been encountered due to complications associated with diabetes. Diabetic foot ulcer is the leading cause of hospitalization, disability and death among diabetic patients (Commons *et al.*, 2018). The ulcers are prone to fungal infection with *Candida species* presenting as the common fungal agent. The deep fungal diabetic wound infection may contribute to bone infection, candidaemia and delay in wound healing.

Adequate therapy for patient management is difficult to achieve because of the narrow spectrum of antifungal drugs/classes, toxicity associated with some of the drugs, the cost of the drugs and emergence of antifungal resistance. The rising trends of antifungal resistance reported in *Candida albicans* and non- *Candida albicans* isolates together with the recently revised Clinical and Laboratory Standards Institute (CLSI) antifungal breakpoints necessitates periodic and continuous fungal culture and sensitivity from deep tissue (Zaidi *et al.*, 2018; Fothergill *et al.*, 2014; Ooga, Bii and Gikunju, 2015).

This study identified the species and determined the antifungal susceptibility pattern of *Candida* species isolated from diabetic foot ulcer patients attending Kenyatta National Hospital between June and August 2019. Results from this study may be used in developing treatment and infection control policies in the management of DFU. This may guide clinicians in prescribing appropriate antifungal drugs to curb antifungal drug resistance, reduce hospital admissions and prevent major surgical interventions thus minimizing healthcare cost. Information from the study may also be used as a baseline in determining trends in antifungal susceptibility pattern.

2.6 Study questions

1. What is the prevalence of *Candida* infection of diabetic foot ulcers among patients with diabetes attending Kenyatta National Hospital between June and August 2019?
2. What are the species of *Candida* isolated from the study population?
3. What is the antifungal susceptibility pattern of *Candida* species isolated?

2.7 Study objective

2.8 Broad objective

To determine the prevalence, species and antifungal susceptibility of *Candida* isolates from diabetic foot ulcer patients attending Kenyatta National Hospital for clinical services between June and August 2019.

2.9 Specific objectives

1. To determine the prevalence of *Candida* infection of diabetic foot ulcers among diabetic patients.
2. To identify the species of *Candida* isolated from diabetic foot ulcer.
3. To determine antifungal drug susceptibility of *Candida* species isolated.

CHAPTER THREE

3.0 METHODOLOGY

3.1 Study design

This was a prospective cross-sectional study

3.2 Study site

The study was carried out in Kenyatta National Hospital (KNH) located along Hospital Road, Upper Hill in Nairobi. It's the main teaching hospital for the University of Nairobi, College of Health Sciences. With 50 wards, 22 out-patient clinics, 24 theatres (16 specialized), an Accident and Emergency department and a bed capacity of 1800, the hospital is the largest referral hospital in East and Central Africa. The study was conducted in the medical ward, surgical outpatient clinic, orthopedic ward and diabetic outpatient clinic. On average, 400 diabetic patients and 15 DFU patients are attended to at the clinics per week. The KNH diabetes clinics is managed by consultants, endocrinologists, physicians, graduate resident doctors, nutritionists, nurses and specialized educators.

3.3 Study population

We enrolled diabetic patients presenting with foot ulcer attending KNH between June and August 2019.

3.3.1 Inclusion criteria

- Diabetic patients presenting with foot ulcer
- Aged 18 years and above

3.3.2 Exclusion criteria

- Patients who decline to consent
- Patients on immunosuppressive drugs/state e.g. steroids, HIV/AIDS, cancer

3.4 Sample size

To determine the sample size, Cochran's formula was adopted (Kothari, 2016). The prevalence of *Candida* species among diabetic foot ulcer patients in KNH is unknown. An assumed prevalence of 50% was used to estimate the appropriate sample size. As per KNH records, approximately 3

diabetic foot ulcer patients are attended to in the KNH Diabetes Clinic every day. The total number of DFU patients attended during a three-month study period would be approximately 180. A representative sample was calculated using the finite population correction for proportions.

$$n_0 = Z^2pq/d^2$$

$$n = \frac{n_0}{1+\frac{(n_0 - 1)}{N}}$$

Where:
n_0 = initial estimated sample study size
Z = standard normal deviate at 95% confidence interval (1.96)

p = estimated prevalence of *Candida* species in diabetic foot ulcers patients in KNH.

q = 1-p

d = degree of freedom (0.05)

N= Total population of diabetic foot ulcer patients that will be attended to in KNH diabetic clinic for three months (180)

$$n_0 = \frac{1.96^2 * 0.50\ (1-0.50)}{0.05^2}$$

$$= 384$$

$$n = \frac{384}{1+\frac{(384 - 1)}{180}}$$

= 123

3.5 Sampling technique

Consecutive sampling technique was applied to recruit the patients. The researcher obtained informed consent from suitable patients. Consequently, patients who agreed and signed an informed consent to participate in the study were selected until the desired sample was achieved (Appendix 1).

3.6 Variables

Independent variables measured included age, gender, level of education, occupation, residence, marital status, type of diabetes, medication use, Wagner classification of the ulcer.

Dependent variables included *Candida* isolates, antifungal susceptibility profile, duration of diabetes and duration of diabetic foot ulcer.

3.7 Data collection procedures

Structured questionnaire (Appendix 2) was used by the principal investigator and the trained research assistant to collect information on patient's bio-data, demographic details, history of medication, duration of diabetes, diabetic foot ulcer and pre-existing conditions. Samples were collected with the help of a diabetologist. The samples were collected using two sterile swabs moistened with sterile normal saline from the deep portion of the ulcer wound by a firm rotatory movement after cleaning and debridement. The samples were transported in a labeled cool box to the UoN Microbiology Laboratory for analysis within two hours after collection. The microbiological analysis was carried out by the principal investigator and a laboratory technologist.

3.8 Laboratory procedures

Mycological laboratory procedures were conducted as per the standard operating procedures developed and approved by the Department of Medical Microbiology, UON. Two smears were prepared from the deep tissue sample swab and examined in 10% KOH and gram stain using direct microscopy after inoculation on SDA media supplemented with chloramphenicol and gentamicin. Culture plates were incubated at 37˚C for 18 – 24 hours and examined afterwards for growth. Germ tube production was detected using germ tube test. Identification test and antimicrobial susceptibility testing was done using the VITEK 2 System (YST card and AST-YS08 respectively) and analyzed according to the 2017 Clinical and Laboratory Standards Institute guidelines (CLSI M60). The antifungal agents that were tested for susceptibility included amphotericin B, caspofungin, fluconazole, flucytosine, micafungin and voriconazole. Quality control strains *C. albicans* ATCC 10231 and *C. parapsilosis* ATCC 22019 were used during the laboratory procedure.

3.9 Ethical consideration

Ethical approval was obtained from KNH-UoN Ethics and Research Committee (P290/04/2019). Permission to conduct the study was sought from the Head of Department, Medicine and Laboratory Medicine, KNH and the Chairman, Department of Medical Microbiology, College of Health Sciences, UoN. Informed and signed consent was obtained from each participant. The principal investigator and the research assistant explained to the participants what the study entailed, the benefit, risks, voluntary participation and the confidentiality of the information collected. Patient names, file and clinic number were excluded. The patient identifiers in the questionnaires were recoded to maintain confidentiality. Patients benefitted from microbiological analysis of collected swabs and deep tissue at no cost. The report was communicated to the clinicians on the most appropriate antimicrobial agent for the species isolated. The probable risk during the study involved a slight feeling of pain upon touch on the participants open wound, cross contamination and microbiological analysis of collected swabs. This risk was mitigated by preparing the participant for the event and slight pain anticipated to occur, the use of standard operating procedures and qualified laboratory personnel at the Department of Medical Microbiology, UoN and KNH.

3.10 Data management

Filled questionnaires were stored in a cabinet under lock and key. Data cleaning was done by checking the questionnaires for errors and frequency distribution. The cleaned data was entered in a Microsoft Excel sheet, saved in a password-controlled laptop for security and privacy purposes. A dedicated USB drive under the custody of the principal investigator was used as a back-up. Data was analyzed using WHONET version 5.6 and IBM SPSS Statistics version 21. Univariate analysis was done using frequency distributions and proportions for categorical variables such as antimicrobial susceptibility, gender and age. Bivariate analysis was done using Chi-square test to assess any association between the outcome variable and categorical independent variables such as the type of diabetes and *Candida* species isolated. The percentage resistance for each *Candida* species or antifungal combination was generated by keying the result of the first isolate. At 95%, confidence intervals (binomial proportions) were calculated using the Agresti-Coull interval as recommended in the CLSI M60 (CLSI, 2017). The level of significance for all tests was set at $\leq$ 0.05. Data was presented in tables and graphs.

CHAPTER FOUR

4.0 RESULTS

4.1 Demographic and social characteristics of the study participants

We recruited a total of 152 diabetic patients presenting with active foot ulcers. These patients were drawn from diabetic outpatient clinic, medical ward, orthopedic ward and surgical outpatient clinic (Figure 1).

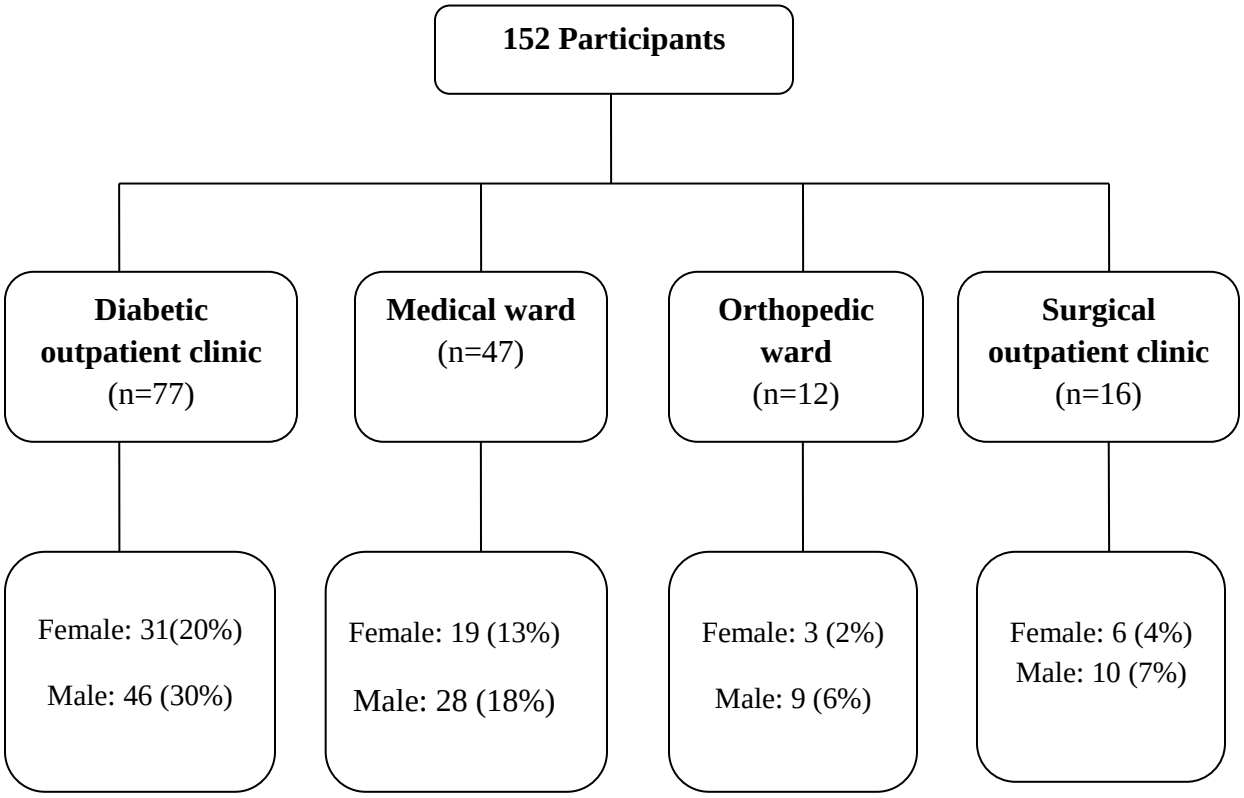

Figure 1. A flow diagram showing the different units we recruited the patients from in relation to the frequency per unit and gender of the patients

Majority of the participants were recruited from Diabetic outpatient clinic (51%, n=152) and Medical wards (30%). One hundred and thirteen (74%) of the study participants were urban residents while 39 (26%) resided in the rural areas. Majority of the patients sampled (30%) aged between 40 and 50 years with 54% having attained secondary education and 78% were on employment (salaried or self employment). Eighty percent of the study participants were married, 11% divorced or widowed and 9% single (Table 1).

Table 1: Socio-demographic characteristics of study participants

Characteristics		Point of Care n (%)		Total
		Diabetic Outpatient Clinic	Other points of care	
Gender	Male	46 (60)	47 (63)	93 (61%)
	Female	31 (40)	28 (37)	59 (39%)
Age group	<40	19 (25)	16 (21)	35(23%)
	40-50	23 (30)	24 (32)	47 (31%)
	50-60	13 (16)	23 (31)	36 (24%)
	>60	22 (29)	12 (16)	34 (22%)
Marital Status	Single	5 (7)	8 (11)	13 (9%)
	Married	62 (80)	60 (80)	122 (80%)
	Divorced/Widow(er)	10 (13)	7 (9)	17 (11%)
Education	Primary	15 (20)	12 (16)	27 (18%)
	Secondary	37 (48)	45 (60)	82 (54%)
	Tertiary	20 (25)	16 (21)	36 (23%)
	Informal	5 (7)	2 (3)	7 (5%)
Residence	Urban	61 (79)	52 (69)	113 (74%)
	Rural	16 (21)	23 (31)	39 (26%)
Occupation	Salaried	4 (5)	5 (7)	9 (6%)
	Self employed	53 (69)	57 (76)	110 (72%)
	Unemployed	20 (26)	13 (17)	33 (22%)

The mean age of the study participants was 50.7 years (SD, 12.9). Patients in diabetic outpatient clinic (n=77) and other points of care (n=75) had an average age of 50.8 years (SD, 14.01) and 50.5 (SD, 11.7) respectively. There was no significant difference in age distribution between patients in diabetic outpatient clinic and other points of care (*p=0.848*).

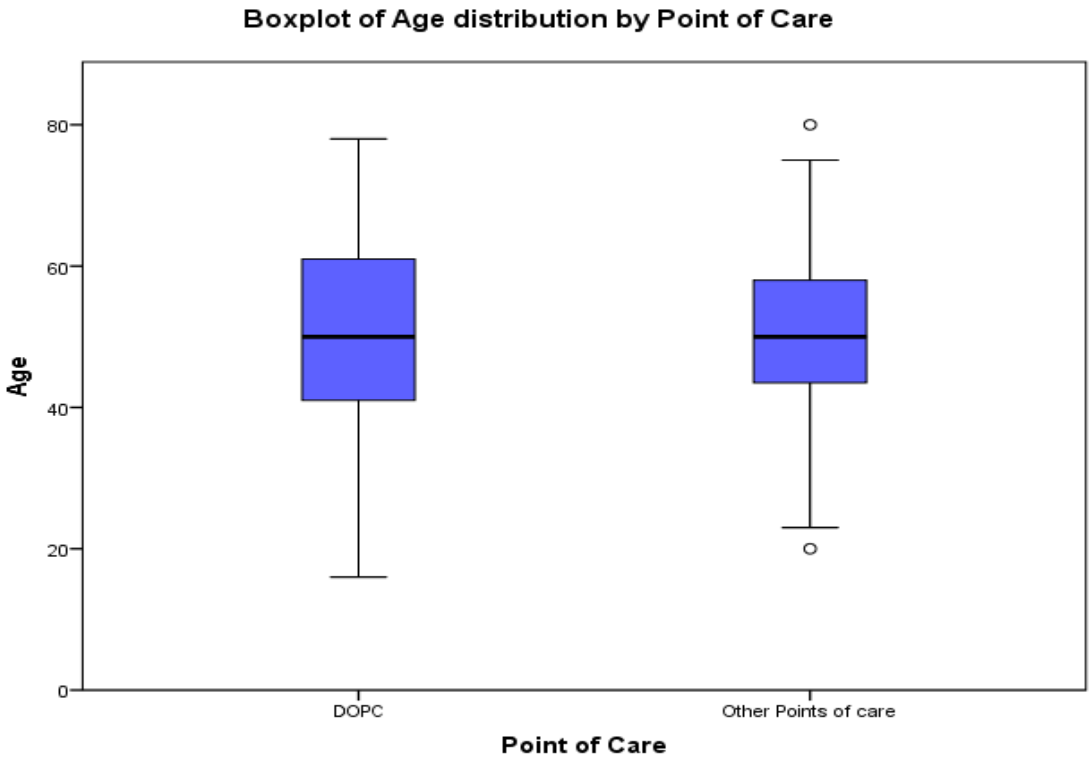

Figure 2. Age distribution by point of care

4.2 Clinical characteristics of the study participants

Nearly all participants had Type 2 diabetes (149, 98%) with type 1 diabetes forming less than 2% of the study population. The median duration of diabetes and diabetic foot ulcers within the study population was 11 years (IQR 5.25-11.0) and 2 months (IQR 1.0-3.0) respectively. Majority of the study participants (97%) had random blood sugar level within reference ranges (<10 mmol/L). Twenty nine percent of the participants had been diagnosed with diabetes for the past 15 years and 21% for the past 5 years. Most of the participants (80%) presented with foot ulcers that had lasted less than 3 months and 10 (7%) for more than 5 months. More than half of the population studied had 2 or more episodes of foot ulcers. A fifth of the study population was under antimicrobial agent

medication with ceftriaxone and metronidazole being the most common prescribed agents. None of the patients was on antifungal medication (Table 2).

Table 2: Clinical characteristics of study participants

Characteristics		n (%)
Type of Diabetes	Type 1	3(2)
	Type 2	149 (98)
Random Blood Sugar (mmol/L)	High	5 (3)
	Within Range	147 (97)
Duration of diabetes (years)	<5	32 (21)
	5-10	34 (22)
	10-15	42 (28)
	>15	44 (29)
Duration of diabetic foot ulcer (months)	<3	123 (80)
	3-5	19 (13)
	>5	10 (7)
Mean duration		2.58 (±1.75)
Median (IQR)		2.00 (2)
Wagner stage	Grade I	40 (26)
	Grade II	93 (61)
	Grade III	13 (9)
	Grade IV	6 (4)
	Grade V	0 (0)
Episode of DFU	Episode 1	58 (38)
	Episode 2	88 (58)
	Episode 3	6 (4)
	Episode 4	0 (0)
On Antibiotics/ Antifungals	Metronidazole	24 (80)
	Ceftriaxone	3 (10)
	Metronidazole & Ceftriaxone	1(3)
	Metronidazole & Amoxycillin	1(3)
	Ciprofloxacin	1(3)
	Antifungals	0(0)

Table 3: Socio-demographic and clinical characteristics of the study population focusing on DFU *Candida* infection

Characteristic		n	*Candida*		P value
			Positive	Negative	
Age (Years)					
	< 40	37	8 (21.6%)	29 (78.4%)	*0.831*
	> 40	115	23 (20.0%)	92 (80.0%)	
Gender					
	Male	93	22 (23.7 %)	71 (76.3%)	*0.210*
	Female	59	9 (15.3%)	50 (84.7%)	
Marital status					
	Single/Divorced	30	5 (16.7%)	25 (83.3%)	*0.572*
	Married	122	26 (21.3%)	96 (78.7%)	
Residence					
	Rural	39	6 (15.4%)	33 (84.6%)	*0.368*
	Urban	113	25 (22.1%)	88 (77.9%)	
Education					
	Primary/Vocational	34	7 (20.6%)	27 (79.4%)	*0.975*
	Secondary/above secondary	118	24 (20.3%)	94 (79.7%)	
Employment					
	Unemployed	33	3 (9.1%)	30 (90.9%)	*0.069*
	Employed	119	28 (23.5%)	91 (76.5%)	
Duration of diabetes (years)					*0.708*
	< 10	69	15 (21.7%)	54 (78.3%)	
	>10	83	16 (19.3%)	67 (80.7%)	
Random Blood Sugar (mmol/L)	High	5	1 (20.0%)	4 (80.0%)	*0.982*
	Within Range	147	30 (20.4%)	117 (79.6%)	
Duration of DFU (months)	< 2	96	20 (20.8%)	76 (79.2%)	*0.861*
	>2	56	11 (19.6%)	45 (80.4%)	
Wagner (Grade)					
	<2	131	26 (19.8%)	105 (80.2%)	*0.676*
	>2	21	5 (23.8%)	16 (76.2%)	
On Antibiotics					
	Yes	27	8 (29.6%)	19 (70.4%)	*0.189*
	No	125	23 (18.4%)	102 (81.6%)	

There was no significant association between *Candida* DFU infection and the various variables studied including gender (*p=0.831*), age group (*p=0.210*), marital status (*p=0.572*), residence (*p=0.368*), education level (*p=0.975*), employment (*p=0.069*), duration of diabetes (*p=0.708*), duration of foot ulcers (*p=0.861*), grading of the ulcers (*p=0.676*), prior antibiotic use (*p=0.189*), and random blood sugar level (*p=0.982)* as shown in Table 3.

4.3 Isolation of *Candida* species and bacterial/other fungal organisms in Diabetic foot ulcer

Out of 152 samples collected, 38 (25%) and 36 (24%) were KOH and gram stain positive respectively for fungal elements. Fifty-nine samples were gram stain positive for bacteria; 9 (15.3%) gram positive cocci in clusters; 30 (50.9%) gram negative rods and 20 (33.9%) mixed bacterial infection of gram positive cocci in clusters and gram negative rods.

Thirty-nine samples (25.7%) showed fungal growth on SDA medium after 18-72 hours of incubation; we observed yeast cells in 31 culture plates and in the other 8 culture plates we observed moulds after an incubation period of 7-14 days at 19-25ºC (3 culture plates had *Penicillium spp*, 2 had *Aspergillus spp*, 2 had *Microsporum spp* and 1 culture plate had *Trichophyton mentagrophytes*). Yeast cells were identified using VITEK 2 System. *Candida albicans* and *C. dubliniensis* species were confirmed by germ tube test (GTT) while the growing moulds were identified and confirmed by colonial morphology on SDA and Lactophenol Cotton Blue (LPCB) staining technique.

Among the 36 *Candida* species isolated, 30 were GTT positive. *Candida albicans* (27; 75%) was the most frequently isolated species (Figure 3). Non-albicans *Candida* species (NAC) identified included *Candida lusitaniae* (3; 8.3%), *C. dubliniensis* (2; 5.6%), *C. glabrata* (1; 2.8%), *C. tropicalis* (1; 2.8%), *C. famata* (1, 2.8%) and *C. parapsilosis* (1; 2.8%). Other yeast cells isolated included *Trichosporon asahii*.

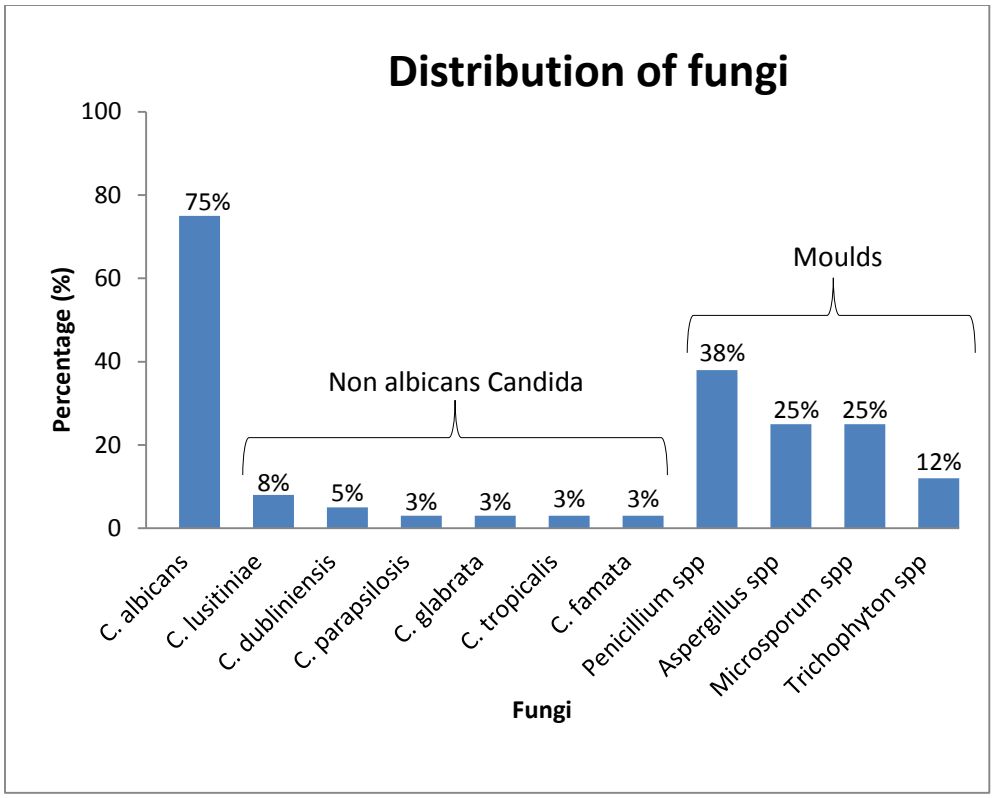

Figure 3. A chart showing the distribution of fungi

4.4 Monomicrobial versus Polymicrobial infection

The pattern of mixed infections is summarized in Table 4. Sample received was analyzed for the presence of bacteria and *Candida* species using gram stain and culture growth respectively. Eighty percent of the *Candida* positive samples had mixed infection of at least two *Candida* species, a gram positive and (or) gram negative bacteria. Approximately 20% of the samples had pure *Candida* species isolated and among these, 13% were *Candida albicans* isolates.

Table 4: Diabetic Foot Ulcer infection profile

Organism	**n (%)**
Fungi only	
C. albicans	4 (66.7)
C. albicans & *C. dubliniensis*	1 (16.7)
C. albicans & *C. tropicalis*	1 (16.7)
Total	**6**
Mixed Infections (*Candida* & bacteria)	
C. albicans, gram positive cocci in clusters & gram negative rods	7 (28)
C. albicans & gram negative rods	6 (24)
C. albicans & gram positive cocci in clusters	4 (16)
C. lusitaniae, gram positive cocci in clusters & gram negative rods	2 (8)
C. glabrata & gram negative rods	1 (4)
C. dubliniensis, gram positive cocci in clusters & gram negative rods	1 (4)
C. albicans, *C. famata*, gram positive cocci in clusters & gram negative rods	1 (4)
C. albicans, *C. parapsilosis*, gram positive cocci in clusters & gram negative	1 (4)
C. albicans, *C. lusitaniae* & gram negative rods	1 (4)
C. albicans, *T. asahii* & gram negative rods	1 (4)
Total	**25**

4.5 Antifungal susceptibility testing

The antifungal susceptibility testing results indicated that *Candida* species (n=35) isolated from DFU showed low resistance rates to flucytosine (3%; 0-17) (%R; 95% C.I), amphotericin B (17%; 7-34), echinocandins (caspofungin and micafungin) (20%; 9-38), fluconazole (20%; 9-38) and voriconazole (23%; 11-41) as shown in Figure 4.

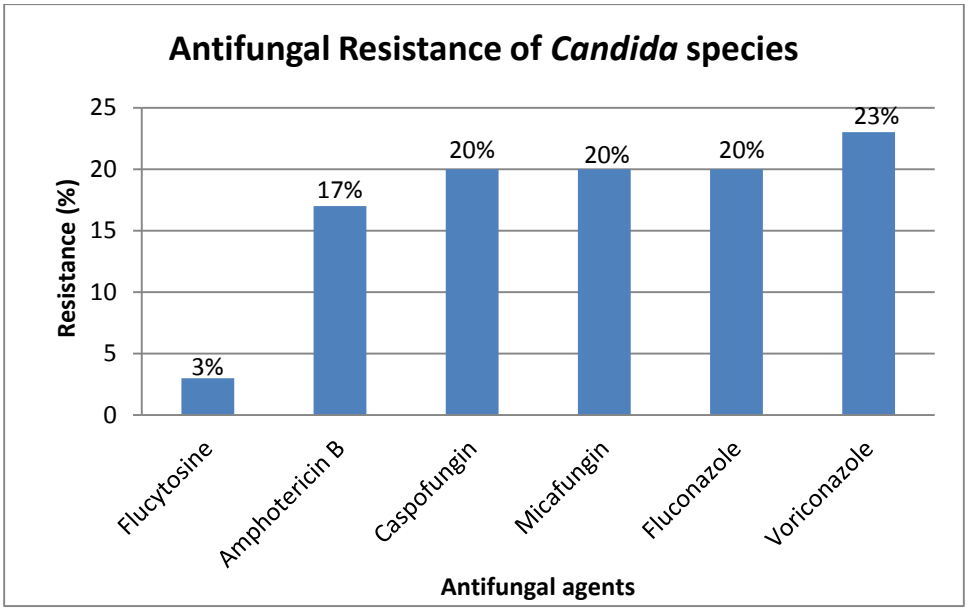

Figure 4. Antifungal susceptibility profile of isolated *Candida* species

Candida albicans (n=27) had high susceptibility to flucytosine (96%), amphotericin B (81%), echinocandins (74%) and triazoles (74%). *Candida parapsilosis* (n=1), *C. tropicalis* (n=1) and *C. glabrata* (n=1) showed 100% susceptibility to amphotericin B, echinocandins, triazoles and flucytosine.

Candida lusitaniae (n=3) showed resistance to amphotericin B (33%) and complete susceptibility to echinocandins, flucytosine and triazoles.

Candida dubliniensis were resistant to voriconazole (50%) and 100% susceptible to caspofungin, amphotericin B, flucytosine, fluconazole and micafungin as indicated in Table 5. Sixteen (46%) *Candida* species isolated mostly *C. albicans* were drug resistant, 4 (11%) multidrug-resistant (MDR), 1 (3%) pandrug-resistant (PDR) and 14 (40%) of the isolates susceptible to all the antifungal agents tested.

Table 5: Antifungal susceptibility profile of different *Candida* species isolated

Candida spp	Antifungal agents (n, % Susceptible)					
	AB	**CAS**	**FCT**	**FLU**	**MCF**	**VRC**
C. albicans	27 (81)	27(74)	27 (96)	27 (74)	27 (74)	27 (74)
C. lusitaniae	3 (67)	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)
C. dubliniensis	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (50)
C. parapsilosis	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
C. glabrata	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
C. tropicalis	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
C. famata	-	-	-	-	-	-

Abbreviations: AB, Amphotericin B; CAS, Caspofungin; FCT, Flucytosine; MCF, Micafungin; VRC, Voriconazole.
(-) drug not indicated.

CHAPTER FIVE

5.0 DISCUSSION

This study presents a mycological survey of diabetic foot ulcer patients treated and managed at Kenyatta National Hospital. The aim of the study was to determine the prevalence, species and antifungal susceptibility of *Candida* species isolated from diabetic foot ulcer.

In this study, we isolated 39 fungal species from diabetic foot ulcers and the most occurring species was *Candida albicans* (75%). Other non-albicans *Candida* (NAC) species were identified in low numbers and included *Candida lusitaniae* (8%), *C. dubliniensis* (5%), *C. glabrata* (3%), *C. tropicalis* (3%), *C. famata* (3%) and *C. parapsilosis* (3%). The prevalence of *Candida* species in this study is 20% comparable to prevalence reported in Turkey, India and Iran ranging from 16-30% (Fata *et al.*, 2011; Abilah *et al.*, 2015; Raiesi *et al.*, 2018; Öztürk *et al.*, 2019; Kareliya *et al.*, 2019). However, isolation rates as high as 44% and as low as 2% of *Candida albicans* have also been documented in diabetes studies in Saudi Arabia and Kuwait respectively (Johargy, 2016; Khalifa, Ahmed and Rotimi, 2012). Our results on species predominance are inconsistent with previous similar studies done in Kenya and India which reported *Candida parapsilosis* as the most common yeast isolated (Gitau *et al.*, 2011; Chellan *et al.*, 2010). Increased glucose concentration in tissues and body fluids, neuropathy, immunological imbalances and vasculopathy are among the factors that predispose to fungal infections, particularly those by *Candida* species in diabetic patients (Mehra *et al.*, 2017). The high frequency of *Candida* isolation from DFU patients may be attributed to the covering of the skin ulcer with dressing material that increases the local temperature and stimulate sweating which favors the growth of *Candida*. In addition, selective administration of antibacterial agents and immunomodulating action of antibiotics supports yeast survival and replication upon interference with skin microbiome including myobiome (Ali, 2013; Mlinaric-Missoni *et al.*, 2005; Malone, 2018).

Other than *Candida* species we also isolated other fungal organisms including *Trichosporon asahii, Trichophyton mentagrophytes, Microsporum, Penicillium* and *Aspergillus species*. This findings concur with results from several previous studies in Africa and other parts of the world. Two independent studies done in India reported *Trichosporon asahii* and *Aspergillus* species among the most common yeast and moulds isolated from diabetic patients presenting with active diabetic foot ulcers (Abilah *et al.*, 2015; Chellan *et al.*, 2010); Punia *et al.*, 2019). In Turkey, Öztürk et al noted

the presence of *Trichosporon asahii* in deep tissues from admitted DFU patients (Öztürk *et al.*, 2019). In Iran, *Trichophyton mentagrophytes* and *Aspergillus* species were the most common moulds isolated during a DFU mycotic study (Fata *et al.*, 2011; Raiesi *et al.*, 2018). A previous similar study in Kenya targeting outpatients attending diabetes clinic reported *Trichosporon asahii, Microsporum, Penicillium, Trichophyton mentagrophytes* and *Aspergillus* species in almost all categories of samples analysed (Gitau *et al.*, 2011). Although moulds are rare in diabetic foot ulcers, there are more progressive than yeast. Incidence of moulds may be associated with poor foot care, recurrent infections and underlying medical conditions.

Diabetic foot ulcer infections are usually of polymicrobial nature, constituting both bacterial and fungal organism. Gram negative rods (51%) and mixed infection of gram positive cocci in clusters and gram negative rods (34%) were the predominant bacterial isolates in this study. Mixed infection of at least two *Candida* species, gram positive and (or) gram negative bacteria was reported in 80% of samples positive for *Candida* species. Polymicrobial nature of DFU infection and isolation predominance of gram negative bacteria has been documented in studies carried out in China, India, Middle East, North Africa, Tanzania and Kenya. All these studies demonstrated that in nearly all cases of DFU the infections were polymicrobial and gram negative bacteria particularly *Escherichia coli, Pseudomonas aeruginosa, Proteus* and *Klebsiella* species were the most frequently isolated organisms (Wu *et al.*, 2018a; Kareliya *et al.*, 2019; Jouhar *et al.*, 2019; Kassam *et al.*, 2017; Gitau *et al.*, 2011; Mutonga *et al.*, 2019). In contrast, similar studies in the United States of America and England reported gram positive bacteria as the most isolated microorganism (Kwon and Armstrong, 2018; Nelson *et al.*, 2018). Polymicrobial DFU infection and predominance of gram negative microorganism is not clear but this may be related to impaired immune system, the wound environment that favors growth of most microorganisms for long period, antimicrobial pre-treatment, ability to respond to selective environmental pressure and the non fastidious nature of the organisms.

Development of antifungal agents is limited and this may be due to several factors which include selective toxicity associated with most antifungal agents. Antifungal susceptibility testing is therefore key for efficient patient management. *Candida* species isolated in this study showed high susceptibility to the antifungal agents tested including flucytosine (97%), amphotericin B (83%), echinocandins (80%) and fluconazole (80%). Some countries have reported susceptibility of upto

100% to amphotericin B, triazoles and echinocandins in majority of the *Candida* species isolates (Khadka *et al.*, 2017; Johargy, 2016). Based on the SENTRY international fungal surveillance program carried out by Messer and colleagues in 2009, 93-99% of the *Candida* isolates were susceptible to echinocandins, amphotericin B and triazoles (Messer *et al.*, 2009).

As much as diverse groups of *Candida* species continue to show high susceptibility to antifungal agents used in treatment, resistance to most of the antifungal agents especially the clinically used azole agent is slowly developing. In this study, we reported resistance rate of 19-33% to most of the antifungal agents tested. Four (11%) *Candida* species isolates were multidrug-resistant while 1 (3%) was pandrug resistant. Multidrug-resistant (MDR) *Candida* was defined as an isolate not susceptible to at least one agent in two or more antifungal classes while PDR was defined as an isolate non-susceptible to all agents in all antifungal classes (Arendrup and Patterson, 2017).

Candida albicans and *C. lusitaniae* isolated had a resistance rate of 19% and 33% respectively to amphotericin B. Our findings on amphotericin B resistance is similar to findings of studies done in India and South Africa which reported resistance rate of 4-10% to amphotericin B (Chellan *et al.*, 2010; Mnge *et al.*, 2017). Higher rates of resistance to amphotericin B (71%) in *C. albicans* have been reported in India (Sugandhi and Prasanth, 2016). This observation contrasts with findings from other studies done in different parts of the world that have reported upto 100% susceptibility rate of amphotericin B to *Candida* species (Munguia-perez *et al.*, 2017; Tasneem *et al.*, 2017; Zaidi *et al.*, 2018; Marak and Dhanashree, 2018). The low incidence of resistance to amphotericin B in *Candida* species is most likely due low usage of the agent among the diabetic patients. Resistance to polyenes in *Candida* species and especially *C. albicans* may also be associated with mutation of the target genes (*ERG 2, 3, 5, 6 and 11*) involved in ergosterol cell membrane synthesis. In addition, genetic strains of *C. albicans* that have defective enzymatic functionality (C5, 6-desaturase) consequently lead to production of less ergosterol reducing the drug binding sites. Different studies have shown *C. lusitaniae* to be intrinsically resistant to amphotericin B which may also be attributed to the resistance noted in our study (Arendrup and Patterson, 2017; Taff *et al.*, 2013).

Additionally, we also observed 26% resistance rate of *C. albicans* to echinocandins, triazoles and voriconazole (50%). *Candida dubliniensis* recorded 100% susceptibility to fluconazole. Our findings on triazole resistance in *C. albicans* are similar to findings of studies done in Europe, India and Kenya that reported resistance rate of 20-48% to triazoles (Minea *et al.*, 2014; Khadka *et al.*, 2017;

Ooga, Bii and Gikunju, 2015). Similarly, Sugandhi and Prasanth in India observed high resistance to triazoles particularly fluconazole (86-100%) in *C. dubliniensis* and *C. albicans* (Sugandhi and Prasanth, 2016) contrary to 100% susceptibility reported in Saudi Arabia and Tunisia (Johargy, 2016; Eddouzi *et al.*, 2013). In concurrence, low rates of resistance to echinocandins (2%) in *C. albicans* were reported in Iran (Badiee *et al.*, 2016). Our results on echinocandins are in contrast with findings from studies done in India and South Africa that reported high susceptibility (96-100%) to echinocandins in *C. albicans* and *C. dubliniensis* (Katsuragi *et al.*, 2014; Mnge *et al.*, 2017). The *C. albicans* and *C. dubliniensis* resistance to triazole and echinocandins could be attributed to the high clinical usage especially of azole derivatives by patients as prophylaxis. This could also be due to activation of the efflux pump encoded by *CDR* and *MDR* genes decreasing drug concentration to the enzyme target site, mutation of *ERG11* gene altering the binding of the azoles to the enzymatic site and finally the mutation of *ERG3* gene preventing the accumulation of the toxic sterol 14–a-methyl-3, 6 diol (Wiederhold, 2017). In echinocandins, resistance maybe attributed to mutations within the conserved regions of *FK1* & *FK2* genes encoding for the enzyme glucan synthetase (Sanguinetti, Posteraro and LassFlorl, 2015).

Flucytosine (5-fluorocytosine), commonly used in combination with other antifungal agents act by inhibiting metabolism of pyrimidine and synthesis of DNA nucleic acid in fungal cells. Our study observed low rates of resistance to flucytosine (4%) in *C. albicans* and high susceptibility (100%) in non-albicans *Candida* species comparable to what has been documented in different tertiary hospitals in Europe (4%), India (4%), Iran (10%) and South Africa (5%) (Schmalreck *et al.*, 2012; Chellan *et al.*, 2010; Sadeghi *et al.*, 2014; Mnge *et al.*, 2017). This observation is contrary to what was observed in a tertiary hospital in Mexico and Middle East where they reported moderate susceptibility rates to flucytosine (50%) in *C. albicans* (Munguia-perez *et al.*, 2017; Johargy, 2016). The low resistance to flucytosine may be attributed to combination of the drug with other antifungal agents for clinical use. Resistance has been noted in monotherapy as shown in our study and this is most likely due to mutations in the *FCY1, 2* and *FUR1* genes associated with actively transportation of the drug into the fungal cell and enzymatic conversion of the drug into 5-fluorouracil or 5-fluorouridine monophosphate (Arendrup and Patterson, 2017). In addition, resistance to antifungal agents in *Candida* species may be due to biofilms formed by the organisms present in chronic wounds (Silva *et al.*, 2017; Bruder-nascimento *et al.*, 2014).

The main limitation in our study was the panel of antifungal agents tested which were pre-determined by the use of VITEK 2 AST cards, therefore excluding other agents (griseofulvin, abafungin and miconazole). Identification of bacteria to genus and species level would have further supported our findings on polymicrobial infections, however, the scope of this study was to highlight fungal infecting agents. Another limitation was lack of clinical information, particularly HBA_1C for correlation with *Candida* infection. We would also have wished to detect the genes coding for resistance to support the resistance pattern observed.

6.0 CONCLUSION

Our study highlights the polymicrobial nature of diabetic foot ulcer infection and the gap in isolation, speciation and antifungal susceptibility of *Candida* isolates from diabetic foot ulcer patients. *Candida albicans* was the predominant species isolated and demonstrated low incidence of resistance to antifungal agents including echinocandins and triazoles, but showed high susceptibility to flucytosine and amphotericin B. We also noted that species identification is vital in determining the appropriate therapeutic agent for treatment of infected wounds.

7.0 RECOMMENDATION

Based on the findings of this study we propose the addition of fungal diagnosis to the routine bacteriological assays of specimens from DFUs patients. Future research should also look into using advanced molecular assays in detecting diverse groups of pathogens in these wounds including the microbiome and further assess the role of biofilms in the disease progressions of the ulcer. Considering the low incidence of resistance reported in this study, hospitals need to strengthen antifungal susceptibility surveillance of clinical isolates and available antimicrobial stewardship programmes to also include antifungal agents.

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APPENDICES

1a. Information and Consent Form – English version

b. Information and Consent Form – Swahili version

2. Questionnaire

3. Diabetic Foot Ulcers

4. Laboratory identification of fungi

5. Ethics clearance

APPENDIX 1a: Information and Consent Form – ENGLISH Version
INFORMATION AND CONSENT FORM

STUDY TITLE: Speciation and Antifungal Susceptibility of *Candida* isolated from Diabetic Foot Ulcer Patients in Kenyatta National Hospital, Nairobi, Kenya

Principal Investigator: Mr. Moses Musyoki (MSc student, University of Nairobi)

Co-Investigators: Prof. Fredrick Otieno (University of Nairobi), Dr. Moses Masika (University of Nairobi), Miss Winnie Mutai (University of Nairobi), Dr. Nancy Ngugi (Kenyatta National Hospital)

Introduction:

I would like to tell you about a study being conducted by the above-listed researchers. The purpose of this consent form is to give you the information you will need to help you decide whether or not to be a participant in the study. Feel free to ask any questions about the purpose of the research, what happens if you participate in the study, the possible risks and benefits, your rights as a volunteer, and anything else about the research or this form that is not clear. When we have answered all your questions to your satisfaction, you may decide to be in the study or not. This process is called 'informed consent.' Once you understand and agree to be in the study, I will request you to sign your name on this form. You should understand the general principles which apply to all participants in a medical research: i) Your decision to participate is entirely voluntary ii) You may withdraw from the study at any time without necessarily giving a reason for your withdrawal iii) Refusal to participate in the research will not affect the services you are entitled to in this health facility or other facilities. We will give you a copy of this form for your records.
May I continue? YES / NO

WHAT IS THIS STUDY ABOUT?

The researchers listed above are interviewing individuals who are diabetic and presenting with foot ulcers. The aim of the research is to identify *Candida* species in diabetic foot ulcer and assess the antifungal agent used for treatment and their susceptibility pattern among diabetic patients in Kenyatta National Hospital. Approximately 262 diabetic foot ulcer patients chosen randomly will participate in this study. We are asking for your consent to consider participating in this study.

WHAT WILL HAPPEN IF YOU DECIDE TO BE IN THIS RESEARCH STUDY?

If you agree to participate in this study, the following things will happen:
You will be interviewed by a trained interviewer in a private area where you feel comfortable answering questions. The interview will last approximately five minutes. The interview will cover topics such as the type of diabetes, age, any other preexisting condition,
After the interview we will get a deep tissue swab once, the swab will be taken to the laboratory to test for candida and antifungal susceptibility. The samples will be stored for five years.

ARE THERE ANY RISKS, HARMS DISCOMFORTS ASSOCIATED WITH THIS STUDY?

Medical research has the potential to introduce psychological, social, emotional and physical risks. Effort should always be put in place to minimize the risks. One potential risk of being in the study is the loss of privacy. We will keep everything you tell us as confidential as possible. We will use a code number to identify you in a password-protected computer database and will keep all of our paper records in a locked file cabinet. However, no system of protecting your confidentiality can be absolutely secure, so it is still possible that someone could find out you were in this study and could find out information about you.

Also, answering questions in the interview may be uncomfortable for you. If there are any questions you do not want to answer, you can skip them. You have the right to refuse the interview or any questions asked during the interview.

It may be embarrassing for you to give some private information. We will do everything we can to ensure that this is done in private. Furthermore, all study staff and interviewers are professionals with special training in these examinations/interviews.

You may feel some discomfort when collecting the deep tissue swab and you may have a small bruise or swelling in your lower limb. In case of an injury, illness or complications related to this study, contact the study staff right away at the number provided at the end of this document. The study staff will treat you for minor conditions or refer you when necessary.

ARE THERE ANY BENEFITS BEING IN THIS STUDY?

You may not benefit directly as an individual, but the study will aid in the selection of appropriate antifungal drugs for the treatment of infected ulcer. We will refer you to a hospital for care and support where necessary. Also, the information you provide will help us better understand the antifungal susceptibility profile of *Candida* isolated from diabetic foot ulcers patients in Kenyatta National Hospital. This information is a contribution to science and aid in curbing the burden of antimicrobial resistance. There will be no direct compensation for participating in this study.

WILL BEING IN THIS STUDY COST YOU ANYTHING?

Participation is free and voluntary.

WILL YOU GET REFUND FOR ANY MONEY SPENT AS PART OF THIS S

There is no expense involved in participating in this study. You will not be compens

CONTACTS: WHAT IF YOU HAVE QUESTIONS IN FUTURE?

If you have further questions or concerns about participating in this study, please call or send a text message to the Principal Investigator, Mr. Moses Musyoki +254 722 488729.

For more information about your rights as a research participant, you may contact the Secretary/Chairperson, Kenyatta National Hospital-University of Nairobi Ethics and Research Committee Telephone No. 2726300 Ext. 44102 email uonknh_erc@uonbi.ac.ke.

The study staff will pay you back for your charges to these numbers if the call is for study-related communication.

WHAT ARE YOUR OTHER CHOICES?

Your decision to participate in research is voluntary. You are free to decline participation in the study, and you can withdraw from the study at any time without suffering any negative consequences. You will continue to receive the care and treatment needed even if you do not wish to participate in this study.

CONSENT FORM (STATEMENT OF CONSENT)

Participant's statement

I have read this consent form or had the information read to me. I have had the chance to discuss this research study with a study counselor. I have had my questions answered in a language that I understand. The risks and benefits have been explained to me. I understand that my participation in this study is voluntary and that I may choose to withdraw at any time. I freely agree to participate in this research study.
I understand that all efforts will be made to keep information regarding my identity confidential.

By signing this consent form, I have not given up any of the legal rights that I have as a participant in a research study.

I agree to participate in this research study:	Yes	No
I agree to have any isolates from my swab preserved for up to 20 years:	Yes	No
I agree that the *Candida* isolates from the swabs be stored (-80ºC) and used for teaching and any other research in future	Yes	No

Participant printed name: _____

Participant signature / Thumb stamp _____ Date _____

Researcher's statement

I, the undersigned, have fully explained the relevant details of this research study to the participant named above and believe that the participant has understood and has willingly and freely given his/her consent.

Researcher's Name: **_____ Date**: **_____**

Signature _____

Role in the study: **_____**

Witness (*If witness is necessary, A witness is a person mutually acceptable to both the researcher and participant*)

Name _____ Contact information _____

Signature /Thumb stamp: _____ Date: **_____**

APPENDIX 1b: Information and Consent Form – SWAHILI Version

MAELEZO KUHUSU UTAFITI/WARAKA WA IDHINI

Aina na Antifungal Kutokubalika kwa *Candida* pekee kutoka kwa Wagonjwa wa Miguu ya Ulinzi wa Diabetic katika Hospitali ya Taifa ya Kenyatta, Nairobi, Kenya

Mtafiti mkuu: Mr Moses Musyoki (Chuo Kikuu cha Nairobi)

Watafiti weza: Prof. Fredrick Otieno (Chuo Kikuu cha Nairobi), Dr. Moses Masika (Chuo Kikuu cha Nairobi), Miss Winnie Mutai (Chuo Kikuu cha Nairobi), Dr. Nancy Ngugi (Hospital Kuu ya Kenyatta)

UTANGULIZI

Ningependa kukueleza juu ya utafiti unaofanywa na watafiti waliotajwa hapo juu. Madhumuni ya fomu hii ya idhini ni kukupa maelezo unayohitaji ili kukusaidia uamuzi ikiwa Utahusishwa kwa utafiti huu au la. Jisikie huru kuuliza maswali yoyote kuhusu madhumuni ya utafiti, kinachotokea ikiwa unashiriki katika utafiti, hatari na faida iwezekanavyo, haki zako kama kujitolea, na kitu kingine chochote kuhusu utafiti au fomu hii ambayo haijulikani. Tunapojibu maswali yako yote kwa kuridhika kwako, unaweza kuamua kuwa katika utafiti au la. Utaratibu huu unaitwa 'kibali cha habari'. Mara unapoelewa na kukubali kuwa katika utafiti, nitakuomba kusaini jina lako kwenye fomu hii. Unapaswa kuelewa kanuni za jumla ambazo zinatumika kwa washiriki wote katika utafiti wa matibabu: i) Uamuzi wako wa kushiriki ni kikamilifu kwa hiari ii) Unaweza kujiondoa kwenye utafiti wakati wowote bila ya kutoa sababu ya uondoaji wako iii) Kukataa kushiriki katika utafiti hauathiri huduma unazostahili kwenye kituo hiki cha afya au vifaa vingine. Tutakupa nakala ya fomu hii kwa rekodi zako.

Naweza kuendelea? NDIO/LA

UTAFITI HUU UNAHUSU NINI?

Mtafiti aliotajwa hapo juu atawaoji watu wenye ugonjwa wa kisukari na wana vidonda vya miguu. Lengo la utafiti ni kutambua aina za *Candida* katika jicho la mguu wa kisukari na kutathmini wakala wa antifungal kutumika kwa matibabu na muundo wao wa kukubalika kati ya wagonjwa wa kisukari katika Hospitali ya Taifa ya Kenyatta. Karibu wagonjwa 100 wa ugonjwa wa mguu wa kisukari waliochaguliwa kwa nasibu watashiriki katika utafiti huu. Tunaomba ridhaa yako kufikiria kushiriki katika utafiti huu.

NI NINI KITAKACHO FANYIKA UKIAMUA KUHUSIKA KWA UTAFITI HUU?

Ikiwa unakubali kushiriki katika utafiti huu, mambo yafuatayo yatatokea:
Utashughulikiwa na mhojiwaji mwenye mafunzo katika eneo la kibinafsi ambako unajisikia kujibu maswali. Mahojiano itaendelea dakika takriban tano. Mahojiano itafikia mada kama vile aina ya ugonjwa wa kisukari, umri, hali nyingine yoyote ile,
Baada ya mahojiano, atashika na swabu uvungu wa tishu mara moja, swabu itachukuliwa kwa mahabara ya kutahini *Candida* na uwezekano wa antifungal. Sampuli zitahifadhiwa kwa miaka mitano.

KUNA MADHARA YOYOTE YANAYOTOKANA NA UTAFITI HUU?

Utafiti wa matibabu una uwezo wa kuanzisha hatari za kisaikolojia, kijamii, kihisia na kimwili. Jitihada zinapaswa kuwekwa daima ili kupunguza hatari. Hatari moja ya kuwa katika utafiti ni kupoteza faragha. Tutaweka kila kitu unachotuambia kama siri iwezekanavyo. Tutatumia namba ya nambari ili kukutambua kwenye darasani ya kompyuta iliyohifadhiwa na nenosiri na tutahifadhi rekodi zote za karatasi kwenye baraza la mawaziri lililofungwa. Hata hivyo, hakuna mfumo wa kulinda siri yako inaweza kuwa salama kabisa, kwa hiyo bado inawezekana kwamba mtu anaweza kujua wewe ulikuwa katika utafiti huu na anaweza kupata habari kukuhusu.
Pia, kujibu maswali katika mahojiano inaweza kuwa na wasiwasi kwako. Ikiwa kuna maswali yoyote utaki kujibu, unaweza kuruka. Una haki ya kukataa mahojiano au maswali yoyote yaliyoulizwa wakati wa mahojiano.
Inaweza kuwa aibu kwa wewe kutoa maelezo ya kibinafsi. Tutafanya kila kitu tunaweza kuhakikisha kuwa hii imefanywa kwa faragha. Zaidi ya hayo, wafanyakazi wote wa utafiti ni wataalamu wenye mafunzo maalum katika mitihani/mahojiano haya.
Unaweza kujisikia wasiwasi wakati wa kukusanya tamba la kina la tishu na huenda ukawa na kuvuta au kuvimba kwenye sehemu yako ya chini. Ikiwa kuna jeraha, ugonjwa au matatizo yanayohusiana na utafiti huu, wasiliana na wafanyakazi wa kujifunza mara moja kwa namba iliyotolewa mwishoni mwa hati hii. Wafanyakazi wa utafiti watawafanyia kwa hali ndogo au kukutaja wakati unahitajika

KUNA MANUFAA YOYOTE KWA KUHUSIKA KWA UTAFITI HUU?

Huwezi kufaidika moja kwa moja kama mtu binafsi, lakini utafiti huu utasaidia katika uteuzi wa madawa sahihi ya kuzuia ugonjwa wa kidonda cha kuambukizwa. Tutakupeleka kwenye hospitali kwa ajili ya huduma na msaada ikiwa inahitajika. Pia, taarifa unazoyatoa itatusaidia kuelewa vizuri zaidi maelezo ya kuambukizwa ya antifungal ya *Candida* pekee kutoka kwa wagonjwa wa mguu wa kisukari katika Hospitali ya Taifa ya Kenyatta. Taarifa hii ni mchango kwa sayansi na msaada katika kuzuia mzigo wa upinzani wa antimicrobial. Hutakuwa na fidia moja kwa moja ya kushiriki katika utafiti huu.

KUHUSIKA KWA UTAFITI HUU KUTAGHARIMIA CHOCGOTE?

Hakuna malipo ila tutachukua muda wa dakika kumi

UTAPATA MALIPO YOYOTE AU FIDIA

Hakuna malipo au fidia ili kuhusika kwa utafitu huu

UKITAKA KUULIZA SWALI BAADAYE KUHUSU UTAFITI HUU?

Wasiliana na Mtafiti mkuu, bwana Moses Musyoki kwa nambari ya simu: +254 722 488 729. Ama mwenyekiti au katibu msimamizi, utafiti, Hospitali ya Kitaifa ya Kenyatta na Chuo kikuu cha Nairob kupitia nambari 2726300/44102; au kwa anuani uonknh_erc@uonbi.ac.ke. Watafiti watakurejeshea pesa zilizotumika kwa mawasiliano kuhusu utafiti huu

HUNA HIARI GANI?

Uamuzi wako wa kushiriki katika utafiti ni wa hiari. Una uhuru wa kushiriki katika utafiti na unaweza kujiondoa kwenye utafiti wakati wowote bila mateso yoyote mabaya. Utaendelea kupata huduma na matibabu zinahitajika hata kama hutaki kushiriki katika utafiti huu.

IDHINI

Nimesoma au kusomewa waraka huu na nimweulewa kabisa. Nimepata nafasi ya kujadiliana na mtafiti na akajibu maswali yangu kwa lugha ninayoelewa. Niemarifiriwa kuhusu faida na madhara ya utafiti huu na kwamba nitapewa nakala ya waraka huu baada ya kutia sahihi. Pia naelewa kuwa nahusika kwa hiari yangu na ninaweza kujitoa kwa utafiti huu wakati wowote.

Kwa kusaini fomu hii ya kibali, sijaacha haki yoyote ya kisheria niliyoshiriki katika utafiti huu.

Nakubali kushiriki katika utafiti huu:	Ndio	La
Nakubali kuwa swabu yangu ihifadhiwa kwa miaka 20:	Ndio	La
Nakubaliana kwamba *Candida* inatengwa kutoka swabu kutumika kwa mafundisho na utafiti zaidi	Ndio	La

Jina la kuchapishwa la Mshiriki:_____

Sahihi ya Mshiriki: _____ Tarehe:_____

KAULI YA MTAFITI

Nimemueleza mhusika taarifa zinazofaa kuhus utafiti huu na naamini kuwa ameelewa vyema na kukubali kuhusika kwa hiari yake.

JINA:_____ **TAREHE**:_____

SAHIHI:_____

JUKUMU LAKO KWA UTFITI HUU:_____

SHAHIDI (*Ikiwa atahitajika kama vile kutasfiri)*_____

Sahihi:_____ Tarehe:_____

APPENDIX 2: Questionnaire

QUESTIONNAIRE

STUDY TITLE: Speciation and Antifungal Susceptibility of *Candida* isolates from Diabetic Foot Ulcer Patients in Kenyatta National Hospital, Nairobi

Patient study no …….. Date……..

I. DEMOGRAPHICS

1. Age (years) …….

2. Gender:

   Male ☐ Female ☐

3. Marital status:

   Single ☐ Married ☐ Divorced ☐ Separated ☐ Widow(er) ☐

4. Education

   Primary ☐ Secondary ☐ Tertiary ☐ Informal ☐

5. Residence:

   Rural ☐ Urban ☐ County: _____

6. Religion:

   _____

7. Occupation:

   _____

II. CLINICAL INFORMATION

8. Point of care:

   Medical ward ☐

   Surgical outpatient clinic ☐

   Diabetic outpatient clinic ☐

   Orthopedic ward ☐

9. Type of diabetes: Type I ☐ Type II ☐

10. Year diabetes was diagnosed: …….

11. Duration of foot ulcer (months): ……

12. Current Medications:

   i. ……

   ii. ……..

   iii. ……..

13. Today's blood sugar level: …… HbA1c (if available) ……

14. Classification of the ulcer wound (Wagner staging)

   1. ☐ 2. ☐ 3. ☐ 4. ☐ 5. ☐

15. Episode of Diabetic foot ulcer

   1. ☐ 2. ☐ 3. ☐ 4. ☐ >5. ☐

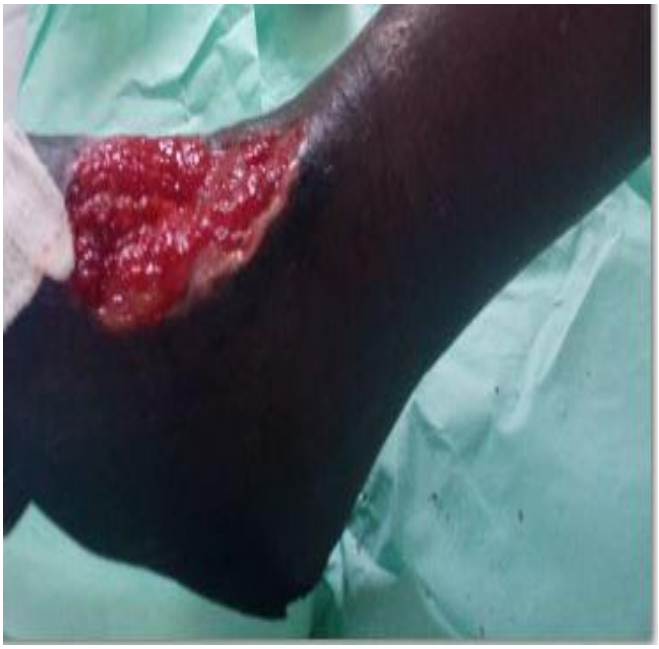
16. Prescribed antifungal

Lab Result Form

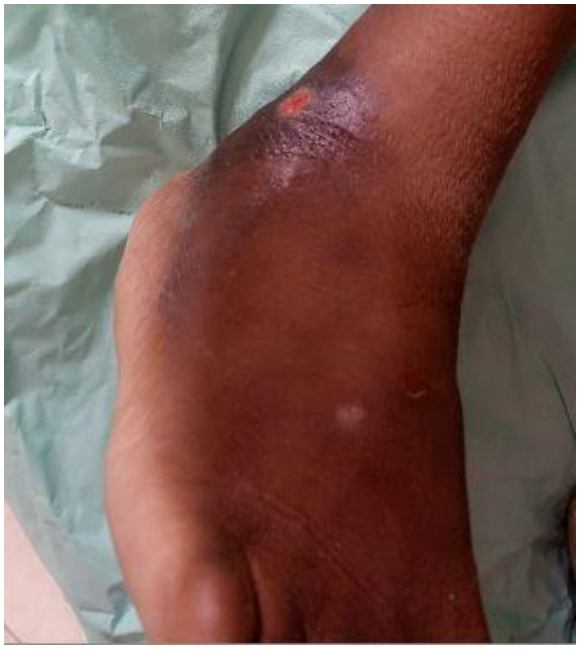
III. SPECIES OF *Candida* ISOLATED

Species	n (%)

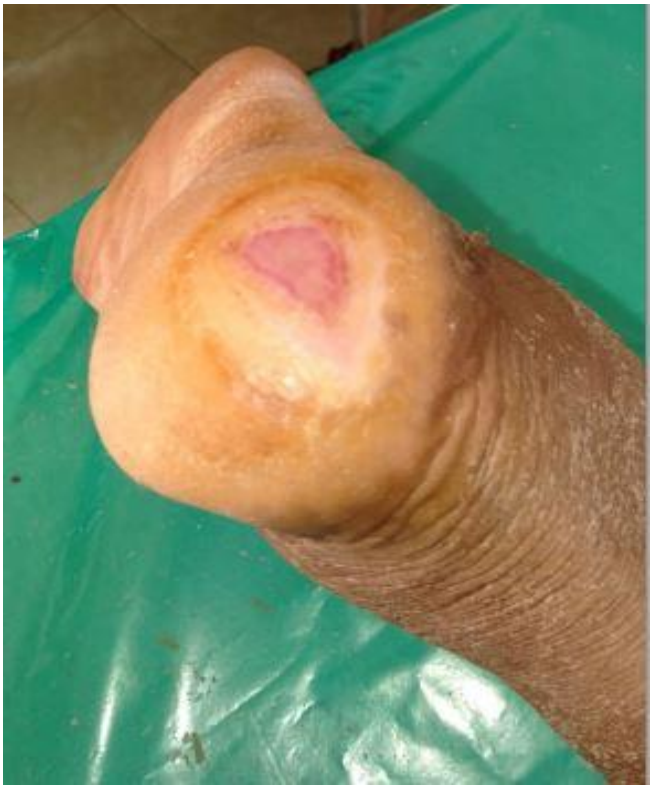
APPENDIX 3: Diabetic Foot Ulcers



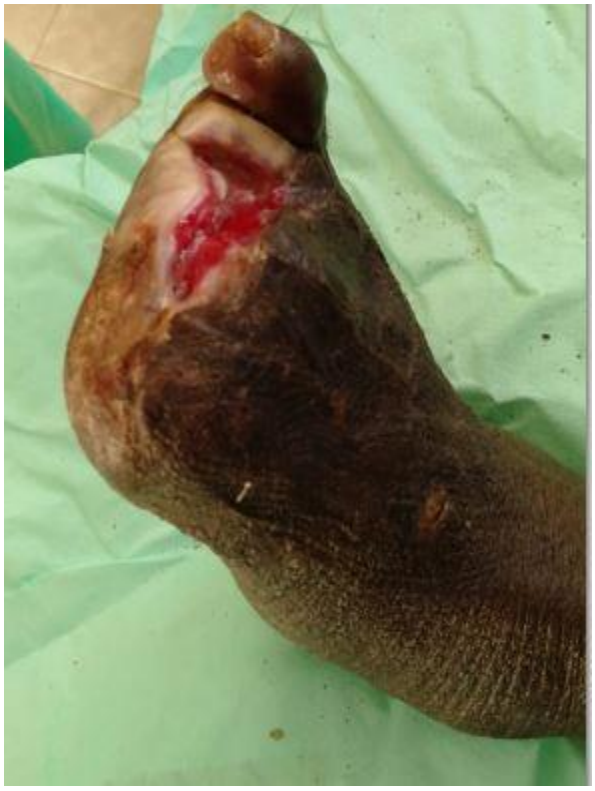
a



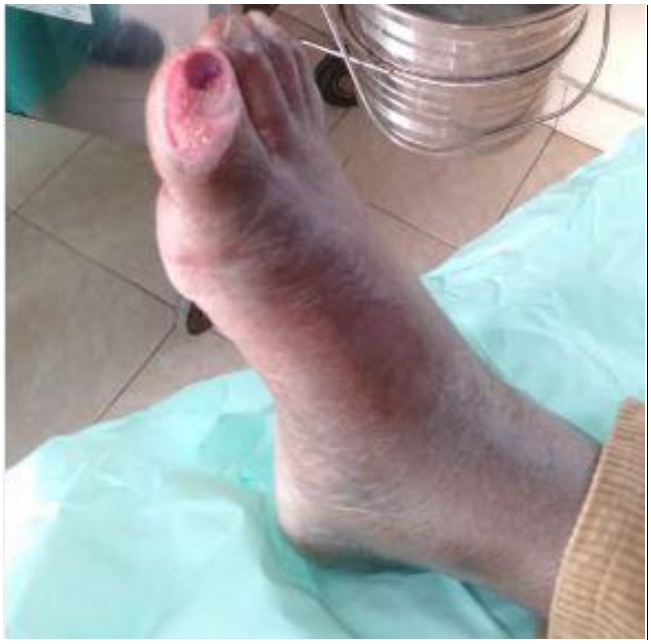
b



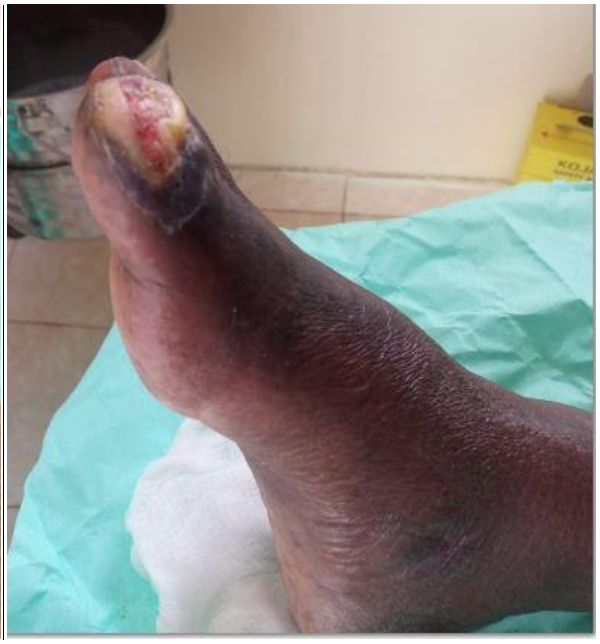
c



d



e



f

g

Figure 5 (a) Deep ulcer without abscess or bone involvement; wagner stage 2. (b,c) Healing superficial ulcer; wagner stage 1. (d,e) Deep ulcer without abscess or bone involvement; wagner stage 2. (f) Healing deep ulcer of the big toe, wagner stage 2. (g) Deep ulcer without abscess or bone involvement, wagner stage 2

APPENDIX 4: Laboratory identification of fungi

1. Gram stain: Gram positive yeasts cells

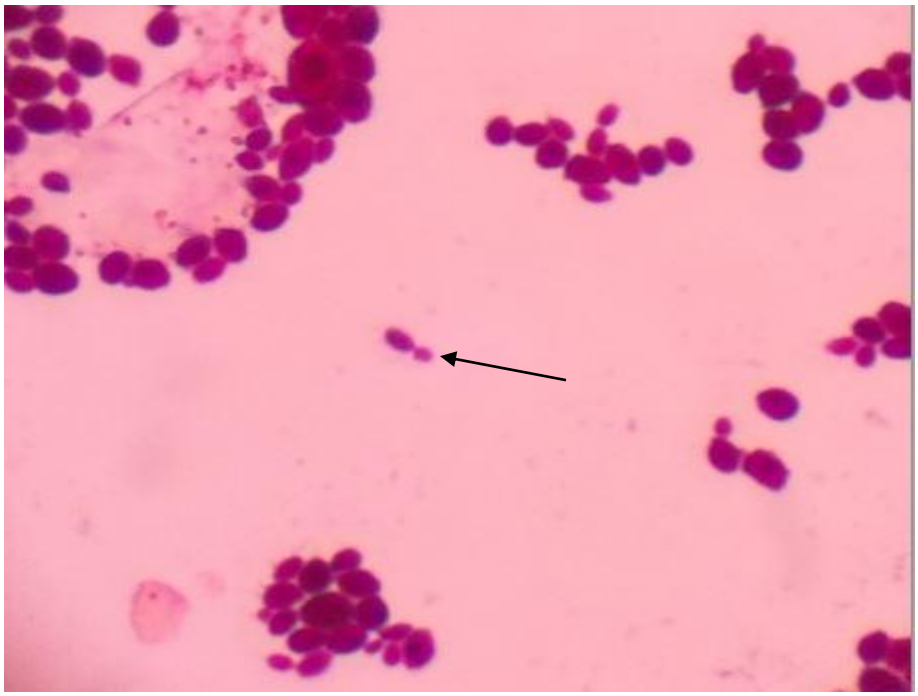
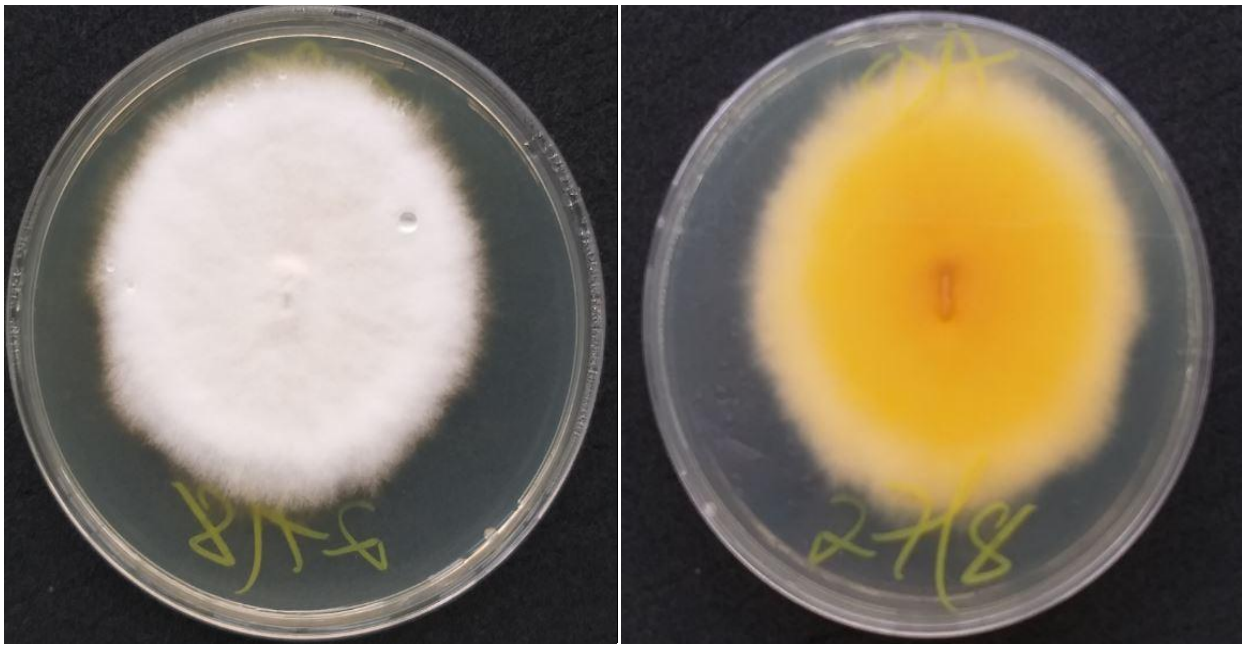
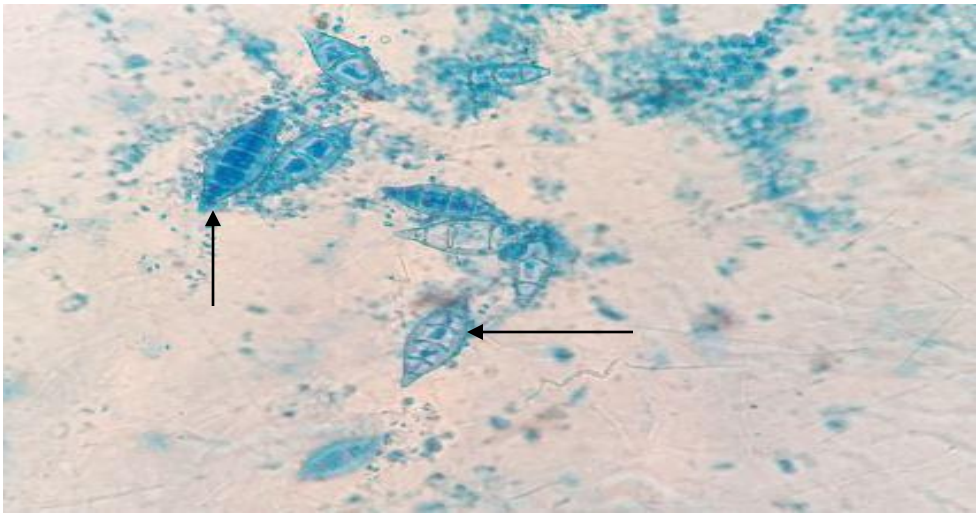


Figure 6.1 Microscopic appearance of budding *Candida* species yeast cells (arrow pointer) in a Gram-stained Diabetic Foot Ulcer smear (Magnification X100)

2. *Microsporum* species on SDA and Lactophenol Cotton Blue stain



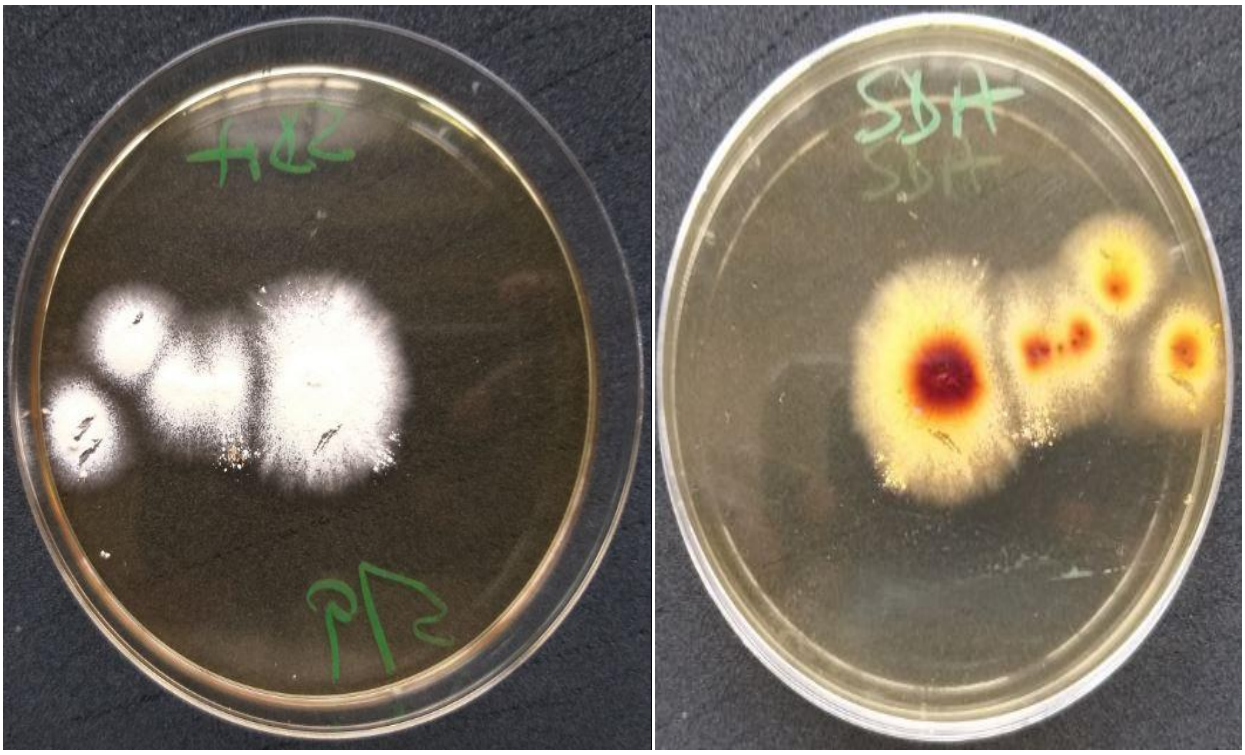
a **b**



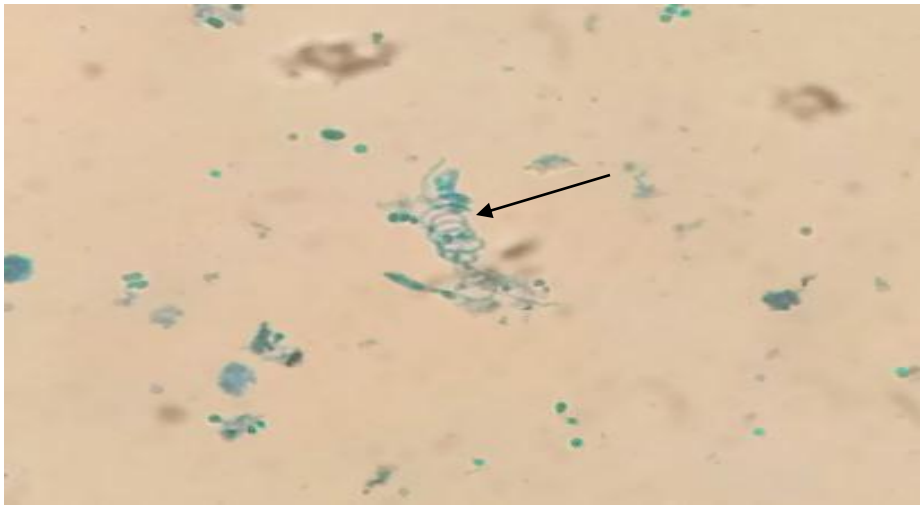
c

Figure 6.2 (a) White flat to sparsely spreading colony of *Microsporum canis* with woolly or cotton feathery texture growing on Sabouraud Dextrose Agar. (b) Lemon-tinged reverse pigmentation. (c) Lactophenol cotton blue (LPCB) staining preparation of the culture showing spindle shaped, thick walled macroconidia (arrow pointer) with 5-6 septa cells (Magnification X40)

3. *Trichophyton mentagrophytes* on SDA and Lactophenol Cotton Blue stain



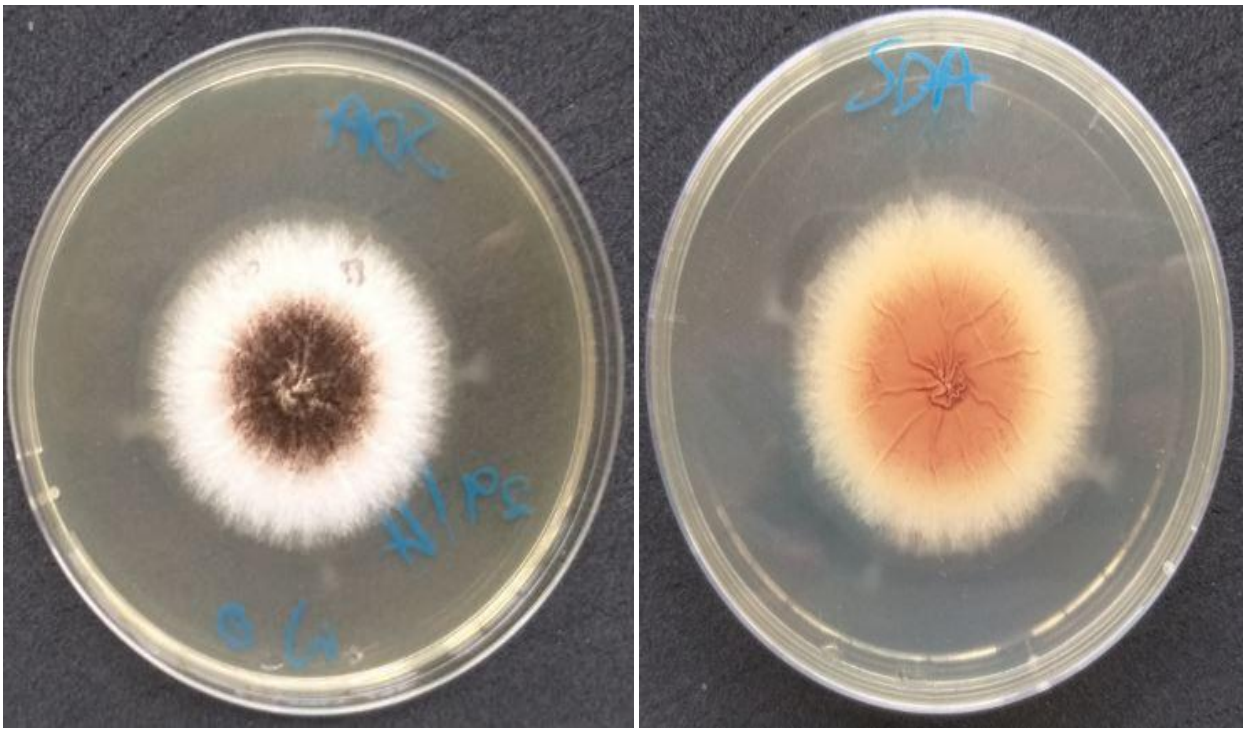
a **b**



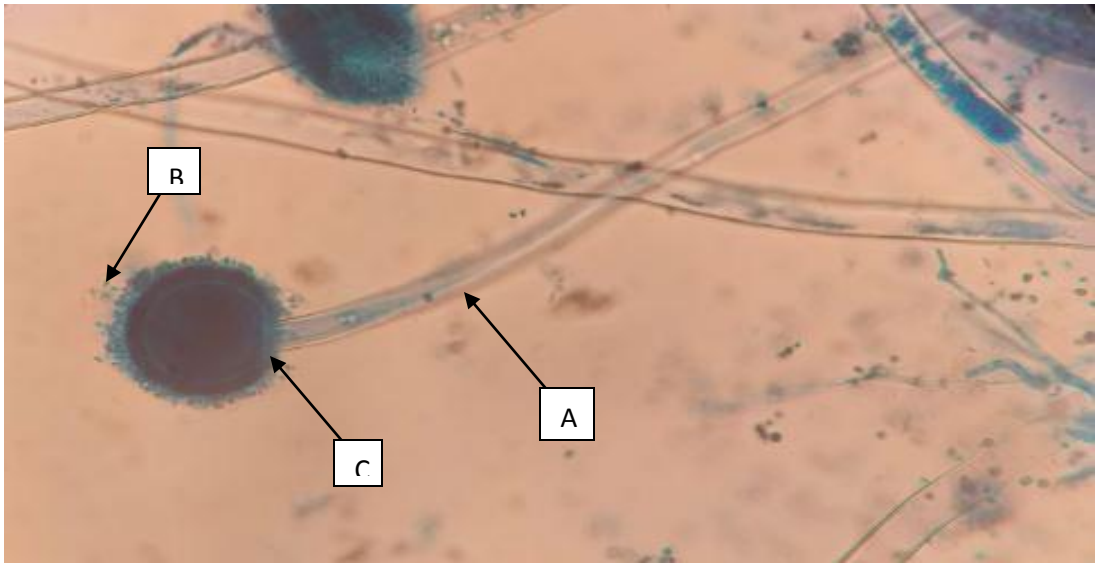
c

Figure 6.3 (a) Flat cream white colonies of *T. mentagrophytes* with powdery to granular surface texture. (b) Reverse pigmentation of the culture showing yellow brown colour. (c) Spiral hyphae on a lactophenol cotton blue staining preparation (arrow pointer) (Magnification X40)

4. *Aspergillus* species on SDA and Lactophenol Cotton Blue stain



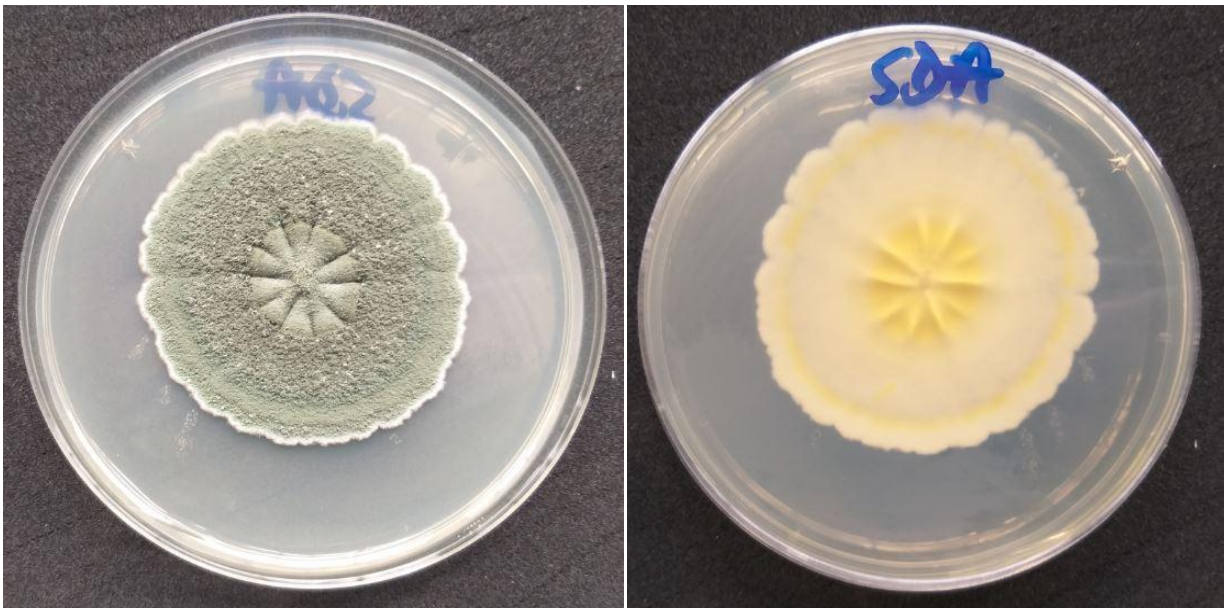
a **b**



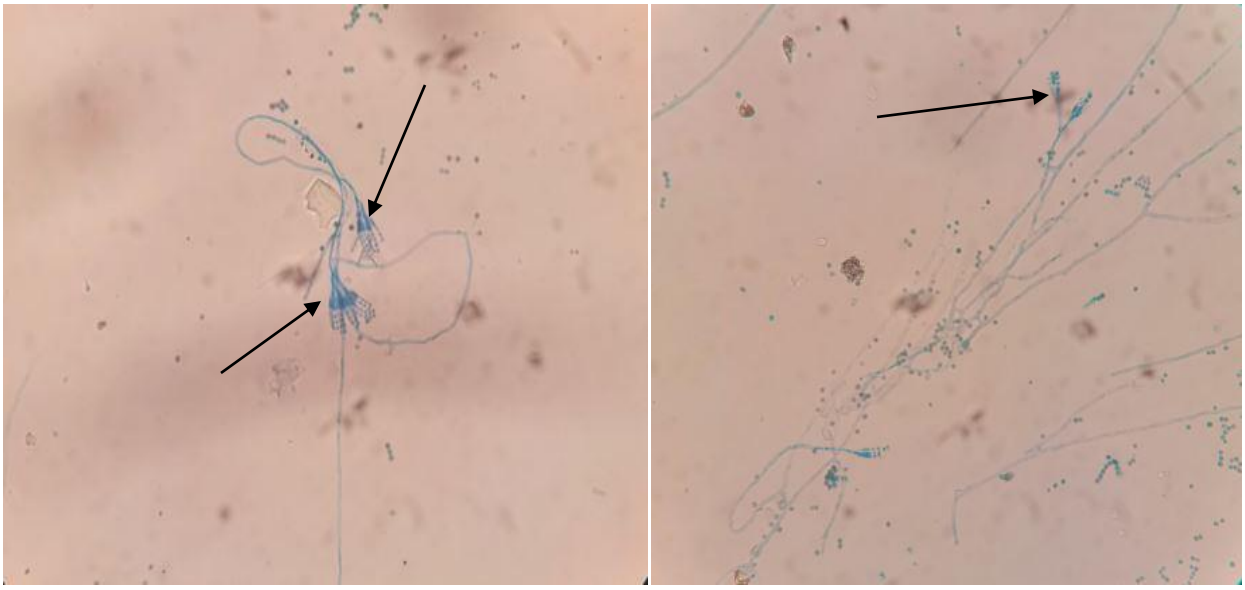
c

Figure 6.4 (a) Colony of *Aspergillus niger* presenting with deep brown, densely stippled surface. (b) The reverse of the fungi showing light gray pigmentation. (c) Conidiophore [A] of *A. niger* with a bulging vesicle [C]. Chains of conidiospores [B] on sterigmata of conidiophore (Magnification X40; LPCB)

5. *Penicillium* species on SDA and Lactophenol Cotton Blue stain



a **b**



c **d**

Figure 6.5 (a) Colonies of *Penicllium* species appearing blue green with velvet-powdery surface. (b) Cream-white reverse pigmentation. (c,d) Microscopic examination of lactophenol cotton blue culture preparation showing brush like arrangement of conidia, sterigmata and conidiophore (fingerlike) (arrow pointer) (Magnification X40)

APPENDIX 5: Ethical clearance

UNIVERSITY OF NAIROBI
COLLEGE OF HEALTH SCIENCES
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Twitter: @UONKNH_ERC https://twitter.com/UONKNH_ERC

KENYATTA NATIONAL HOSPITAL
P O BOX 20723 Code 00202
Tel: 726300-9
Fax: 725272
Telegrams: MEDSUP, Nairobi

Ref: KNH-ERC/A/206

31st May, 2019

Victor Moses Musyoki
Reg. No. H56/9607/2018
Dept. of Medical Microbiology
School of Medicine
College of Health Sciences
University of Nairobi

KENYATTA NATIONAL HOSPITAL APPROVED 31 MAY 2019 KNH/UoN-ERC P.O. BOX 20723 - 00202 NAIROBI

Dear Victor,

RESEARCH PROPOSAL: SPECIATION AND ANTIFUNGAL SUSCEPTIBILITY OF *CANDIDA* ISOLATES FROM DIABETIC FOOT ULCER PATIENTS IN KENYATTA NATIONAL HOSPITAL (P290/04/2019)

This is to inform you that the KNH- UoN Ethics & Research Committee (KNH- UoN ERC) has reviewed and **approved** your above research proposal. The approval period is 31st May 2019 – 30th May 2020.

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. Clearance for export of biological specimens must be obtained from KNH- UoN ERC for each batch of shipment.
f. 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*).
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**

Yours sincerely,

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

c.c. The Principal, College of Health Sciences, UoN
The Director, CS, KNH
The Chairperson, KNH- UoN ERC
The Assistant Director, Health Information, KNH
The Dean, School of Medicine, UoN
The Chair, Dept. of Medical Microbiology, UoN
Supervisors: Prof. Fredrick Otieno (Dept. of Clinical Medicine and Therapeutics, UoN)
Dr. Moses Masika (Dept. of Medical Microbiology, UoN)
Dr. Nancy Ngugi (Consultant Endocrinologist/ Diabetologist, KNH),
Ms. Winnie Mutai (Dept. of Medical Microbiology, UoN)