PREVALENCE, ANTIFUNGAL SUSCEPTIBILITY AND MOLECULAR CHARACTERIZATION OF ISOLATED *CANDIDA* SPECIES FROM BLOODSTREAM OF CRITICAL CARE UNIT PATIENTS IN NAIROBI HOSPITAL, KENYA

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JOMO KENYATTA UNIVERSITY OF

AGRICULTURE AND TECHNOLOGY

2021

Prevalence, Antifungal Susceptibility and Molecular Characterization of Isolated *Candida* Species from Bloodstream of Critical Care Unit Patients in Nairobi Hospital, Kenya

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A Thesis Submitted in Partial Fulfilment of the Requirements for the Degree of Master of Science in Infectious Diseases and Vaccinology of the Jomo Kenyatta University of Agriculture and Technology

2021

DECLARATION

This thesis is my original work and has not been presented for a degree in this or any other University.

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To my beloved husband Pawlos Semere Tsegay and to my parents, Andemichael Solomon, and Mulu Tewelde who sacrificed a lot for me to reach here.

ACKNOWLEDGEMENT

First and foremost, I would like to express my gratitude to the almighty God for the strength and perseverance he gave me to accomplish this research. Secondly, I would like to thank Africa *ai* Japan project for funding this research. I am also deeply grateful to my supervisors Dr. Andrew.K. Nyerere, Dr. Caroline Wangari Ngugi, and Dr. Alice Kanyua for their full guidance and advisory to make the research successful. My extended appreciation goes out to my husband Mr. Pawlos Semere Tsegay and my parents, Andemichael Solomon and Mulu Tewelde as well as all my family for their continuous support and motivation throughout the research and without whom I would not have had the forbearance to accomplish this research. My special thanks goes to Nairobi Hospital, especially Microbiology Department and Ms. Felister Musyoki for their material and physical support in the hospital. I would also like to convey my appreciation to One Health, HORN project for their material support. Finally, I am very thankful to Pawlos Semere Tsegay, Dr. Douksouna Youmma, James Gachugia, and all other people who assisted me in different stages of my research with physical or intellectual support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

LIST OF ACRONYMS

AST	Antifungal Susceptibility Test
BSIs	Bloodstream Infections
CAU	*Candida auris*
CCU	Critical Care Unit
CDR	*Candida* Drug Resistance
CP	*Candida parapsilosis*
CT	*Candida tropicalis*
DNA	Deoxyribonucleic Acid
ERG	Ergosterol
FKS1 &2	Glucosyltransferase
FUR	Uracil phosphoribosyl transferase
GTP	Guanosine Triphosphate
HDU	Highly Dependent Unit
ICU	Intensive Care Unit
JKUAT	Jomo Kenyatta University of Agriculture and Technology
MDR	Multidrug Resistance
MIC	Minimum Inhibitory Concentration
PCR	Polymerase Chain Reaction
RBC	Red Blood Cells
RNA	Ribonucleic Acid
SDA	Saboraud Dextrose Agar

SNP Single Nucleotide Polymorphism

SPSS Statistical Package for the Social Sciences

YBC Yeast Biochemical Card

ABSTRACT

Upsurge of candidemia in the past years has been a burden on public health and the number of deaths caused by candidemia particularly in critical care unit patients has been increased. *Candida* species account for 30-60% mortality rate and compared to healthy individuals or those with less serious illnesses, the incidence is increased to 60 to 80% in those who are chronically ill. Grounded on a recent report from Nairobi Hospital showing the emergence of previously unobserved species; *Candida auris,* this study aimed to determine the prevalence, antifungal susceptibility profile and genotypic characterization of candidemia in critical care unit patients of the hospital. A total of 378 Critical Care Unit patients were enrolled for the study. Positive archived isolates were sub-cultured using Saboraud Dextrose Agar. *Candida* species were identified utilizing API20C AUX and Vitek-2. Antifungal susceptibility testing was conducted using the Liofilchem MIC Test strip. Genomic DNA was extracted using QUICK DNA miniprep extraction kit, amplified using PCR and outsourced to macrogen for purification and sequencing. Out of 378 patients, thirty-one presented a positive culture for *Candida* species. The prevalence of Candidemia was **8.2%** with **9 (29.03%)** *Candida auris,* **8 (25.81%)** *Candida albicans,* **6 (19.35%)** *Candida parapsilosis,* **3 (9.68%)** *Candida famata,* **3 (9.68%)** *Candida tropicalis,* **1 (3.23%)** *Candida duobushaemolumonii,* and **1 (3.23%)** *Candida lusitaniae.* A resistance pattern to Fluconazole was observed among *Candida auris,* and *Candida parapsilosis,* and resistance to Flucytosine was observed in *Candida tropicalis,* whereas susceptible MIC values were obtained for the other drugs. Sequencing of resistant isolates' ERG 11 and FUR1 gene obtained 6 SNPs (Erg11C374T, Erg11C376T, Erg11A395T, Erg11T729C, Erg11T1126A, FUR1C297T) and 4 amino acid substitutions (A125V, L126F, Y132F, L376V). There is an increase in candidemia among critical care unit patients in Nairobi Hospital and onset of new species; *Candida auris*. The uniform resistance of *Candida auris, Candida parapsilosis,* and *Candida tropicalis* towards Fluconazole and Flucytosine and the mutation analysis obtained three previously undocumented amino acid substitutions and one substitution which has been documented in two continents other than Africa of which the latter is elucidated in causing resistance to fluconazole and one of the previously undocumented substitutions might also have a role in the resistance patterns of fluconazole. Based on the results obtained in this study there is a necessity of constant drug monitoring for empirical treatment regime. Therefore, the high potency of Echinocandins demonstrate them as the drug of choice.

CHAPTER ONE

INTRODUCTION

1.1. Background of the Study

Fungal infections, especially those associated with *Candida* species have been the major cause of mortality and morbidity in hospitalized patients and predominantly in the Critical Care Units (Cortegiani *et al.*, 2018; Kullberg & Arendrup, 2015). Immunocompromised patients have several risk factors, mainly attributed to graft transplantation, broad-spectrum antibiotic therapy, catheter implantation, or digestive surgery (Sandt *et al.*, 2003). Hospital fungal infections contribute to the upsurge of critically ill patients in numerous sites of the world, the onset of hospital fungal infections is dramatic (Bonfim-mendonça *et al.*, 2013). Consequently, high-risk patients are those undergoing intensive care in ICU and HDU as they have several of the above risk factors (Sandt *et al.*, 2003). High mortality rate, which surpasses 50% has been attributed to invasive fungal infections, (Brown *et al.*, 2012). In most cases, *Candida* species has been the main cause of invasive fungal infections in accordance with the lengthy stay of patients in hospitals (Bonfim-mendonça *et al.*, 2013).

Candida species contribute 30-60% mortality rate of hospitalized patients (Bonfim-mendonça *et al.*, 2013). According to the National Nosocomial Infections Surveillance System and the European Prevalence of Infection in Intensive Care, nosocomial infections caused by *Candida* species rates third or fourth from all hospital-acquired infections (Sandt *et al.*, 2003). Several risk factors of the origin of the *Candida* infections in hospitalized patients could be endogenously brought by the patients themselves or could be from instruments in the hospital. It might also be contributed to contaminations of hospital surroundings, or cross-infection from health workers which would attribute to the exogenous cause (Al-obaid *et al.*, 2017; Suleyman Alangaden, 2016). According to several studies, 50 to 70% of the infections caused by *Candida* species are by *Candida albicans*, and the implication of its endogenous origin is present (Bonfim-mendonça *et al.*, 2013).

Patients in critical care units contribute to an increased rate of candidemia. In comparison to healthy individuals or those with less serious illnesses, this ranges from 60 to 80% of those who are chronically ill patients (Sadrossadati *et al.*, 2018). This, in turn, puts in place the need for proper treatment of the yeast. However, results of anti-fungal resistance have been causing epidemiological unsustainability which brings the issue of identification and attaining anti-fungal susceptibility patterns of the species for further implementation of an accurate drug for the resistant strain. This has contributed to many deaths due to *Candida* infections in the past 20 years (Ghahri, Mirhendi, Zomorodian, & Kondori, 2013; Sadrossadati *et al*., 2018).

Candida species are considered as normal flora of the human urinary tract, the oral cavity as well as the skin and gastrointestinal tract thus, they have a big role in opportunistic infections. They are able to cause invasive infections with a high rate of mortality and morbidity threatening the life of humans by causing bloodstream or urinary tract infections; however, they can also inflict mild superficial infections which are easily managed (Al-obaid *et al.*, 2017). The state of immunocompromised patients brings these commensal microorganisms to pathogenicity (Sardi *et al.*, 2012). Prominently causing nosocomial infections, *Candida* spp. rates the fourth in causing all bloodstream infections and third of bloodstream infections in critically ill patients. Worldwide in tertiary care hospitals, the above results attribute to mortality in 15±35% adults and 10±15% neonates (Al-obaid *et al.*, 2017).

Among the significant fungal infections caused by *Candida* species is Candidemia (Ghahri *et al.*, 2013; Sadrossadati *et al.*, 2018). Based on studies in several hospitalized patients, including intensive care unit, candidemia causes mortality in 47% of the patients. Moreover, as a result of these infections, increased costs in healthcare are incurred, ranging from $35,000 to $68,000 for a single candidemia case in the United States (Suleyman *et al.*, 2016). Therefore, more information is highly required on the prevalence, antifungal susceptibility, and molecular epidemiology of *Candida* infections for adequate prevention, treatment of nosocomial infections, mainly among intensive care unit patients (Bonfim-mendonça *et al.*, 2013).

1.2. Statement of the Problem

A major cause of mortality and morbidity in hospitalized patients and especially CCU patients is observed from the less noticed fungal infections, specifically *Candida* species (Cortegiani *et al.*, 2018; Ruan *et al.*, 2016). A significant rise in clinical manifestations is seen as a result of yeasts of genus *Candida* infections increasing the rate of bloodstream infections (BSIs) (Cortegiani *et al.*, 2018; Cortegiani *et al.*, 2017; Le *et al.*, 2016). The increase in infections caused by *Candida* species have been observed in the last decades and is usually attributed to the number of invasive measures taken in hospitalized patients when handling their medical care.

According to literature from Kenya, the major agent for hospital infections is considered to be *Candida albicans* (Guto *et al.*, 2016; Kangogo *et al.*, 2011). However, the increase in onset of several species of *non-albicans* has brought high treatment failures due to the misleading perception of *Candida albicans* being the primary target. *Candida auris* has been noted frequently in Nairobi Hospital based on several lab reports from ICU and HDU patients. Swift dissemination of this multi-drug resistant species is seen in different parts of the world (Cortegiani *et al.*, 2018). It was first reported in 2009, and since then, it has been observed in five continents of the world, causing serious hospital-acquired infections (Cortegiani *et al.*, 2018).

Critically ill patients face the threat of morbidity and mortality as a result of the wide use of broad-spectrum anti-fungal drugs where there is a development of resistance by *Candida* species (Cortegiani *et al.*, 2018). Patterns of anti-fungal drug resistance by *Candida* species have been causing stern public health challenges and were encompassed in CDC's 2013 Antibiotic Resistance Threat Report (Centers for Disease Control and Prevention, 2013; Toda *et al.*, 2019). Critically ill patients undergo invasive treatments and consume several anti-fungal drugs, however, results of anti-fungal resistance have been causing epidemiological unsustainability (Ghahri *et al.*, 2013; Sadrossadati *et al.*, 2018). Certain *Candida* strains are progressively resistant to commonly used antifungal drugs. Based on recent data from CDC a discernable shift is observed in candidemia occurrences with augmented drug resistance to first-line and second-line antifungal drugs such as Azoles and Echinocandins (Centers for Disease

Control and Prevention, 2013). Inherent causes of resistance are also noted on top of the usual acquired pattern of drug resistance after antifungal drugs are administered to patients. This intrinsic nature of resistance is usually seen in *non-albicans*, which warrants further research (Cortegiani *et al.*, 2018; Mandelblat *et al.*, 2017).

1.3. Justification of the Study

The isolation and identification of *Candida* species will prompt in studying the recent and updated prevalence of *Candida* infections from the bloodstream of patients in critical care units of Nairobi Hospital. The literature present on *Candida* infections' prevalence from clinical sources dates back to 2000-2005 archived isolates of *Candida* species in Nairobi Kenya (Kangogo *et al.*, 2011) as well as in 2015-2016 in Mombasa county of Kenya where in the latter study isolates from the bloodstream were not incorporated (Subira *et al.*, 2018). A solitary study conducted by Adam *et al.* focused on the risk factors of *Candida auris'* Fungemia in a single facility in Kenya (Adam *et al.*, 2019). The former two studies which conducted a thorough prevalence study never encountered the prevalence of *Candida auris*, whereby, the latter study was limited to risk factors of the species without a comprehensive study of the prevalence and species distribution as well as molecular basis of resistance. *Candida auris* has started to be seen globally since 2009 as a virulent species and its occurrence in Kenya is unprecedented. Therefore, an update in the prevalence research is justified, and as the most affected population are critically ill patients, the need to target those patients specifically is very vital.

Furthermore, there is an increase in anti-fungal resistance by *Candida species* highly affecting the treatment strategies routinely used in hospitals. As critically ill patients are at major risk of mortality caused by this fungal infection giving the proper treatment and ensuring the sensitivity of the drug is very crucial in decreasing the rate of morbidity and mortality. Therefore, anti-fungal susceptibility tests should be conducted continuously to prevent unheeded resistance of drugs.

This study is significant as it incorporates the genotypic characterization of the resistant *Candida species* to determine the molecular changes present in the mechanism of resistance. The genotypic study for establishing the molecular basis of resistance has

never been established in Kenya and doing such research will appeal to the scientific community by providing an utmost important knowledge of the pattern of resistance and if variation in mutation patterns is available locally in Kenya. Moreover, having a baseline study of the molecular mechanism of resistance of *Candida* infections in CCU patients will aid in decreasing the rate of mortality by conducting further research in creating a sensitive drug to the pathogens. All in all, as CCU patients are immunocompromised, early identification of any opportunistic infection like *Candida* infections has a great impact on the fast recovery of patients and decreasing mortality.

1.4. Research Questions

This study is expected to answer the following questions.

i) What is the prevalence of *Candida* infections in the bloodstream of CCU patients in Nairobi Hospital, Kenya?

ii) What is the antifungal susceptibility profile among the *Candida* species isolated from blood stream of CCU patients in Nairobi Hospital, Kenya?

iii) What is the gene sequence of the resistant *Candida* species from CCU patients in Nairobi Hospital, Kenya?

1.5. Objectives

1.5.1. General Objective

To determine the prevalence, anti-fungal susceptibility, and molecular characteristics of *Candida* species isolated from the blood stream of CCU patients in Nairobi Hospital, Kenya.

1.5.2. Specific Objectives

i) To determine the prevalence of bloodstream *Candida* Infections in CCU patients attending Nairobi Hospital, Kenya.

ii) To determine the Anti-Fungal Susceptibility profile of the isolated *Candida* species to Azoles, Echinocandins, and Amphotericin B.

iii) To determine the genotypic characteristics of the isolated resistant *Candida* species.

CHAPTER TWO

LITERATURE REVIEW

2.1. Candida Species

Candida species are found on the host as part of the normal flora and dwell in the oral cavity of healthy human beings. However, the state of immunocompromised patients brings these commensal microorganisms to pathogenicity. These are due to the changes in the individuals' physiology, which result in *Candida* infections (Sardi *et al.*, 2012). In the USA *Candida species* are considered as the third or fourth most causative agent of healthcare-acquired infections. Among those infections, candidemia is seen frequently, exclusively in patients at Critical Care Units (Al-obaid *et al.*, 2017).

2.1.1. Candida albicans

One of the numerous commensals dwelling within the human body is *Candida albicans* and it is an imperative part of normal flora of the human body. As an individual faces an ailment and is immunocompromised, however, *Candida albicans* is able to cause infections ranging from superficial to perilous systemic infections, instigating increased mortality (Sardi *et al.*, 2012). *Candida albicans* produce creamy, soft colonies on SDA agar. The presence of germ tube and chylamdospores on corn meal agar and also the pseudo hyphae and true hyphae observed microscopically makes it unique from the other non *albicans* (Kangogo *et al.*, 2011; Nadeem *et al*, 2010). Moreover, they produce green colonies on CHROMagar (Kangogo *et al.*, 2011). The pathogenic capacity of this fungus is aided by the presence of molecules which are key factors in the adhesion and invasion of the fungus to a host. It also has the capacity of secreting hydrolases and biofilm formation which has a role in causing severe infection in a host. In addition, the phenotypic switching, and yeast to alpha transition contributes to the pathogenicity of the microorganism (Sardi *et al.*, 2012).

2.1.2. Candida tropicalis

Accounting for the second most encountered *Candida* species, *Candida tropicalis* is a major cause of infections in the bloodstream (Al-obaid *et al.*, 2017). *Candida tropicalis*

is a yeast with creamy and soft colony appearance on SDA agar. Moreover, it is seen to have a dark blue colonies that diffuse into surrounding agar in CHROMagar when incubated for 48 hours at 35 ^{o}C (Kangogo *et al.,* 2011). Moreover, it has pseudo hyphae with blastoconidia in corn meal agar (Nadeem *et al.,* 2010). The presence of several commercial kits like API 20C AUX have made identification of *Candida tropicalis* easier by incorporating carbohydrate assimilation tests and giving it a unique numbering identification (SA, BioMérieux, 2010). It harbors a great threat of nosocomial fungal infections, predominantly in individuals suffering from hematologic malignancies (Al-obaid *et al.,* 2017). This is seen frequently in older patients who are chiefly critical care patients due to the prolonged usage of catheterization. In addition, the resistance of *Candida tropicalis* towards azoles, particularly fluconazole has increased to approximately 10%, in certain countries in Asia (Al-obaid *et al.,* 2017).

2.1.3. Candida auris

Candida auris is an emerging multi-drug resistant species of *Candida*, perceived in many nations contemporarily. Since its first onset seen in 2009, it has been identified globally as a hospital-associated infection (Cortegiani *et al.*, 2018). *Candida auris* has resulted in increased mortality rates due to its progressive drug resistance property. *Candida auris* infection mostly attacks critical care patients and is characterized by difficult microbiological identification, drug-resistant property, and high virulence (Cortegiani *et al.,* 2018). The absence of *candida auris* in modern technique database like APIweb and the misidentification of the species with *candida famata* makes it difficult to identify clearly, however, recent technologies like Vitek-2 and MALDI-TOF have made it easier to identify the organism *(Fakhim et al.,* 2018) This species has turned into a global threat due to its widespread and resistant property to several anti-fungal drugs (Cortegiani *et al.*, 2018).

2.1.4. Candida parapsilosis

Candida parapsilosis is prominently seen over *Candida albicans* in some European and South American Hospitals (Trofa *et al.,* 2008). With the intense increase in the onset of *Candida parapsilosis* in the last ten years, it is clarified that *Candida parapsilosis* is usually the second most encountered *Candida* infection (Trofa *et al.,* 2008). Ashford

primarily identified *C. parapsilosis* with a species name of *Monila* indicating its inability to ferment Maltose (Trofa *et al.,* 2008). The isolation was done from a patient's stool sample back in 1928 (Trofa *et al.,* 2008). *Candida parapsilosis* is presented by its formation of pink colonies in CHROMagar (Kangogo *et al.,* 2011). Additionally, it shows clusters of blastophores in corn meal agar (Nadeem *et al.*, 2010). *Candida parapsilosis* caused fatal endocarditis in an intravenous drug user in 1940 which ruled out its identification as non-pathogenic. *C. parapsilosis* is considered to be transmitted by an exogenous cause that presages its association with invasive medical instrumentation (Trofa *et al.*, 2008).

2.1.5. Candida glabrata

C. glabrata was reflected as a commensal to the human body for a lot of years. Nevertheless, the onset of candidemia due to *C. glabrata* has been enormous (Chakrabarti, 2015). This is partially caused by the usage of broad-spectrum antifungals as well as the extensive use of immunosuppressive drugs (Chakrabarti, 2015). *Candida* glabrata is often differentiated phenotypically from *albicans* by the light pinkish color formation on CHROMagar, however, similar to other *candida* species it produces creamy colonies on SDA agar (Kangogo *et al.*, 2011). In contrast to *candida albicans, candida glabrata* doesn't possess pseudo hyphae (Nadeem *et al.*, 2010). The mechanism of host evasion in *C. glabrata* is different from *C. albicans* in that *C. glabrata* uses persistence when attacked by macrophage and evade host defense rather than using belligerent strategy seen in *C. albicans*. *C. glabrata* has been seen significantly to cause candidemia in Northern Europe and the USA for the last ten years (Chakrabarti, 2015).

2.1.6. Candida famata

Candida famata is usually found in the environment, dairy products as a commensal yeast. However, on hospitalized patients especially dealing with catheterization, it can cause severe infections in humans (Beyda *et al.,* 2012). Nevertheless, its onset in Bloodstream infection is very rare (Castanheira *et al.,* 2013). as per several antifungal surveillance studies conducted concerning *Candida famata* as a cause of candidiasis, it

was rated from 0.2%-2% in causing *Candida* infections in humans (Beyda *et al.*, 2012). *Candida famata* present with white to light pink colonies on CHROMagar and its absence of pseudo hyphae in corn meal agar differentiates it from other *candida* species (Nadeem *et al.*, 2010). *Candida famata* is also known as Debaryomyces hansenii and Torulopsis *Candida* (Beyda *et al.*, 2012).

2.1.7. Candida duobushaemolumonii

Candida duobushaemolumonii is a rare pathogen grouped under the species called *Candida haemulonii* (Ramos *et al.*, 2018). According to their species category, *Candida duobushaemolumonii* and *Candida auris* are closely related *Candida* species of which *Candida auris* have been identified as a multidrug-resistant species in many studies (Khan *et al.*, 2018; Ramos *et al.*, 2018). However, as of recent *Candida duobushaemolumonii* has been categorized under its own species group and tends to cause few infections in humans (Ramos *et al.*, 2018).

2.1.8. Candida rugosa

Candida rugosa's emergence as a cause of opportunistic infections in critically ill patients has posed a great threat (Pfaller *et al.*, 2006). It has also occurred as a causative agent of bloodstream infections in North America and Europe (Pfaller *et al.*, 2006). *Candida rugosa* had 0.4% prevalence from isolates of *Candida* species in the program of antifungal surveillance conducted by ARTEMIS DISK (Pfaller *et al.*, 2006). However, this species is seen frequently in different parts of the world. Studies show that its occurrence has reached 44% in critically ill patients in Brazil out of 32 cases of candidemia. Moreover, its onset in India is exceedingly noted as a survey conducted in the country showed its foremost encounter accounting for 20% of critically ill patients (Tay *et al.*, 2011).

2.1.9. Candida lusitaniae

Candida lusitaniae is regarded as a normal flora found in the gastrointestinal tract of warm-blooded animals (Khan *et al.*, 2019), however, on the onset of an immunocompromised state it can cause human infections and It was first found with septicemia regarded as a variant of *Candida tropicalis* (Khan *et al.*, 2018). *Candida*

lusitaniae produces pink gray purple colonies on CHROMagar and presents branched pseudo hyphae on corn meal tween agar (Nadeem *et al.*, 2010). *Candida lusitaniae* is highly associated with resistance to Amphotericin B (Wawrysiuk *et al.*, 2018) with an increasing number of cases infected with *Candida lusitaniae*, the most infected people are patients with cancer and undergoing chemotherapy (Khan *et al.*, 2019). There are also several risk factors like neutropenia and long term usage of broad-spectrum antibiotic in addition to *Candida lusitaniae*'s high association with fungal infection of the bloodstream (Wawrysiuk *et al.*, 2018).

2.1.10. Candida krusei

Candida krusei is one of the rare non *albicans* occurring as a nosocomial infection. The infection is associated with hematological patients. *Candida Krusei* has been causing increased anti-fungal resistance in the past years.in patients admitted to hospitals its presence is often related to prophylactic use of antifungal drugs (Ooga., 2011). Moreover, its appearance as whitish rose with white edges in CHROM agar after incubation of 48 hours at 35 ^{0}C differentiates it from the other *candida* species (Kangogo *et al.*, 2011; Nadeem *et al.*, 2010).

2.2 Candidemia

Candidemia is a crucial nosocomial infection in the world rated as the fourth most encountered fungal infection. According to a recent literature, the number one cause of bloodstream infections was found to be candidemia (Bassetti *et al.*, 2015). Candidemia accounts for a mortality rate greater than 40% and prevails severe morbidity. This, in turn, results in increased costs for healthcare including a prolonged stay in the hospital (Bassetti *et al.*, 2015). Of all species, *Candida albicans* has been the most encountered species, however, non-*albicans* have harbored global attention as emerging invasive fungal infections (Li *et al.*, 2017).

Severe outcome of mortality has been seeking the attention of researchers to investigate the importance of prompt diagnosis and the availability of appropriate treatments of candidemia (Bassetti *et al.*, 2015). The usage of invasive instruments has led to an increase in the onset of hospital-acquired infections, mainly infections of the

bloodstream, and the increase is mostly as a result of *Candida* infections. This has put into perspective the different causes of the increase in candidemia (Suleyman *et al.*, 2016). Moreover, the occurrence of drug resistance either inherently or acquired has been primarily seen in azoles and Echinocandins, which opt for appropriate therapeutic strategies (Bassetti *et al.*, 2015).

2.2.1 Diagnosis of Candidemia

Candidemia is the occurrence of *Candida* infection in the bloodstream. The diagnosis mainly takes place using a blood sample. Different methods are available for diagnosis, including culture methods, biochemical tests, and new non-culture methods.

2.2.1.1 Blood Culture for Identification of *Candida Species*

A gold standard of diagnosis in candidemia has been blood culture. Nevertheless, culture is time-consuming in obtaining results as it might take several days to give results of the growth of microorganisms (Ahmad & Khan, 2012). Culturing has become easier through the usage of different techniques like lysis-centrifugation tubes and automated supervision of blood culture bottles (Deorukhkar & Saini, 2014). The lysis helps in aggrandizing the sensitivity of the culture method by increasing the amount of *Candida* species detected in the blood using a detergent to release the fungi to the extracellular surface from inside the host phagocytic cells. It is also helpful in reducing the time elapsed between inoculation and the growth of the fungi. Even though this procedure takes a lot of time and energy, it has the sole advantage of improvements to an automated feature that enables the continuous supervision of growth (Deorukhkar & Saini, 2014).

Non-fastidious organisms such as *Candida species* are able to grow in most media easily; however, the most utilized media in these fungi remains Saboraud Dextrose Agar. The growth of *Candida* species is enabled by SDA, whereas the growth of many bacteria is inhibited (Deorukhkar &Saini, 2014). This is with respect to its low PH. The appearance of smooth, pasty, creamy, and convex shapes colonies indicates *Candida* species growth in SDA. SDA is not regarded as either selective or differential media and can be supplemented with antibiotics like tetracycline, chloramphenicol, and/or gentamicin to make it selective for only the growth of *Candida* and inhibit bacterial

growth. Moreover, the addition of cycloheximide prevents the growth of saprotrophic fungal contaminants (Deorukhkar *et al.*, 2014). However, certain species of *Candida* like *C. krusei*, *C. tropicalis*, and *C. parapsilosis* are sensitive to cycloheximide. Therefore, the addition of cycloheximide is not usually recommended. The culturing process is done by incubating the inoculated organisms in SDA at 28 ^{o}C to 37 ^{o}C for 24 to 72 hours (Deorukhkar & Saini, 2014).

2.2.1.2 BACT/ALERT for Identification of *Candida* Species

A programmed instrument BACT/ALERT (Organon Teknika Corp., Durham, North Carolina) works to detect microorganisms using a colorimetric method by recognition of microorganism formed $CO_{2.}$ (Thorpe *et al.*, 1990). Based on the assessment of its detection algorithm as well as the media and sensor evaluation, this automated machine is considered to produce reliable results. Moreover, it gives accurate detection of different fungi and bacteria grown on this machine. This system eliminates the possibility of radioactive wastes as it is entirely non-radioactive. In addition, this machine is self-sufficient, thereby eliminating the necessity of other devices such as incubators, detectors, or agitators to complete the growth mechanism. (Thorpe *et al.*, 1990).

2.2.1.3 Biochemical Characterization of *Candida* Species

The identification and characterization of *Candida species* can be made by their properties of carbohydrate utilization (Deorukhkar & Saini, 2014). In *Candida species*, there is a pattern of anaerobic carbohydrate fermentation and aerobic assimilation, which makes it easier to identify the species biochemically. There are also several easy ways to identify the assimilation property of *Candida species* such as auxano graphic method, rather than the classical way by Wickerham and Burton. Other available methods also include the API-20C system, Fungi Chromo1, VITEK as well as API - Yeast Indent and Yeast Biochemical Card (YBC) system. Invention and usage of those commercial technologies have made the identification process easier (Deorukhkar & Saini, 2014).

2.2.1.4 VITEK-2 for Identification of *Candida* Species

An automated instrument VITEK is essential in the identification of microorganisms through the usage of inoculum of pure culture (Pincus, 2013). Incorporating a large number of databases VITEK-2 identifies microorganisms using the inoculum introduced to a card in a machine. It has made the identification process a lot faster than classical methods. In addition to that, VITEK-2 is also used to test the drug susceptibility of microorganisms to different anti-microbial drugs, specifically to the organism of interest. Holding 30 cards, this system utilizes fluorogenic method to identify the organism (Pincus, 2013). This fast output machine also uses turbidimetry to test drug susceptibility of microorganisms incorporating 64 cards for such use. The technologies incorporated in this instrument have made patient diagnosis easier and faster. This, in turn, makes the hospital stay of patients shorter (Pincus, 2013).

2.2.1.5 API 20C AUX for Identification of *Candida* Species

API 20 C AUX is a system for the precise identification of the most frequently encountered yeasts. The complete list of those species in the Identification Table at the end of the package insert makes it possible to identify the organisms with this system. The API 20 C AUX strip consists of 20 cupules containing dehydrated substrates which enable the performance of 19 assimilation tests. The cupules are inoculated with a semi-solid minimal medium. The yeasts will only grow if they are capable of utilizing each substrate as the sole carbon source. The reactions are read by comparing them to growth controls. Identification is obtained by referring to the Analytical Profile Index or using the identification software (SA, BioMérieux, 2010).

2.3 Antifungal Susceptibility Testing

Antifungal susceptibility testing methods are available to detect antifungal resistance and to determine the best treatment for a specific fungus. To distinguish the national and global epidemiology of anti-fungal resistance, clinical microbiology depends upon the different anti-fungal susceptibility tests available in laboratories (Alastruey-Izquierdo *et al*, 2015).

2.3.1 Liofilchem MIC Test Strip

Liofilchem MTS is an antifungal susceptibility testing method that works through a direct count of the antimicrobial susceptibility of microorganisms. It defines the minimum inhibitory concentration (MIC) and thus determining the susceptibility of a microorganism to certain drug actions. The Liofilchem MTS is a non-penetrating plastic material designed to have 15 gradients of antibiotic concentrations (Liofilchem, 2018). These are put stationery in the strip and have MIC scale on the other side of the strip, which is made of nylon. Liofilchem MTS is used worldwide for testing anti-microbial and anti-fungal susceptibility and its similar MIC strips have been described with its principle in 1988. The conventional MIC strips have been in commercial use since 1991 by AB BIODISK. The principle of detecting the MIC of the drugs towards the organisms has been crucial in therapeutic strategies for several patients, especially critical care patients. It is both cost-wise effective as well as has different dilutions of the MIC concentrations which makes it easier to use than disk diffusion methods. The resistance identification can also be enhanced using the macro method (Liofilchem, 2018)

2.4 Antifungal Drugs in Clinical Treatments

Despite the presence of diverse and numerous anti-fungal drugs for clinical treatments, only a few classes of antifungal agents are currently available to treat mucosal or systemic infections with *Candida* spp.

2.4.1 Azoles

Azole families are the leading anti-fungal drugs in the treatment of fungal infection. Their mechanism of action involves disrupting the cell membrane and suppress the activity of lanosterol 14- α-demethylase (Spampinato & Leonardi., 2013). This is an enzyme synthesizing Ergosterol which is the cholesterol of the fungi. Being the biggest sterol, ergosterol is part of the fungal cell membrane (Spampinato & Leonardi, 2013). However, the structural differences of ergosterol of fungi and cholesterol of animal cells prevent antifungal drugs from attacking the cholesterol of the host cell when taken

as treatment of fungal infection and targeted to bind the ergosterol component of the fungi cell membrane (Spampinato & Leonardi., 2013).

Usage of azoles can be topical and for prophylaxis of invasive fungal infections. Approved by the US food and drug administration, this family of anti-fungal drugs remains routinely used drugs for fungal infections (Spampinato & Leonardi, 2013). There are several types of azoles including, imidazoles (miconazole, econazole, clotrimazole, and ketoconazole) and triazoles (fluconazole, itraconazole, and the latest agent voriconazole (second-generation, synthetic triazole derivative of fluconazole) and posaconazole (hydroxylated analog of itraconazole) (Spettel *et al.*, 2019).

2.4.2 Echinocandins

Echinocandins include drugs like caspofungin, anidulafungin, and micafungin which inhibit the synthesis of glucan (Spampinato & Leonardi, 2013). On the other hand, they are lipopeptides and inhibitors of fungal wall synthesis due to the obstruction of (1,3)-β-D-glucan synthase. Incorporating fungicidal activity which is concentration based, the glucan inhibitors have an anti-fungal activity to most of *Candida* species. These drugs have the approval of the FDA and are utilized for most candidiasis like esophageal, invasive candidiasis as well as candidemia (Spampinato & Leonardi, 2013).

2.4.3 Polyenes

These anti-fungal drugs include nystatin and amphotericin B which are isolated from *Streptomyces* species (Spampinato & Leonardi, 2013). Their major role in anti-fungal treatment is to bind ergosterol and cause the disorder of the fungal cell membrane, especially its lipidic component (Spampinato & Leonardi, 2013). This, in turn, results in the formation of aqueous pores which enhances the reformation of the cell membrane permeability and contributes to seepage of cytosolic components and ultimately death of the fungi (Spampinato & Leonardi, 2013).

2.4.4 Flucytosine

Flucytosine (5FC) is an effective antifungal compound (M. Cuenca-Estrella *et al.*, 2001). The mode of action of Flucytosine is different from the commonly used

antifungal drugs like azoles and polyenes. Deamination of 5FC to antimetabolite 5-fluorouracil activates it with in fungal cells thereby converting into specific inhibitors of DNA/RNA synthesis (M. Cuenca-Estrella *et al,*, 2001). Flucytosine is a water soluble drug and can be given either oral or intravenous. Flucytosine is used as a treatment for many yeasts including *candida, aspergillosis,* and chromoblastomycosis (M. Cuenca-Estrella *et al.*, 2001). However, patterns of primary resistance to Flucytosine have been observed in certain yeasts and molds, and this agent is subject to developing secondary resistance in patients undergoing 5FC monotherapy. Therefore, the usage of Flucytosine have been only I n combination antifungal therapy (M. Cuenca-Estrella, *et al.*,, 2001).

2.4.5 Other Antifungal Agents

Other anti-fungal agents are present which are not routinely used. These include Allylamines and thiocarbomates which majorly have a role in the reformation of the cell membrane through the inhibition of squalene-epoxidase and another enzyme that synthesizes ergosterol (Spampinato & Leonardi, 2013). Another drug called Griseofulvin is also an inhibitor of fungal mitosis using the disturbance of spindle and cytoplasmic microtubule production. This drug was isolated from penicillium (Spampinato & Leonardi, 2013).

2.5 Mechanism of Drug Resistance in *Candida* Species

Long-term treatment of candidiasis and the emergence of resistance to azole and polyene drugs usually results in treatment failure. However, the pathway for fungal sterol biosynthesis is still a known and confirmed target for antifungal drug development (Lotfali *et al.*, 2017). Resistance to azoles is acquired when mutations occur in the pharmacological target which mainly is able to alter the structure of the enzyme and thus decrease the affinity of binding to ERG 11 (Spettel *et al.*, 2019). Moreover, the sequestration of intracellular azoles is reduced by efflux pumps (Spettel *et al.*, 2019). Resistance to echinocandins occurs when mutations that are targeted in the hotspot regions of FKS proteins are present. Susceptibility to echinocandins can be decreased when point mutations are present in those regions making the drug less susceptible to 1,3-β-D-glucan synthase (Healey & Perlin, 2018).

2.5.1 Ergosterol 11 (ERG 11)

Ergosterol is a sterol that is similar to cholesterol and found in most fungi. Its synthesis is inhibited by the usage of Azoles and thus destroying the main component of cell membranes of fungi (Xiang *et al.*, 2013). This destruction is done by Azoles by inhibiting the 14-α-sterol demethylase enzyme which is the product of the ERG11 gene. This enzyme is also called CYP51A1 (Xiang *et al.,* 2013). Therefore, azoles have a capacity of occupying an active site located in this enzyme as they possess basic nitrogen that collaborates with the heme group of an iron atom of the enzyme produced by ERG 11 (Xiang *et al.,* 2013). This resistance to azoles is, however, caused due to point mutations in the ERG 11 gene which increases the number of CYP51A1 as ERG 11 is overexpressed (Xiang *et al.*, 2013)**.**

2.5.2 Ergosterol 3 (ERG 3)

ERG3 and ERG6 are other genes of ergosterol biosynthesis which have not been entirely explored (Lotfali *et al.,* 2017). Mutations of ERG3 cause decreased sequestration of azoles and polyenes and as such *C. albicans* develop resistance to polyene and azole treatment. Through the examination of sterol content, the resistance mechanism leads to a build-up of sterol intermediates. These intermediates are 14-methyl fecosterol, which have the ability to damage the ergosterol pathway (Lotfali *et al.*, 2017). On the other hand, ERG6 has not been sequenced thoroughly, but upon its mutation, the sequence might give an outcome of missense mutation which indicates the replacement of cysteine with phenylalanine. (Lotfali *et al.*, 2017).

2.5.3 Candida Drug Resistance 1 and 2 (CDR 1 and CDR 2)

Overexpression of CDR1 and CDR2 efflux proteins dispose of drug resistance in *Candida* species (Yadav *et al.*, 2014). These are characterized as major efflux pumps from the ABC transporters. Other efflux proteins include MFS efflux proteins (MDR1). As the first ABC transporter in *Candida albicans* CDR1 need regulation and the regulation process is very complicated. In the regulation of this efflux protein, numerous cis and trans factors are found. The comprehension of drug resistance mechanism is necessary for successful regulation of multidrug resistance (MDR) in *Candida albicans* (Yadav *et al.*, 2014).

2.5.4 Multi-Drug Resistance 1 (MDR 1)

Alterations in the genetic makeup of *Candida albicans* can predispose to overexpression of some efflux pumps resulting in multiple drug resistance. Decreased drug sequestration inside the cell of resistant *Candida albicans* is associated with increased expression of resistance genes. Usually, MDR is the major facilitator of the resistance genes along with ABC Transporters CDR1 and CDR2 (Wirsching *et al.*, 2000). The prove of resistance is displayed in the undetectable state of MDR1 in fluconazole susceptible C. *albicans* while it is increased tremendously in those species which have developed resistance to fluconazole. Molecular basis of identification of the resistance mechanism remains unexplored. However, there are suggestions of mutation or irregular expression of the MDR1 gene (Wirsching *et al.*, 2000).

2.5.5 Glucosyltransferase (FKS 1 and FKS 2)

Echinocandins drugs which were first put to use in 2001 have a mechanism of blocking the synthesis of β-1,3-glucan, thus, acting as a very vigorous drug in eliminating *Candida* infections (Healey & Perlin, 2018). They inhibit the activity of β-1-3-d-glucan synthase which incorporates the catalytic subunit prearranged by *FKS* genes whereas, its activity is controlled by Rho, a GTP-binding protein. Alteration in the FKS subunits results in resistance of the species to the drug (Healey & Perlin, 2018). Moreover, mutations in most *Candida* species in the hot spot regions of FKS 1 while FKS2 in *C. glabrata* results in increased MIC values. In addition to that, glucan synthase sensitivity might be decreased by >3000 fold. (Healey & Perlin, 2018).

CHAPTER THREE

MATERIALS AND METHODS

3.1. Study Site

The study site was Nairobi Hospital. Nairobi Hospital is a large hospital in Kenya that has been officially operational since 9th April 1954 (The Nairobi Hospital, 2016). It is located on Argwings Kodhek Road, in the neighborhood of Upper Hill, in Kenya's capital city of Nairobi. A total number of 355+ beds are present in the hospital while 16+ beds are in the ICU facility and 8 are in the HDU facility. This hospital serves different patients from the whole country (The Nairobi Hospital, 2016).

3.2. Research Design

The study was cross-sectional research design. This research design was followed since the data collection was done at one point in time and no further follow-up was done for the patients (Levin, 2006).

3.3. Study Population

The study participants were enrolled from the intensive care unit and highly dependent unit of Nairobi hospital. The study population included patients of all age groups who were tested from January 2019 to January 2020 for *Candida* bloodstream infections.

3.3.1. Inclusion Criteria

- Patients who were admitted to CCU (ICU and HDU) in Nairobi Hospital.
- Patients who were requested by the physician to do a blood culture.

3.3.2. Exclusion Criteria

- Patients under any circumstance who were not able to give a blood sample.
- Patients who were on antifungal medication

3.4. Sample Size Determination

The sample size required was a minimum of 167 which was determined based on Naing *et al.* (2006).

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

Where: n = Sample size

Z = the Confidence Interval at 95% (1.96)

P = estimate of the proportion or anticipated prevalence used 12.42% candidiasis from a population-based study in Africa (Omrani *et al.*, 2014).

d = margin of error at 5% (0.05)

$$n = \frac{Z^2(P)(1-P)}{d^2} = \frac{1.96^2(0.1242)(1-0.1242)}{0.05^2} = 167$$

A total number of 378 patients were tested. The sample size was increased to make the study population more representative and aggrandize the prevalence study's validity in representing the patients of the critical care units in Nairobi Hospital.

3.5. Sampling Technique

Purposive sampling method was used to select the patients admitted to the CCU and who met the inclusion criteria of the research while excluding those patients who did not meet the criteria for inclusion (Etikan, 2016).

3.6. Data Collection

3.6.1. Demographic Data Collection

Based on the inclusion and exclusion criteria above patients in the Critical Care Unit facility of Nairobi hospital were selected. Patients' age, gender, and ward were documented for analysis from the health facility's records and patient identification was coded in numbers to avoid the leak of patient history.

3.6.2. Sample Collection

Samples were collected by licensed lab personnel. The sample collected was venous blood of 5ml drawn in a test tube from the patient and immediately inoculated in an enriched media before transporting it to the lab. Then the sample was incubated at 37 ^{o}C for 72 hours to see any growth of microorganisms. The incubation was done in BACT/ALERT (Organon Teknika Corp., Durham, North Carolina) where the temperature was held in parallel with the body temperature to make an amiable environment for the microorganisms (Thorpe *et al.*, 1990). This process selected the samples which had general growth of microorganisms from those samples which did not have any growth. Sub culturing was done in Saboraud Dextrose Agar (SDA) to detect the presence of *Candida* species. Specimens were inoculated in SDA agar and incubated at 35 to 37 ^{o} C for 24 to 72 hours to observe the growth of *Candida* species and isolates which were positive for candidemia were archived.

3.6.3. Culture of Isolates for Identification of *Candida Species*

Archived isolates were stored in glycerol for the timeline of the study. Fresh Culture was obtained for confirmatory identification and antifungal susceptibility testing. Sub-culture was done by inoculating the isolates to a selective media, Saboraud Dextrose Agar (SDA). Specimens were then incubated at 35 to 37 ^{o} C for 24 to 48 hours to observe the growth of *Candida* species. The appearance of creamy white colonies with soft texture (***Plate 3.1***) was considered positive and proceeded to anti-fungal susceptibility tests (Deorukhkar *et al.*, 2014).

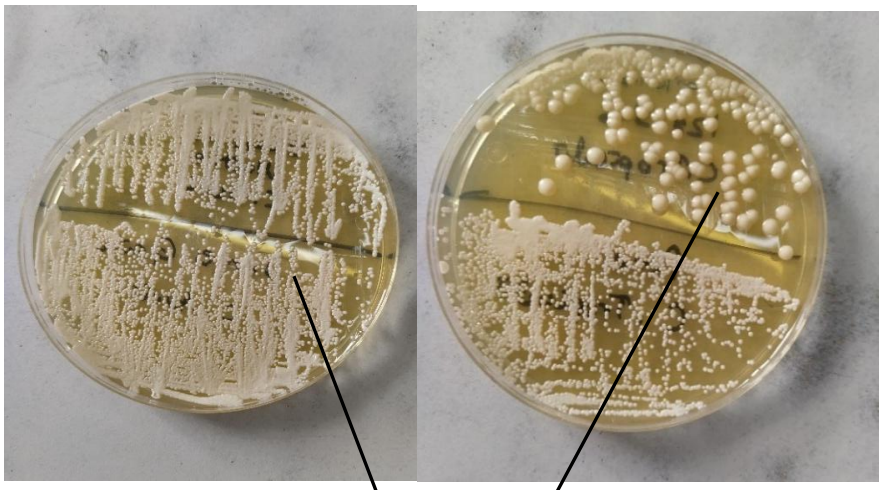


Plate. 3.1. A colony of *Candida* Species' after 24-48 hours of incubation on SDA agar

Pictures of plates was taken from the lab work done in Nairobi Hospital to subculture the isolates for *candida* specific growth.

3.6.4. API 20C AUX test for *Candida* species Identification

API 20C AUX (Biomerieux, USA) was used for the identification of the *Candida* species. Isolated *Candida* colonies were inoculated in the API 20C cupules for positive or negative growth check in the 19 assimilation tests. This method incorporates 20 cupules with 19 assimilation tests that aided to identify the type of *Candida* species accurately (***Figure 3.1.***).

First, the incubation tray was prepared by putting in 5.0 ml of distilled water to create a humid atmosphere. Then the strip was put in the tray ready for inoculating the culture into it. The inoculum was prepared by mixing 2.0 ml of normal saline with a portion of the yeast colony to prepare a turbid solution of 2 McFarland. The McFarland was checked to make sure it didn't exceed or was lower than 2. Afterward, 100µl of the turbid colony suspension was introduced to a 7 ml ampule of API C medium.

Inoculation of suspension into the cupules which contains 20 tests of identification was done using a pipette carefully without creating bubbles (SA, BioMérieux, 2010). After the suspension was transferred from the ampule to the strip, the tray was closed with a lid and incubated at 29 ^{o}C +/- 2 ^{o}C for 48-72 hours.

Reading was done by observing the positive and negative growth of the inoculum in response to the assimilation tests. The reaction was measured by comparing with the 0 cupule which served as a negative control. Any cupule which was more turbid than the 0 cupule was considered to have a positive reaction. The interpretation was then done by adding up the three numerical values assigned to the positive reaction. The interpretation was done by using the database with the Analytical Profile Index.

Negative Control Cupule **19 Assimilation test Cupules**

Figure 3.1. API 20C AUX test strip used for Identification of *Candida* species

3.6.5. VITEK-2 Automation for *Candida* species Identification

VITEK-2 version 8.1 (Biomerieux, USA) was used for confirmatory identification of the *Candida* species which were identified as *Candida famata* using API 20C AUX. Studies show *Candida auris and Candida duobushaemolumonii* are often misidentified as *Candida famata* in API 20 C AUX (Wang *et al.*, 2018). Therefore, confirmatory identification using VITEK-2 (Biomerieux, USA) was done to distinguish between true *Candida famata* and *Candida auris* or *Candida duobushaemolumonii*. Isolated *Candida* colonies were suspended in 2.0 ml sterile saline to make a 2.0 McFarland turbidity. Turbidity was measured using a turbid meter in McFarland (Pincus, 2013). Saline suspended colonies were then inoculated in the VITEK-2 Card which contains 10-15 cards and identification was done by using a vacuum instrument (Pincus, 2013). The identification of the species takes place in a percentage format and the system identified the species by rating it in the percentage of accuracy (Pincus, 2013).

Vitek-2 Cassettes loaded with *Candida* isolates **Turbidometer measuring McFarland of *Candida* colonies**

Figure 3.2. Vitek-2 loading cassettes and Turbidometer used in identification of *Candida* Species

3.6.6. Antifungal Susceptibility Testing

Anti-fungal drug susceptibility was performed using Liofilchem MIC test strip (Liofilchem S.R.I., Italy) for Fluconazole, Micafungin, Caspofungin, Amphotericin-B, Flucytosine, and Voriconazole (Liofilchem, 2018). The Liofilchem MIC test strip is a non-penetrating plastic material designed to have 15 two-fold dilutions of antibiotic concentrations (Liofilchem, 2018). The strip was placed in an SDA agar inoculated with 0.5 McFarland pure colonies' suspension of the positive isolated species. Afterward, the agar was incubated at 37 ^{o} C and observed for Minimum Inhibitory Concentration (MIC) reading after 24 hours (***Plate 3.2)***. The interpretation was done classifying the organisms as sensitive or resistant. The breakpoint for classification of the sensitivity or resistance was done following the guidelines set out by CLSI MA-27 (Cantón & Peman, 2009).

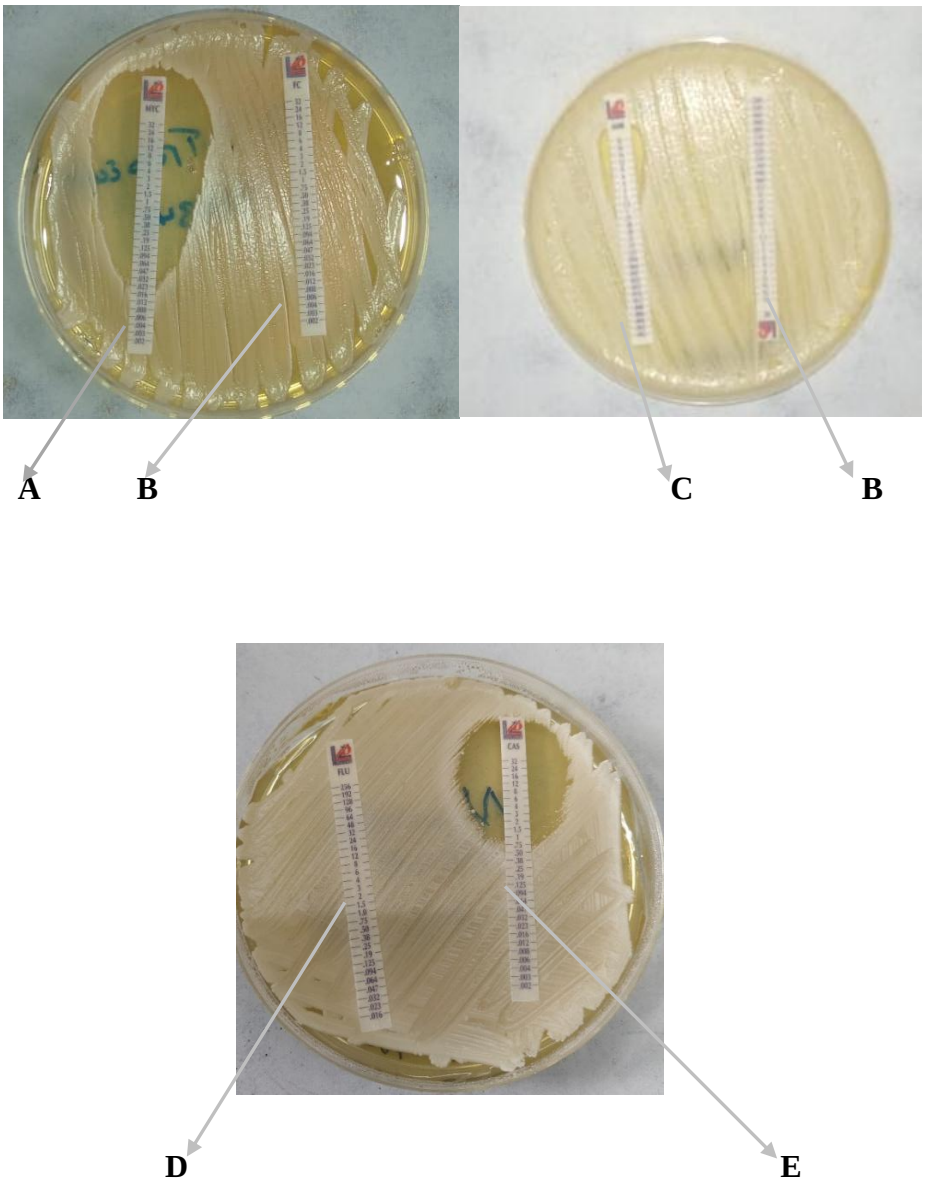


Plate 3.2. Liofilchem MIC test results for six Antifungal Drugs after incubation for 24 hours on SDA

Keywords: A: Micafungin, B: Flucytosine, C: Amphotericin-B, D: Fluconazole, E: Caspofungin. The anti-fungal susceptibility MIC test strip pictures were captured while performing the tests in Nairobi Hospital.

3.6.7. DNA Extraction and Amplification of Resistant *Candida* Isolates

Resistant *Candida* species which were determined using the antifungal tests were subcultures in SDA to get pure cultures and colonies were used for DNA extraction within seven days.

The DNA extraction was done using a QUICK DNA Mini prep extraction kit from Zymo research (Zymo Research). First, 50-100 mg of the colonies were suspended in 2 ml distilled water to make an aqueous solution. The solution was mixed thoroughly using a sonicator (QSONICA, LLC, Newtown, Connecticut) to destruct the cell walls of the *Candida* colonies. Afterward, 100 µl of the suspension was put into a bashing bead and 750 µl of bashing bead buffer was added to it. The solution in the bashing bead was then fitted in the vortex of 2 ml and processed at maximum speed for 5 minutes (Zymo Research). Subsequently, the ZR Bashing Bead™ Lysis Tube (0.1 & 0.5 mm) was Centrifuged in a micro centrifuge at 10,000 x g for 1 minute. After centrifugation up to 400µl supernatant was transferred to a Zymo-Spin™III-F Filter in a Collection Tube and centrifuged at 8,000 x g for 1 minute. A 1,200 µl of Genomic Lysis Buffer was added to the filtrate in the collection tube from the previous step. After thoroughly mixing this mixture 800 µl of it was transferred to a Zymo-Spin™ IICR Column in a Collection Tube and centrifuged at 10,000 x *g* for 1 minute. Afterward, the flow-through was discarded from the Collection Tube and centrifuged again at 10,000 x *g* for 1 minute. Then 200 µl DNA Pre-Wash Buffer was added to the Zymo-Spin™ IICR Column in a new Collection Tube and centrifuged at 10,000 x *g* for 1 minute. After which 500 µl genomic DNA Wash Buffer was added to the Zymo-Spin™ IICR Column and centrifuged at 10,000 x *g* for 1 minute. The Zymo-Spin™ IICR Column was transferred to a clean 1.5 ml micro centrifuge tube and 50 µl DNA Elution Buffer was added directly to the column matrix and centrifuged at 10,000 x *g* for 30 seconds to elute the DNA. Ultra-pure DNA was then extracted and ready for PCR.

3.6.7.1. Quantification of Genomic DNA

The quantification of genomic DNA was performed by Nanodrop Spectrophotometer (Model PCR Max Lambda, Thermo Fisher Scientific, Waltham, MA, USA). The concentration of genomic DNA was measured in µg/ml and the quality and purity of

DNA was measured in 260nm/280nm ratio. The concentration of the genomic DNA ranged from 10.519µg/µl to 265.15 µg/µl with a mean of 79.1 and standard deviation of 70.9 giving a similar range of concentration in most of the DNA samples.

3.6.7.2. DNA amplification using PCR

A total of 18 (58.06%) isolates were found resistant and subjected to DNA amplification and sequencing of 23 samples and 39 reactions for mutation analysis was performed. Nine (9) resistant *Candida auris* isolates were amplified using forward and reverse primers obtained from a study conducted by Chowdhary *et al* (Chowdhary *et al.*, 2018) Moreover, Six (6) resistant *Candida parapsilosis* and three (3) *Candida tropicalis* underwent the same amplification procedure using individual primers attained from similar studies (***Table 3.1.***) (Desnos-Ollivier *et al.*, 2008; Souza *et al.*, 2015)

Table 3.1: Oligonucleotide primer sequences used in this study

PRIMER NAME	SEQUENCE (5^1 - 3^1)	PURPOSE
C.AU.ERG11.F1	TGGGTGGGTTCAGCTGTTG	ERG11 PCR amplification &Sequencing
C.AU.ERG11.R1	TTCAGCTGGTTCCATTGG	
C.AU.ERG11.F2	TGGGTTGGCTCTGCTGTTG	
C.AU.ERG11.R2	TTCTGCTGGCTCCATTGG	
C.T. FUR.F	TCATCAAAACCATGTCTGCTG	FUR PCR amplification &Sequencing
C.T. FUR.R	AAGTGTATGTAGTGATAATTGCTATGC	
C.P.ERG11.F1	CGAGATAATCATCAACGAACATTC	ERG11 PCR amplification &Sequencing
C.P.ERG11.R1	CGTTTAAAACATCCAAAGACCTTA	
C.P.ERG11.F2	AATCTGAGGGTTTCCTTGATGGT	
C.P.ERG11.R2	AAAGACCGCATTGACTACCGAT	

PCR analysis of the extracted DNA was performed in a 30µl reaction mixture comprising 1.5µl of genomic DNA, 0.75 µl of each forward and reverse primer, 18.75 µl of One Taq, Quick Load 2x Master Mix with Standard Buffer (New England Biolabs Inc, MA, USA), and 8.25 µl of PCR water. Amplification was conducted in a Proflex PCR System (Model 4483636, Thermo Fisher Scientific, Waltham, MA, USA) with the below conditions (***Table 3.2.***).

Table 3.2: PCR amplification conditions

Set	PCR Fragment	Initial Denaturation		Denaturation		Annealing		Elongation		Final Elongation		Number of Cycles
1	CAU ERG11F1,R1	94 ^{0}C	5 min	94 ^{0}C	60 s	56^0 C	60 s	68^0 C	60 s	68^0 C	5min	30
2	CAU ERG11 F2R2	94 ^{0}C	5 min	94 ^{0}C	60 s	57.4^0 C	60 s	68^0 C	60 s	68^0 C	5min	30
3	CT FUR	94 ^{0}C	5 min	94 ^{0}C	60 s	57.4^0 C	60 s	68^0 C	60 s	68^0 C	5min	30
4	CP ERG11 F1R1	94 ^{0}C	5 min	94 ^{0}C	60 s	56.7^0 C	60 s	68^0 C	60 s	68^0 C	5min	30
5	CP ERG11 F2R2	94 ^{0}C	5 min	94 ^{0}C	60 s	58.6^0 C	60 s	68^0 C	60 s	68^0 C	5min	30

Keywords: CAU: *Candida auris*, CT: *Candida Tropicalis*, CP: *Candida Parapsilosis*, F: Forward, R: Reverse, ERG11: Ergosterol 11 gene, FUR: Uracil phosphoribosyl transferase gene

3.6.7.3. Resolution of Amplified DNA

Separation of amplified PCR products was done using gel electrophoresis in a 1.5% agarose (Sigma, St. Loaus, MO, USA) gel stained in 2 µL of GelRed® Nucleic Acid Gel Stain (Biotium, USA). After the mixture of agarose gel and GelRed dye were made to dry in a tray with wells created on it through combs, the amplified PCR products were loaded into the agarose gel. The first well was loaded with 10µl of 1Kb DNA ladder (New England Biolabs Inc, MA, USA) followed by negative control in the next well. Then 5µl of the PCR products were loaded in the middle wells followed by negative control and DNA ladder at the end. The DNA bands resolved on agarose gel were visualized in the UV Documentation system with a camera transilluminator (Model UV Doc. HDS UITEC Cambridge, UK). The estimation of the desired bands was done comparing with the DNA ladder marker in base pair measurement.

The Amplified DNA samples were then quantified using NanoDrop and concentrations were recorded in µg/ml. Subsequently, 23 samples and 39 reactions were outsourced for purification and sequencing to macrogen (Macrogen Europe *B.V,* Netherlands*).* Both forward and reverse primers were used for sequencing the DNA samples. The DNA samples were sequenced using standard sanger sequencing by 3730xl DNA analyzer and Big dye v3.1 (ABI, USA) and Blasted on NCBI to compare their Identity from the database. Reference sequences with the accession number of XM_029033208, MK513971, and XM_002548346 were used for *Candida auris, Candida parapsilosis,* and *Candida tropicalis* respectively for sequence analysis.

3.7. Quality Control

Quality control was performed along with each test conducted in this study. Quality control tests help validate test results and make the outcomes of the study reliable. Therefore, the quality control of tests in this study was maintained using reference strains of *Candida albicans* ATCC: 14053 and *Candida parapsilosis* ATCC 22019.

3.8. Data Analysis

Data collected was documented in Microsoft Excel (Microsoft Corp, USA) before transferring to SPSS version 20 (IBM, USA) for analysis. Descriptive Statistics was done to determine the frequencies of the species distribution. Cross Tabulation was used to determine the Prevalence of *Candida* species in relation to age, ward, and gender. Chi-square was used for the level of significance. T-test was used to calculate the p-value association between HDU and ICU based on age, gender, and prevalence. Minimum and maximum susceptibility values were calculated for all the drugs tested to obtain the MIC range.

All the sequences obtained were trimmed and edited using ChromasPro® Version 2.1.9 (Technelysium Pty Ltd, South Brisbane, Australia) to eliminate errors which occurred during sequencing and to obtain the real SNPs. Chromatograms were visually inspected to rule out any ambiguities in contiguous sequences using base call scores. The consensus sequences attained underwent a multiple sequence alignment using MEGAX (Kumar *et al.*, 2018) with MUSCLE to detect the presence of synonyms and non-synonyms Single Nucleotide Polymorphism by comparing them to reference strains obtained from NCBI and amino acid translation was performed using standard genetic code to visually observe any mutation which resulted in amino acid changes. Subsequent changes in amino acid were viewed using Jalview (Waterhouse *et al.*, 2009).

Analysis of the obtained SNPs on the effect of the expected protein structure was conducted using STRUM (Quan *et al*,, 2016) whereby the stability of the predicted protein was calculated using Gibbs free Energy (G). STRUM determines the distinction between Gibbs free Energy of the unfolded (Gu) and folded (Gf), (Gu) (ΔG ¼ Gu – Gf), and afterwards the difference between ΔG of wild type and mutant protein structure (ΔΔG ¼ ΔGm- ΔGw). The main foundation of this calculation is in its thermodynamics whereby SNPs affect the protein stability by reduction in free energy difference between the conformations of folded and unfolded protein. Therefore, ddG below zero indicates the mutation destabilizes the protein structure while value greater than zero

implicates stabilization of the predicted protein structure by the mutation present (Quan *et al.*, 2016).

3.9. Ethical Consideration

Ethical approval was obtained from Jomo Kenyatta University of Agriculture and Technology's Ethics Review Committee and the Nairobi hospital's Ethics Review Committee. No patient was recruited solely for the study, but rather the study was part of the normal patient care process. In addition, there was a huge benefit of knowledge gained by the patients in regards to their current situation of *Candida* infections. Access to the data was strictly prohibited to maintain the confidential information of the patient. Patient Identification was done in a way that doesn't disclose the real information of the patient to avoid the leak of patient history.

3.10. Disposal of Samples

After sample analysis had been performed, samples were stored for a year until results were analyzed and interpreted. Following the thesis completion, samples were disposed of according to the laboratory guidelines. For biological hazardous samples, the samples were accumulated in bags that were marked "Bio-Hazard". The bag was leak-proof and with solid marking (US Environmental Protection, 2010). Samples were treated to render them non-biological waste and not hazardous or they were incinerated at an offsite facility for safety (US Environmental Protection, 2010)

CHAPTER FOUR

RESULT

4.1. Characteristics of the Study Population

A total of 378 patients were recruited to the study. The study was conducted from January 2019 to January 2020. The study population was patients admitted to ICU and HDU of the tertiary care hospital. Out of 378 patients, 203 were from ICU and 105 from HDU. The median age for ICU was 55 years (2mon-95years) and 61 years (16-101 years) in HDU. The majority of the patients were males at 158 (57.9%) and 60 (57.1%), while females were 115 (42.1%) and 45 (42.9%) in ICU and HDU respectively (***Table 4.1.***).

Table 4.1: Characteristics of the Study Population

		ICU	HDU
Age			
Median (Range)		55 (2 months-95years)	61 (16 years-101 years)
Gender	**M**	158	60
	F	115	45
Total (%)		273 (72.2%)	105 (27.7%)

4.2. Prevalence and Distribution of *Candida* Species

Out of the 378 patients tested 31 (**8.2%**) patients were found to have candidemia The highest prevalence was *Candida auris at 9 (*29.03%) and the lowest prevalence in *Candida duobushaemolumonii* and *Candida lusitaniae,* at 1 (3.23%) each (***Figure 4.1.***).

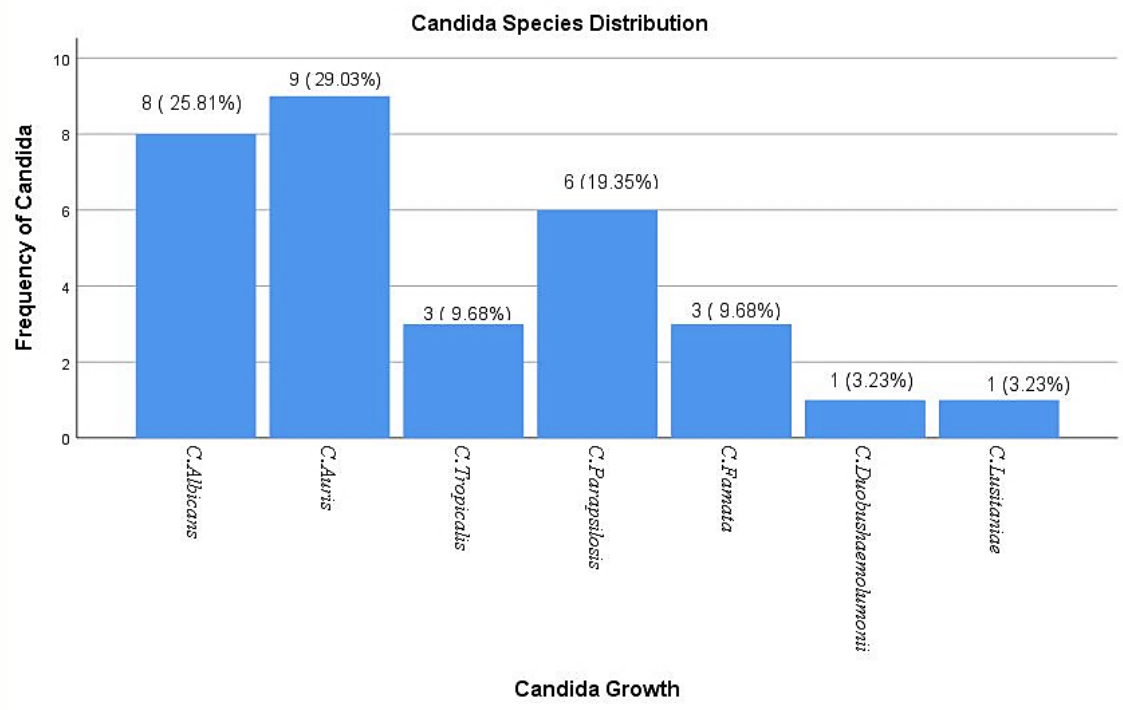


Figure 4.1: Distribution of *Candida* Species amongst the study population.

4.3. Distribution of *Candida* species based on age, gender and ward

An overall higher prevalence was observed in ICU compared with HDU. In patients under the age of 20 years, there were two positive cases in male patients of HDU. In patients between the age of 20- 40, there were four positive cases, three being among the male patients and 1 in the female patient of ICU. A total of seven patients (male and female) were positive for *Candida* species in ICU in the age group of 41-60 while only two male patients were positive in HDU. The most noteworthy prevalence was seen in patients above the age of 60 with females having 29.2% (7) and male patients having an incidence of 25% (6) in ICU. In HDU the prevalence for males was 28.5% (2) and females 14.3% (1) (***Table 4.2.***). A chi-square test on both sides showed there was no significant association of the prevalence results with age, gender, and ward giving a p-value of .543, .401, .436 respectively.

Table 4.2. Distribution of *Candida* Species based on Age, Ward, and Gender

ICU								
Age Group (Years)	**<20**		**20 – 40**		**41 – 60**		**>60**	
Gender	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Organisms								
Candida auris	0 (0%)	0 (0%)	1 (4.2%)	1 (4.2%)	0 (0%)	0 (0%)	0 (0%)	4 (16.6%)
Candida albicans	0 (0%)	0 (0%)	1 (4.2%)	0 (0%)	0 (0%)	2 (8.3%)	2 (8.3%)	2 (8.3%)
Candida parapsilosis	0 (0%)	0 (0%)	1 (4.2%)	0 (0%)	2 (8.3%)	0 (0%)	2 (8.3%)	0 (0%)
Candida tropicalis	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (4.2%)	1 (4.2%)
Candida famata	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2 (8.3%)	0 (0%)	1 (4.2%)	0 (0%)
Candida duobushaemolumonii	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Candida lusitaniae	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (4.2%)	0 (0%)	0 (0%)
Candida auris	0 (0%)	0 (0%)	1 (4.2%)	1 (4.2%)	0 (0%)	0 (0%)	0 (0%)	4 (16.6%)

HDU				
Age Group (Years)	**<20**	**20 – 40**	**41 – 60**	**>60**
Gender	**M**	**F**	**M**	**F**
Organisms				
Candida auris	1 (14.3%)	0 (0%)	0 (0%)	0 (0%)
Candida albicans	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Candida parapsilosis	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Candida tropicalis	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Candida famata	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Candida duobushaemolumonii	1 (14.3%)	0 (0%)	0 (0%)	0 (0%)
Candida lusitaniae	0 (0%)	0 (0%)	0 (0%)	0 (0%)

4.4. Antifungal Susceptibility of Isolated *Candida* Species

Antifungal Drug Susceptibility tests were carried out on 31 isolates. The lowest MIC value was observed in Echinocandins, Micafungin at 0.023 µg/mL for *Candida famata* while the highest MIC value obtained was in Fluconazole resistance at 256 µg/mL and was observed in *Candida auris* and *Candida parapsilosis* strains. The test results were interpreted based on the classification of CLSI breakpoints (Cantón & Peman, 2009).

4.4.1. Antifungal Susceptibility Patterns of Azoles and Flucytosine against isolated *Candida* Species

Out of 31 isolates *candida* tested for antifungal susceptibility to Fluconazole, 16 (51.61%) isolates showed sensitive results with varied MIC results ranging from 0.5-4µg/ml. Fifteen (48.39%) isolates were found resistant with MIC range from >64-256µg/ml. The effectiveness of Fluconazole was decreased in tested species of *Candida auris* and *Candida parapsilosis* with all isolates presenting MIC value >64 µg/ml **(*Table 4.3.*)**. All tested isolates of *Candida* species isolates in this study were sensitive to Voriconazole with a minimum MIC range of 0.047µg/ml and a maximum of 1.5µg/ml. A total of 28 (90.32%) *Candida* species were found susceptible to Flucytosine with varied MIC ranging from 0.5µg/ml to 1.5µg/ml while 3(9.68%) were found to be resistant with all of them showing a MIC value >32µg/ml (***Table 4.3.***).

4.4.2. Antifungal Susceptibility Patterns of Echinocandins and Amphotericin B Against Isolated *Candida* species

All the *Candida* isolates tested for Micafungin drug susceptibility showed a sensitive range of MIC with the minimum value obtained being 0.023µg/ml and the highest MIC value obtained was 1µg/ml. Similarly, Caspofungin also showed a highly effective performance with 31(100%) of the isolates showing sensitive antifungal drug susceptibility results with <1.5 µg/ml MIC values. The MIC of Caspofungin towards the *Candida* species of this study ranged from 0.012µg/ml-1µg/ml (***Table 4.3.***). Amphotericin B showed a high potency as an antifungal drug in this study. A total of 31(100%) of the isolated *Candida* species were found sensitive to Amphotericin B with varied MIC values ranging from 0.047µg/ml-1µg/ml ***(Table 4.3.)***.

Table 4.3:Anti-fungal Susceptibility Results and Minimum Inhibitory Concentration (MIC) Range Against Six Antifungal Drugs as Evaluated by the Liofilchem MIC Test Strip and Distribution of Isolates of *Candida spp*.

Candida Species	**No. (%) Sensitivity Profile**			
	Total (%)	**MIC Range (µg/mL)**	**Sensitive (%)**	**Resistant (%)**
Candida auris	**9 (29.03%)**			
Fluconazole		>64-256	0	9 (100%)
Voriconazole		0.19-1.5	9 (100%)	0
Caspofungin		0.19-1	9 (100%)	0
Micafungin		0.06-1	9 (100%)	0
Amphotericin-B		0.064-0.75	9 (100%)	0
Flucytosine		0.50-1	9 (100%)	0
Candida albicans	**8 (25.81%)**			
Fluconazole		0.5-4	8 (100%)	0
Voriconazole		0.125-1	8 (100%)	0
Caspofungin		0.012-0.94	8 (100%)	0
Micafungin		0.06-1	8 (100%)	0
Amphotericin-B		0.25-0.50	8 (100%)	0
Flucytosine		1-1.5	8 (100%)	0
Candida tropicalis	**3 (9.68%)**			
Fluconazole		1-1	3 (100%)	0
Voriconazole		0.125-0.75	3 (100%)	0
Caspofungin		0.016-0.25	3 (100%)	0
Micafungin		0.50-0.75	3 (100%)	0
Amphotericin-B		0.38-0.50	3 (100%)	0
Flucytosine		>32	0	3 (100%)
Candida famata	**3 (9.68%)**			
Fluconazole		0.50-1	3 (100%)	0
Voriconazole		0.094-1	3 (100%)	0
Caspofungin		0.25-1	3 (100%)	0
Micafungin		0.023-0.38	3 (100%)	0
Amphotericin-B		0.047-0.5	3 (100%)	0
Flucytosine		0.38-1	3 (100%)	0
Candida parapsilosis	**6 (19.35%)**			
Fluconazole		64-256	0	6 (100%)
Voriconazole		0.047-1	6 (100%)	0
Caspofungin		0.50-1	6 (100%)	0
Micafungin		0.25-1	6 (100%)	0
Amphotericin-B		0.25-1	6 (100%)	0
Flucytosine		0.75-1	6 (100%)	0
Candida Lusitaniae	**1 (3.23%)**			
Fluconazole		0.5	1 (100%)	0
Voriconazole		0.125	1 (100%)	0
Caspofungin		0.12	1 (100%)	0
Micafungin		0.06	1 (100%)	0
Amphotericin-B		0.25	1 (100%)	0
Flucytosine		1	1 (100%)	0
Candida Duobushaemolumonii	**1 (3.23%)**			
Fluconazole		32	1 (100%)	0
Voriconazole		1	1 (100%)	0
Caspofungin		0.25	1 (100%)	0
Micafungin		0.12	1 (100%)	0
Amphotericin-B		0.25	1 (100%)	0
Flucytosine		1	1 (100%)	0

Classification of sensitive and resistant according to the rules of the Clinical and Laboratory Standards Institute M27-A3 (Cantón & Pemán, 2009): Fluconazole (S: 8 µg/mL; R: 64 µg/ml); Voriconazole (S: 1 µg/mL; R: 4 µg/mL); Flucytosine (S: 4 µg/mL; R: 32 µg/mL); Amphotericin B (S < 1 µg/mL; R >2µg/mL) ; Echinocandins (S< 2µg/ml, sensitive breakpoint only). S, sensitive; R, resistant

4.5. Gel Bands of PCR Products Verified with Agarose Gel Electrophoresis

The PCR products showed a clear band that parallels the DNA ladder with the desired size. The band size revealed the expected fragment sizes of approximately 1400 base pairs for *Candida auris*' ERG 11 gene, 900 base pairs for *Candida tropicalis*' FUR gene, and 1569 and 780 base pairs for *Candida parapsilosis*' ERG 11 gene (***Figure 4.2.***).

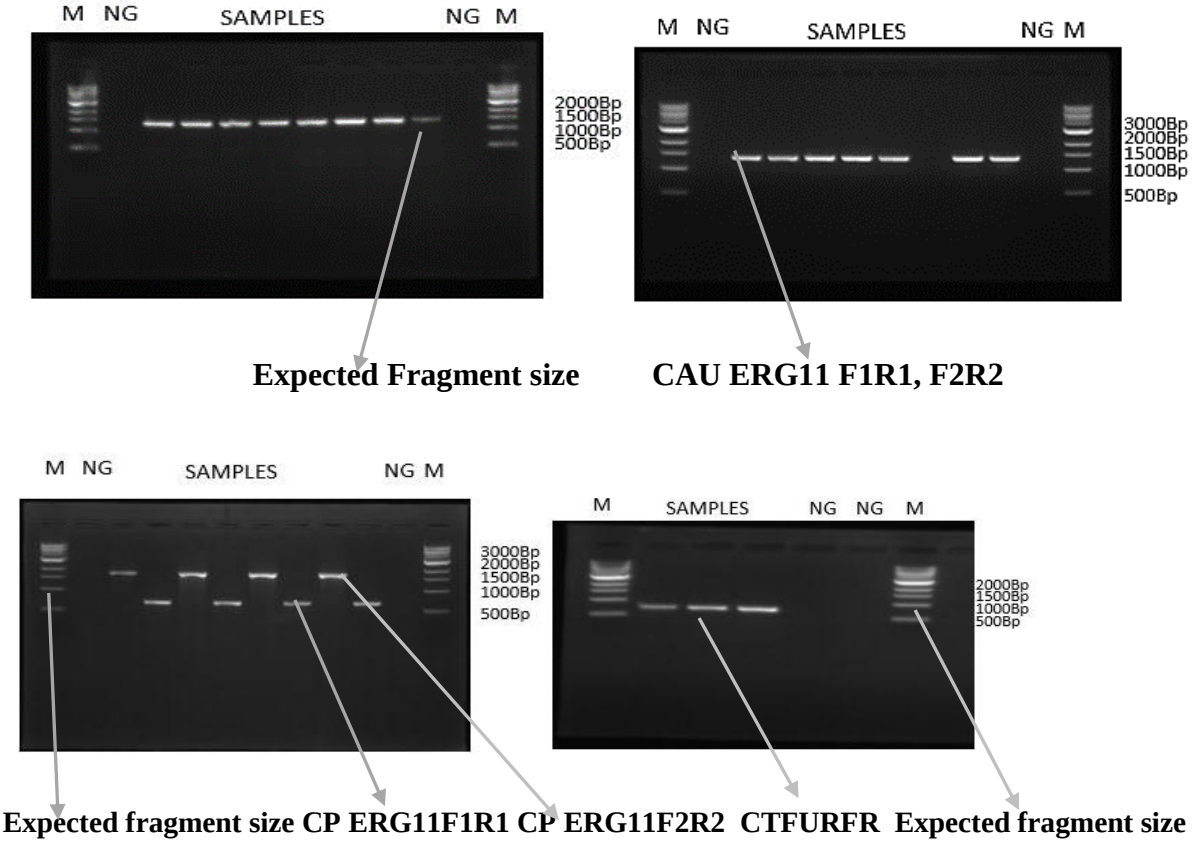


Figure 4.2: Gel Bands of Resistant *Candida* PCR Products verified using Agarose Gel Electrophoresis

Keywords: CAU: *Candida auris*, CP: *Candida Parapsilosis*, CT: *Candida Tropicalis*, ERG11: Ergosterol 11, FUR: Uracil phosphoribosyl transferase, F: Forward primer, R: Reverse primer, M: DNA ladder marker, NG: Negative Control

4.6. Sequence Results of Resistant *Candida* Isolates

4.6.1. Identified Single Nucleotide Polymorphisms

Multiple Sequence Alignment of ERG11 gene and FUR1 gene identified the presence of six Single Nucleotide Polymorphisms (***Table 4.4.***) namely; Erg11C374T, Erg11C376T, Erg11A395T, Erg11T729C, Erg11T1126A, FUR1C297T. These SNP contributes to four non-synonymous mutations (Erg11C374T, Erg11C376T, Erg11A395T, Erg11T1126A) and two synonymous substitutions (FUR1C297T, Erg11T729C) (***Figure 4.3.***)(***Table 4.4***).

Table 4.4: SNPs Identified in Resistant Strains of *Candida*

SNP	Ref	A.A Change	SNP Phenotype	Isolate count
Erg11C374T	C	A125V	Non-synonymous	1
Erg11C376T	C	L126F	Non-synonymous	1
Erg11A395T	A	Y132F	Non-synonymous	3
Erg11T1126A	T	L376V	Non-synonymous	1
Erg11T729C	T	Y243Y	Synonymous	1
FUR1C297T	C	C99C	Synonymous	3

Abbreviations: SNP: Single Nucleotide Polymorphisms, Ref: Reference Sequences, A.A: Amino acid, A: Alanine, V: Valine, L: Leucine, F: Phenylalanine, Y: Tyrosine, C: Cysteine

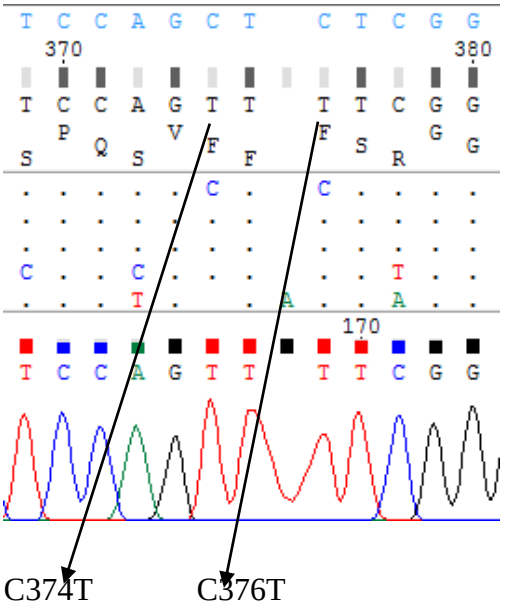


Fig. 4.3A

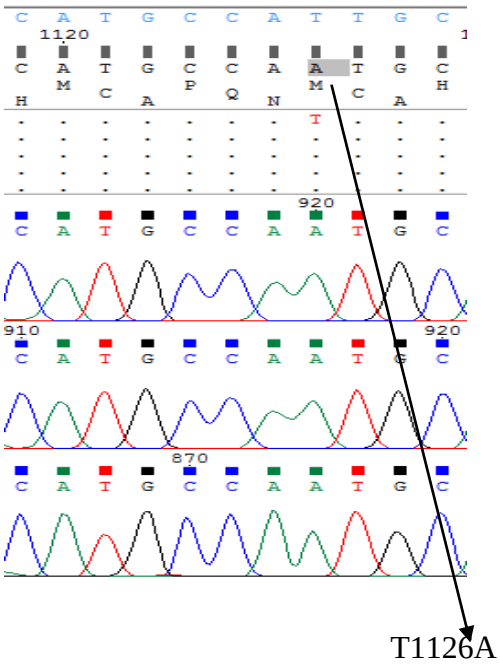


Fig 4.3B

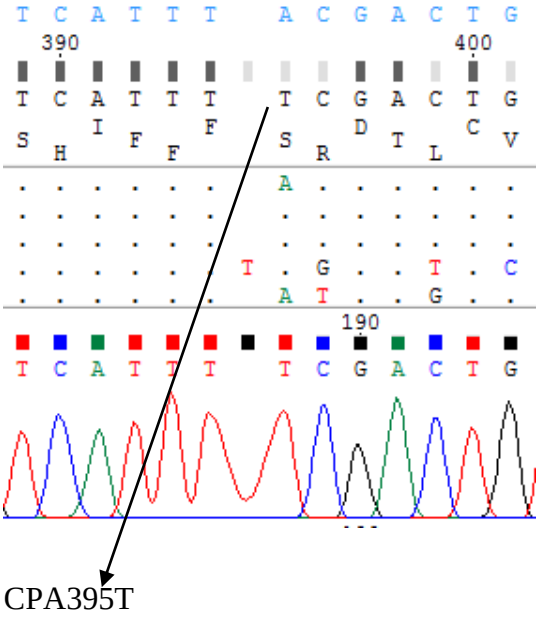


Fig 4.3C

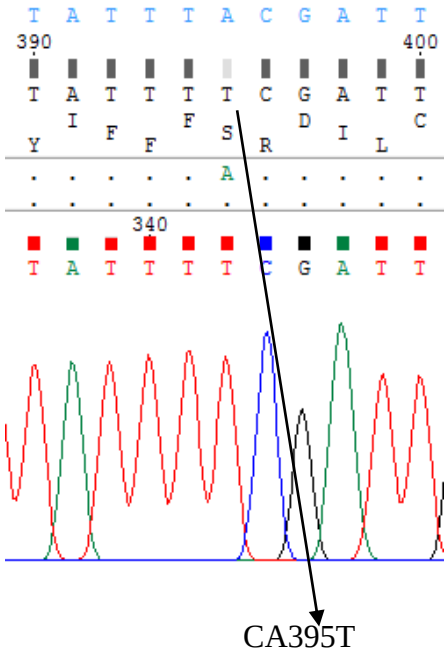


Fig 4.3D

Figure 4.3: Non-Synonymous Single Nucleotide Polymorphism's Chromatogram obtained by sequencing amplified and purified PCR products

Keywords: A: Adenine, T: Thymine, C: Cytosine, CP: *Candida Parapsilosis*, CA: *Candida Auris*

4.6.2. Amino Acid Substitutions of Resistant *Candida* Isolates and Gibbs Free Energy Gaps

Four amino acid changes were found in *Candida auris* Erg11 gene sequences resulting from non-synonymous nucleotide substitution. A125V, L126F, Y132F were observed in one isolate as multiple amino acid substitutions of one strain (***Figure 4.4.***). Additionally, another contig sequence of *Candida auris* resulted in one synonymous SNP (Erg11T729C) and single non-synonymous SNP resulting in the amino acid substitution of L376V. An amino acid substitution of Y132F was visualized in two isolates of *Candida parapsilosis'* Erg11 sequence (***Figure 4.4.)*** whereas, *Candida tropicalis* isolates had no amino acid in their FUR1 gene with obtained SNP being a synonymous substitution. Based on the calculation of free Gibbs Energy using STRUM (Quan *et al.*, 2016) three of the amino acid substitutions (L126F, Y132F, L376V) showed negative Free Gibbs energy gap with -1.23Kcal/mol, -0,53 Kcal/mol, and -1.61 Kcal/mol results respectively which indicates the destabilization of the predicted protein structure (***Table 4.5.)***. One of the amino acid substitution (A125V), however, showed a positive free Gibbs energy gap (0.22 Kcal/mol) indicating an increased stability of the protein structure (***Table 4.5.)***

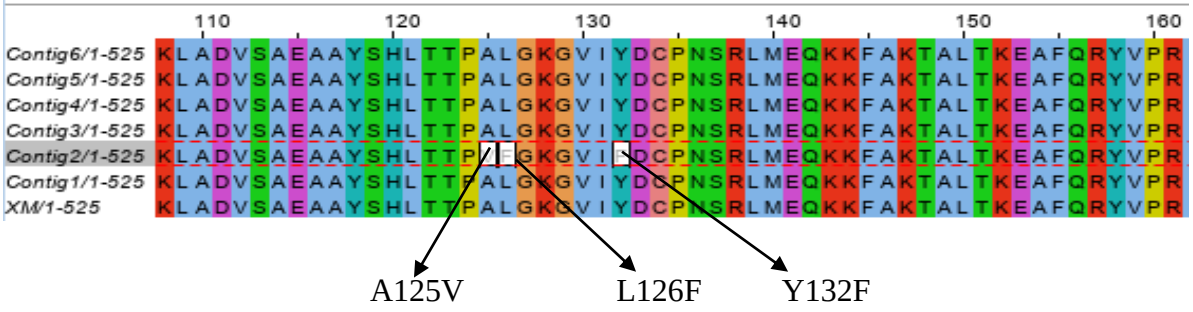
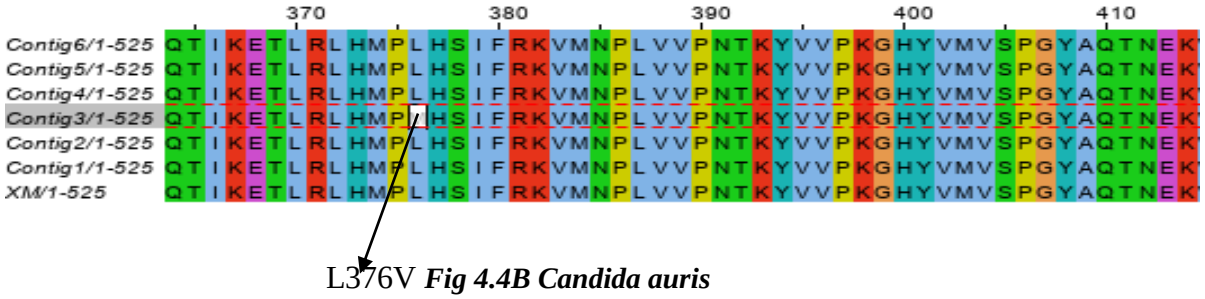


Fig 4.4A Candida auris



L376V ***Fig 4.4B Candida auris***

Y132F

Fig 4.4C: Candida Parapsilosis

Figure 4.4: Amino acid Substitutions in *Candida parapsilosis* and *Candida auris*

Table 4.5: Gibbs Free Energy Gaps (ddG) of nsSNPs in ERG11 for Prediction on the Effect of the Protein Structure

Amino Acid Position	Wild Type	Mutant Type	ddG (Kcal/mol)
125	A	V	0.22
126	L	F	-1.23
132	Y	F	-0.53
376	L	V	-1.61

Key Words: ddG: Change in Gibbs Energy, Binary Classification: ddG<0: Decrease Stability, ddG>0: Increase Stability, ddG=0 Neutral Stability

CHAPTER FIVE

DISCUSSION

5.1. Prevalence and Species Distribution of *Candida* Species

This study documents the prevalence of candidemia among patients admitted to the critical care unit of Nairobi Hospital, a tertiary care hospital in Nairobi, Kenya. The results showed a higher prevalence of 8.2% compared to a study previously conducted in Kenya showing a rate of 5 cases of candidemia per 100,000 patients (Guto *et al.*, 2016). The increased candidemia could be attributed to the number of invasive measures taken in hospitalized patients when handling their medical care and the immunocompromised state of the patients also plays a role in the severity of the infection. This ascent in prevalence demonstrates a public health challenge, especially to those who are hospitalized in ICU and HDU and have relatively severe illnesses.

Existing publications of *Candida* infections in Kenya states that the most frequently isolated *Candida* species was *Candida albicans* (Kangogo *et al.*, 2011; Ooga, 2011; Subira *et al.*, 2018), however, this study found, *Candida auris* in higher prevalence. The former studies conducted, however, looked at various clinical sources like swabs, urine samples, but limited research was done for candidemia in previously conducted studies in Kenya (Kangogo *et al*., 2011; Ooga, 2011; Subira *et al.*, 2018). Moreover, different techniques were used in each of the former studies and the usage of API 20C AUX for identifying the organisms was only utilized in this study. *Candida auris* was first isolated in 2009 and recorded cases are portrayed by elevated levels of total mortality (Cortegiani *et al.*, 2018; Rudramurthy *et al.*, 2017; Sekyere, 2018) and high antifungal resistance rates (Ronen Ben-Ami *et al.*, 2018). Of note, the vast majority of the infections reported involved patients that are critically ill (Chowdhary *et al.*, 2016; Rudramurthy *et al.*, 2017). Furthermore, identification has been difficult by microbiological techniques (Cortegiani *et al.*, 2018; Kathuria *et al.*, 2015; Kim *et al.*, 2016) notwithstanding *Candida auris* being one of the most prevalent species which increases the risk of misidentification and hence improper treatment. *Candida auris*'s high virulence (Fakhim *et al.*, 2018; Sarma Upadhyay, 2017), a profile of multi-drug

resistance (Sharma *et al.*, 2016), and rapid outbreak and global propagation marks it as a worldwide threat (Cortegiani *et al.*, 2018).

5.2. Antifungal Susceptibility Patterns of *Candida* Isolates

The antifungal susceptibility tests performed showed a susceptible profile of all isolates towards Echinocandins, Amphotericin B, and Voriconazole. As evidenced in certain reports *Candida* species are usually susceptible to Amphotericin-B as an antifungal drug (Bassetti *et al.*, 2011), and the resistance to Echinocandins is in very low percentages to *Candida albicans* and non-*albicans* except for *Candida glabrata* (Perlin, 2015). Nevertheless, a study conducted in Mombasa county in Kenya demonstrated resistance patterns to Amphotericin-B (15.2%) and slight resistance patterns in Caspofungin (3%), Micafungin (3%), and Voriconazole (6.1%) (Subira *et al.*, 2018). Furthermore, resistance to Fluconazole in *Candida albicans* has been documented in several instances in Kenya. A study conducted in Kenya by Ooga *et.al*. showed a resistance pattern of *Candida albicans* towards Fluconazole at a rate of 26% (Ooga, 2011) and another study by Subira *et.al*. indicated mild resistance patterns of *Candida albicans* especially in patients with HIV (Subira *et al.*, 2018). Interestingly, there was no *Candida albicans* isolate that was resistant to any of the anti-fungal drugs used in this study. Aware of the latter assertion, the increased susceptibility of *Candida albicans* might indicate the enhancement of drug regime towards this species as research was done massively targeting it in Kenya (Bii *et al.*, 2009; Guto *et al.*, 2016; Kangogo *et al.*, 2011).

An increased resistance pattern in three species was observed in this study. *Candida auris* was seen to have a high resistance pattern up to 256 µg/ml for fluconazole indicating no susceptible isolate from all the 9 isolates tested against fluconazole. Several studies have documented the antifungal resistance of *Candida auris* towards fluconazole (Adam *et al.*, 2019; Cortegiani *et al.*, 2018; Navalkele *et al.*, 2017) ,and the power of *Candida auris* to establish uniform resistance to fluconazole might increase the mortality rates of immunocompromised patients (Cortegiani *et al.*, 2018; Navalkele *et al.*, 2017). This study additionally acquired a drug resistance pattern towards Flucytosine by *Candida tropicalis* with 3(100%) isolates showing increased MIC

values as was classified resistant by CLSI breakpoints. A study conducted in Paris indicated the increased resistant activity of *Candida tropicalis* towards Flucytosine (Desnos-Ollivier *et al.,* 2008), however, this has not been documented in Kenya previously. Despite certain reports expressing *Candida parapsilosis* as frequently susceptible to azoles (Eksi *et al.,* 2013; Lotfali *et al.,* 2016), several pieces of research have indicated its increased resistance towards Fluconazole (Cantón & Peman, 2009; Cleveland *et al.,* 2012; Souza *et al.,* 2015). In this study, all isolated *Candida parapsilosis* were found to be resistant to Fluconazole. The increased resistance of the species predisposes a high risk to hospitalized patients as *Candida parapsilosis* is an easily transmitted species upon the usage of contaminated medical devices or via health care workers (Cleveland *et al.,* 2012; Souza *et al.,* 2015)

The drug susceptibility results exhibited a resistance pattern in the entirety of the isolated species of *Candida parapsilosis, Candida auris,* and *Candida tropicalis* towards Fluconazole in the former two species and to Flucytosine in the latter. Critically ill patients face the threat of morbidity and mortality as a result of the wide use of broad-spectrum anti-fungal drugs where there is a development of resistance by *Candida* species (Cortegiani *et al.,* 2018). Inherent causes of resistance are also noted on top of the usual acquired pattern of drug resistance after antifungal drugs are administered to patients (Cortegiani *et al.,* 2018; Mandelblat *et al.,* 2017). This intrinsic nature of resistance is usually seen in *non-albicans,* as is documented in this study. Despite the fact that the onset of drug resistance happening endogenously in several cases the pattern of complete resistance in all three species might show transmission of the *Candida* species among the patients. Infection control in patients with *Candida* has never been a challenge previously since it was comprehended that *Candida* infections derive essentially from the translocation of host flora to usually sterile locations, such as the bloodstream. Similar patterns of resistance, however, may suggest that the same strains circulate across the patients attributed to hospitalized patients' transmission as is confirmed by numerous studies (Schelenz *et al.,* 2016; Schwartz *et al.,* 2018; Ture & Alp, 2018).

5.3. Sequences of Resistant Isolates of *Candida* Species

Sequencing of isolates provides the genetic analysis of isolates for a better understanding of the mechanism of resistance. In this study, resistances were observed in Fluconazole and Flucytosine whereby studies have explained the main causes of resistance are attributed to mutations in Erg11 and FUR1 genes respectively (Chowdhary *et al.*, 2018; Lockhart *et al.*, 2016; M.Cuenca-Estrella *et al.*, 2001; Marie *et al.*, 2008). The output of the multiple sequence alignment in this study obtained four non-synonymous single nucleotide polymorphisms resulting in amino acid substitutions in the Erg11 gene. The amino acid substitution Y132F has been previously elucidated in India, Pakistan and Venezuela (Chowdhary *et al.*, 2018; Lockhart *et al.*, 2016) resulting in protein function change and thus accrediting to azole resistance. However, *Candida auris* in Africa have not been documented with this amino acid substitution to present as studies show the amino acid substitution observed in South Africa was F126T rather than Y132F (Lockhart *et al.*, 2016). However, the results of this study document Y132F amino acid substitution in two isolates of *Candida parapsilosis* and one isolate of *Candida auris* marking it as the first observation in Africa to the best of our knowledge. Additionally, three amino acid substitutions namely; A125V, L126F, L376V which have never been documented before were found in this study. The presence of the former two amino acid substitutions in the hot spot regions of Erg11 predisposes the isolates to resistance towards fluconazole. Studies have shown the resistance towards azoles are mainly attributed to mutations in the three hotspot regions of Erg 11 denoting the amino acids from 105 to 165, 266 to 287, and 405 to 488 which are the active site of the protein and might bring about a functional change resulting in resistance of the strain (Flowers *et al.*, 2015). L126F and L376V exhibited a negative free Gibbs energy gap destabilizing the protein structure and the presence of the former amino acid substitution in in the hot spot region of Erg11 on top of its destabilizing effect on the protein makes it a priority in causing fluconazole resistance. If the above mutations have a role in the fluconazole resistance, the possible mechanism of resistance is the absence of inhibiting activity of 14-α-sterol demethylase enzyme due to mutation in the ERG11 gene. L376V, however, was observed outside the hotspot regions and therefore, the negative Gibbs free energy gaps value cannot be conclusive of its cause in

Fluconazole resistance. However, further analysis on its attribution towards resistance could prompt to distinguish the presence of additional hotspot regions previously overlooked. In contrary, the amino acid substitution A125V had a stabilizing effect on the protein structure with positive Gibbs free energy gaps value. All of the amino acid substitutions recorded in this study exhibited a conservative change with changes of the amino acids being from neutral, non-polar amino acid side chains to neutral, non-polar amino acids. Regardless, their implication on the resistance towards fluconazole remains high as previously explained mutation K143R (Lockhart *et al.*, 2016) which contributed to resistance in Fluconazole also revealed a change from polar to polar amino acid chains. Moreover, the effect of the mutation in resistance depends on several other factors like position of the mutation and its effect on stability of the protein structure as explained above.

Conversely, two resistant isolates of *Candida parapsilosis* demonstrated a complete match to the reference sequence with accession number (MK513971) whereas four strains of *Candida auris* presented an identical sequence of Erg11 with the reference sequence (XM_029033208) used in this study. The manifestation of strains with no mutation in the Erg11 gene expresses the presence of a mutation in other genes of *Candida* associated with fluconazole resistance. Even though several studies indicate the main mechanism of fluconazole resistance in *Candida parapsilosis* and *Candida auris* originate from mutations in Erg11 (Berkow & Lockhart, 2017; Chowdhary *et al.*, 2018; Khan *et al.*, 2018; Sarma & Upadhyay, 2017; Souza *et al.*, 2015; Xiang *et al.*, 2013), there are also certain researches which feature the resistance of fluconazole to other factors like overexpression of CDR1&2 genes as well as MDR gene (Berkow & Lockhart, 2017). Moreover, with occasional occurrences, a loss of function mutation in Erg3 might also attribute to fluconazole resistance (Berkow & Lockhart*et al.*, 2017). Thus, the alternative possibilities of fluconazole resistance as opposed to the frequently observed mechanism of fluconazole resistance by point mutations in Erg11 could stand as the cause of resistance for the isolates observed with no mutation in their Erg11 gene.

The isolates of *Candida tropicalis* in this study showed a synonymous single nucleotide polymorphism resulting in no alteration of amino acids when aligned with a reference sequence (XM_002548346) from the Gene bank. The presence of synonymous SNP

with no amino acid substitution indicates, the possible cause of resistance of the isolates lies in other genes rather than FUR1. Even though scarce publications are existing in *Candida tropicalis'* mechanisms of Flucytosine resistance, a study conducted in Philadelphia, Pennsylvania documented mutations in FCY1 and FCY2 on top of the usually seen mutations of FUR1 as a cause for Flucytosine resistance in *Candida glabrata* and they underlined the study as a framework for understanding resistance to Flucytosine in other *Candida* and fungal species as well (Edlind & Katiyar, 2010).

The multiple sequences of the isolates resulting in a significant match with each other raise a concern of hospital spread of similar strains of *Candida*. With the frequent usage of invasive medical devices, critical care unit patients are easily liable to the transmission of hospital-acquired *Candida* infections and the exogenous causes of *Candida* infection are opting to be greater than the endogenous mechanisms of infection. Moreover, an epidemiological analysis of resistance in *Candida auris* on three continents second the above speculation as their results documented a possible outcome of hospital spread of *Candida auris* in agreement with the other *Candida* species infection (Lockhart *et al.*, 2016).

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1. Conclusion

1. There is an increase in candidemia cases in the critical care units of Nairobi Hospital and dependent on the outcomes acquired, the study determined an exceptionally pervasive occurrence of new *Candida* species, *Candida auris,* and its drug-resistant property.
2. Additionally, Echinocandins and Amphotericin-B showed high potency as antifungal drugs against *albicans* and non-*albicans* whereas Fluconazole and Flucytosine were proved to be less effective drugs in the conspicuously seen species of *Candida auris, Candida parapsilosis, and Candida tropicalis.*
3. Sequence analysis of the isolated resistant strains confirmed a previously recorded amino acid substitution marking the visualization, however, as the first time in Kenya and possibly the region of Africa. The study also documented three new amino acid substitutions which have not been described previously and two of those substitutions exhibited a destabilizing effect on the predicted protein structure.

6.2. Recommendation & Limitation

The increased prevalence of candidemia and the occurrence of new *Candida* species is a high threat to the public. Thus, measures ought to be taken to control the infection and transmission to diminish the mortality and morbidity of critically ill patients. Moreover, limited studies are present on Candidemia in Kenya and other researchers should explore that topic more in the future for successful treatment for this systemic infection. Consequently, for an appropriate drug regime, physicians should be alert of the drug susceptibility patterns of *Candida* species in the locality before prescribing any of the anti-fungal drugs as variations in susceptibilities might be presents in different counties. With the onset of new species and increased drug resistance patterns warrant constant drug monitoring.

As the study was limited to Nairobi Hospital, researches of *Candida* bloodstream infections in critical care units should be done in hospitals in other hospitals in Nairobi and other counties of Kenya for updated prevalence study and to monitor the spread of *Candida auris* in the country. Moreover, the presence of substantial drug resistance and new amino acid substitutions insinuates researchers to intensively explore the existing mutations regularly for the provision of proper and empirical drugs to patients. Additionally, other mechanisms of resistance as explained above should also be established in future studies for more effective treatment.

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APPENDICES

Appendix I. Demography of Patients with *Candida* Isolates

Patient ID	Age	Gender	Ward	Organism Name
1	82	F	ICU	*C.albicans*
2	82	F	ICU	*C.auris*
3	75	F	ICU	*C.albicans*
4	72	F	ICU	*C.auris*
5	94	M	ICU	*C.famata*
6	71	M	ICU	*C.parapsilosis*
7	18	M	HDU	*C.duobushaemolumonii*
8	44	M	HDU	*C.tropicalis*
9	34	M	ICU	*C.parapsilosis*
10	37	M	ICU	*C.auris*
11	18	M	HDU	*C.auris*
12	42	F	ICU	*C.albicans*
13	61	F	ICU	*C.auris*
14	73	M	ICU	*C.parapsilosis*
15	36	F	ICU	*C.auris*
16	47	M	ICU	*C.parapsilosis*
17	40	M	ICU	*C.albicans*
18	60	M	HDU	*C.auris*
19	62	M	ICU	*C.albicans*
20	41	M	ICU	*C.famata*
21	79	F	ICU	*C.tropicalis*
22	50	M	ICU	*C.famata*
23	66	F	ICU	*C.auris*
24	50	F	ICU	*C.albicans*
25	78	M	HDU	*C.albicans*
26	50	F	ICU	*C.lusitaniae*
27	72	M	ICU	*C.albicans*
28	81	M	ICU	*C.tropicalis*
29	70	M	HDU	*C.auris*
30	51	M	ICU	*C.parapsilosis*
31	61	F	HDU	*C.parapsilosis*

KEYWORDS
HDU: Highly Dependent Unit
ICU: Intensive Care Unit

Appendix II: Interpretive Standards of Liofilchem MIC Test Strip as Per Standards of CLSI

ANTIFUNGAL DRUG	RANGE OF MIC PROFILE	SENSITIVE	RESISTANT
Fluconazole	0.016-256mg/l	<8µg/ml	>64µg/ml
Voriconazole	0.02-32mg/l	<1 µg/ml	>4 µg/ml
Caspofungin	0.02-32mg/l	<2 µg/ml	>2 µg/ml
Micafungin	0.02-32mg/l	<2 µg/ml	>2 µg/ml
Amphotericin-B	0.02-32mg/l	<1 µg/ml	>2 µg/ml
Flucytosine	0.02-32mg/l	<4 µg/ml	>32 µg/ml

Appendix III: Minimum Inhibitory Concentration Profile in Liofilchem-Test

Organism Name	**AMP- B**	**CAS**	**FC**	**FLU**	**MYC**	**VRC**
C.albicans	0.5	0.25	1	4	0.06	1
C.auris	0.25	0.25	0.5	64	1	1.5
C.albicans	0.25	0.25	1.5	1	0.06	0.125
C.auris	0.064	0.5	1	256	0.047	0.5
C.famata	0.5	0.25	1	0.5	0.38	0.75
C.parapsilosis	1	1	1	>64	0.5	0.047
C.duobushaemolumonii	0.25	0.25	1	32	0.12	1
C.tropicalis	0.75	0.25	>32	1	0.5	0.125
C.parapsilosis	0.5	1	1	64	1	0.25
C.auris	0.25	0.19	0.75	256	0.125	0.19
C.auris	0.75	1	1	256	0.38	1
C.albicans	0.5	0.012	1	2	0.094	0.5
C.auris	0.19	0.5	1	96	0.25	1
C.parapsilosis	0.5	0.75	1	256	0.5	0.25
C.auris	0.25	0.125	0.5	256	0.19	1
C.parapsilosis	0.5	0.5	1	64	1	1
C.albicans	0.25	0.125	1.5	0.5	0.032	0.19
C.auris	0.125	1	1	>64	0.125	0.75
C.albicans	0.5	0.94	1	4	0.19	0.25
C.famata	0.047	0.5	0.38	1	0.023	0.094
C.tropicalis	0.5	0.125	>32	1	0.75	0.75
C.famata	0.5	1	1	0.5	0.25	1
C.auris	0.125	0.75	0.5	256	0.25	1.5
C.albicans	0.25	0.38	1	1	1	0.5
C.albicans	0.5	0.5	1	0.5	0.5	1
C.lusitaniae	0.25	0.12	1	0.5	0.06	0.125
C.albicans	0.25	0.25	1.5	1	0.06	0.125
C.tropicalis	0.5	0.016	>32	1	0.016	0.5
C.auris	0.094	1	1	64	0.06	0.75
C.parapsilosis	0.5	0.5	1	64	0.5	1
C.parapsilosis	0.25	1	0.75	256	0.5	0.5

KEYWORDS
AMP-B: Amphotericin-B
FC: Flucytosine
CAS: Caspofungin
FLU: Fluconazole
MYC: Micafungin
VOR: Voriconazole

Appendix IV: Concentration of Genomic DNA Using Nanodrop Spectrophotometer

Patient code	Organism name	Concentration (µg/ml)	260nm/280nm
1	*C.auris*	18.475	1.751
2	*C.auris*	13.028	1.88
3	*C.auris*	13.892	2.061
4	*C.auris*	265.15	1.586
5	*C.auris*	63.194	1.919
6	*C.auris*	39.111	1.923
7	*C.auris*	25.013	1.959
8	*C.auris*	36.534	1.905
9	*C.auris*	67.293	1.897
10	*C.tropicalis*	10.519	1.769
11	*C.tropicalis*	16.123	1.863
12	*C.tropicalis*	32.452	1.923
13	*C.parapsilosis*	154.79	1.903
14	*C.parapsilosis*	124.06	1.882
15	*C.parapsilosis*	135.09	1.992
16	*C.parapsilosis*	122.86	1.873
17	*C.parapsilosis*	135.36	1.919
18	*C.parapsilosis*	151.45	1.899

Appendix V. Publication of Two Objectives

Scientific Research Publishing

Open Journal of Medical Microbiology, 2021, 11, 32-46
https://www.scirp.org/journal/ojmm
ISSN Online: 2165-3380
ISSN Print: 2165-3372

Prevalence, Species Distribution and Antifungal Susceptibility Profile of *Candida* Species Isolated from Bloodstream of Critical Care Unit Patients in a Tertiary Care Hospital in Kenya

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How to cite this paper: Solomon, D.A., Nyerere, A.K., Kanyua, A. and Ngugi, C.W. (2021) Prevalence, Species Distribution and Antifungal Susceptibility Profile of *Candida* Species Isolated from Bloodstream of Critical Care Unit Patients in a Tertiary Care Hospital in Kenya. *Open Journal of Medical Microbiology*, 11, 32-46.
https://doi.org/10.4236/ojmm.2021.111003

Received: January 30, 2021
Accepted: March 8, 2021
Published: March 11, 2021

Abstract

The upsurge of candidemia in the past years has been an immense encumbrance on public health and the number of deaths caused by candidemia particularly in critical care unit patients is devastating. *Candida* species harbor a 30% - 60% mortality rate and compared to stable people or those with less serious illnesses, this ranges from 60% to 80% of those who are chronically ill patients. Grounded on a recent report from a tertiary care hospital in Kenya showing the emergence of previously unobserved species: *Candida auris*, this study aimed to determine the prevalence, species distribution, and antifungal susceptibility profile of candidemia in critical care unit patients of the hospital. 378 Critical Care Unit patients were enrolled for the study from January 2019 to January 2020. Positive archived isolates were sub-cultured using Saboraud Dextrose Agar. *Candida* species were identified utilizing API20C AUX and Vitek-2. Antifungal susceptibility testing was conducted using the Liofilchem MIC Test strip. Out of 378 patients, thirty-one presented a positive culture for *Candida* species. The prevalence of Candidemia was **8.2%** with **9 (29.03%)** *Candida auris*, **8 (25.81%)** *Candida albicans*, **6 (19.35%)** *Candida parapsilosis*, **3 (9.68%)** *Candida famata*, **3 (9.68%)** *Candida tropicalis*, **1 (3.23%)** *Candida duobushaemolumonii*, and **1 (3.23%)** *Candida lusitaniae*. A resistance pattern to Fluconazole was observed among *Candida auris* and *Candida parapsilosis*, and resistance to Flucytosine was observed in *Candida tropicalis*, whereas susceptible MIC values were obtained for the other drugs. There is an increase in candidemia among critical care unit patients in the health facility posing a public health challenge. Moreover, the onset of new species *Candida auris* which is unprecedented in Kenya warrants enhanced infection control, and the uniform resistance of *Candida auris*, *Candida pa-*

rapsilosis, and *Candida tropicalis* towards Fluconazole and Flucytosine necessitate constant drug monitoring for empirical treatment regime. In contrast, the high potency of Echinocandins and Amphotericin-B demonstrate them as the drug of choice.

Keywords

Critical Care Unit, *Candida* Species, *Candida auris*, Candidemia, Antifungal Susceptibility

1. Introduction

Fungal infections, explicitly *Candida* species have been the major cause of mortality and morbidity in hospitalized patients and predominantly in the Critical Care Units [1] [2]. *Candida* species reside inside the host as normal flora and dwell in the oral cavity of healthy human beings, however, in immunocompromised patients, these commensal microorganisms are capable of causing disease [3]. Prominently causing nosocomial infections, *Candida* spp. rates the fourth in causing all bloodstream infections and third of bloodstream infections in critically ill patients [4]. Worldwide in tertiary care hospitals, those results attribute to mortality rates of 15% ± 35% in adults and 10% ± 15% in neonates [5]. Based on studies done in several hospitalized patients, including intensive care unit patients, candidemia causes mortality of 47% among the patients [6].

In the USA, *Candida* species are considered as the third or fourth most causative agent of healthcare-acquired infections [5] and lead to a 30% - 60% mortality rate of hospitalized patients [6]. These are associated with changes in the individuals' physiology and immunocompromised state which results in severe infections [3]. Several risk factors of the origin of the *Candida* infections in hospitalized patients could be endogenously brought by the patients themselves or could be from instruments in the hospital. It might also be contributed to contaminations of hospital surroundings or cross-infection from health workers which would attribute to the exogenous cause [5] [7] [8]. Candidemia is frequently seen among those infections, especially with patients in Critical Care Units [5]. Moreover, as a result of these infections, expanded costs in healthcare are incurred, ranging from $35,000 to $68,000 for a single candidemia case in the United States [8].

Patterns of anti-fungal drug resistance by *Candida* species have been causing stern public health challenges and were encompassed in CDC's 2013 Antibiotic Resistance Threat Report [9] [10]. Critically ill patients undergo invasive treatments and consume several anti-fungal drugs, however, results of anti-fungal resistance have been causing epidemiological unsustainability [11] [12]. Certain *Candida* strains are progressively resistant to commonly used antifungal drugs. Based on recent data from CDC, a discernable shift is observed in candidemia occurrences with augmented drug resistance to first-line and second-line anti-

fungal drugs such as Azoles and Echinocandins [10]. In the United States, 46,000 hospitalized patients face *Candida* infections each year, and approximately 30% of those who harbor drug-resistant *Candida* species are estimated to die during hospitalization [10]. Several reports state that the increase of anti-fungal resistance has been resulting in high mortality and morbidity in critical care patients [2] [13]. This has contributed to many deaths due to *Candida* infections in the past years [11] [12].

According to literature from Kenya, the significant agent for hospital infections is considered to be *Candida albicans* [14] [15]. However, *Candida auris* has recently been observed frequently in a tertiary care hospital in Nairobi, Kenya grounded on several lab reports from ICU and HDU patients. Swift dissemination of this multi-drug resistant species is discerned in different parts of the world [2]. It was first reported in 2009 and since then it has been detected in five continents causing serious hospital-acquired infections [2]. A study conducted in Kenya recorded a drug resistance pattern of *Candida albicans* and *Candida parapsilosis* isolates towards fluconazole [14] [16], however, drug susceptibility patterns of the novel species, *Candida auris* remains unreported up-to-date in Kenya. Therefore, the aim of this study was to determine the prevalence and antifungal susceptibility profile of *Candida* species among critical care unit patients of the hospital.

2. Materials and Methods

2.1. Study Area

The study was conducted in a tertiary care hospital in Nairobi, Kenya. The hospital has been officially operational since 9 April 1954 and is located on Argwings Kodhek Road, in the neighborhood of Upper Hill, in Kenya's capital city of Nairobi. The hospital is a high capacity hospital and serves different patients from the whole country [17].

2.2. Study Population

The study participants were enrolled from the intensive care unit and highly dependent unit of the hospital. The study population included patients of all age groups who had been requested for blood culture by a physician. A total of 378 patients based on Naing *et al.* (2006) [18] formula were tested from January 2019 to January 2020 for *Candida* bloodstream infection in ICU and HDU of Nairobi Hospital. Out of those thirty-one (31), patients showed positive culture for *Candida* and the isolates were archived.

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

where: n = Sample size.

Z = The confidence interval at 95% (1.96).

P = Estimate of the proportion or anticipated prevalence used 12.42% candidiasis from a population-based study in Africa [19].

d = margin of error at 5% (0.05).

$$n = \frac{Z^2(P)(1-P)}{d^2} = \frac{1.96^2(0.1242)(1-0.1242)}{0.05^2} = 167$$

2.3. Culture of Isolates

The archived isolates which had positive blood culture underwent a sub culturing method using Saboraud dextrose agar (SDA). Isolates that were stored in glycerol were inoculated in a Petri dish plate with a Saboraud Dextrose media and they were incubated in 37˚C for 24 - 48 hours. The appearance of creamy white colonies with soft texture was considered positive for *Candida* growth.

2.4. API 20C AUX and VITEK-2 Tests

A confirmatory test was done for the cultured isolates to identify the specific species using API 20C AUX (Biomerieux, USA). Isolated *Candida* colonies were inoculated in the API 20C cupules for positive or negative growth check in the 19 assimilation tests [20]. Incubation of the strip was done for 48 and 72 hours in 31˚C, after which the reactions were compared to the first cupule containing negative control. The numbers recorded were interpreted using apiweb™ software (Biomerieux, SA). Isolates that were identified as *Candida famata* by API 20C AUX (Biomerieux, USA) were confirmed using VITEK-2 version 8.1 (Biomerieux, USA) to differentiate whether they were truly *Candida famata* or they were misidentified *Candida auris* or *Candida duobushaemolumonii*. The test was done following the manufacturer's instructions for VITEK-2 (Biomerieux, USA) [21].

2.5. Antifungal Susceptibility Testing

Anti-fungal drug susceptibility was performed using Liofilchem MIC test strip (Liofilchem S.R.I., Italy) for Fluconazole, Micafungin, Caspofungin, Amphotericin-B, Flucytosine, and Voriconazole [22]. The Liofilchem MIC test strip (Liofilchem S.R.I., Italy) is a non-penetrating plastic material designed to have 15 two-fold dilutions of antibiotic concentrations [22]. The strip was placed in a Saboraud Dextrose Agar (SDA) inoculated with 0.5 McFarland pure colonies' suspension of the isolated species. Subsequently, the agar plates were incubated at 37˚C and observed for Minimum Inhibitory Concentration (MIC) reading after 24 hours. The interpretation was done classifying the organisms as sensitive or resistant based on CLSI MA-27 guidelines [23].

Quality control of the Identification and Antifungal Susceptibility tests in this study was maintained using reference strains of *Candida albicans* ATCC: 14053 and *Candida parapsilosis* ATCC 22019.

2.6. Data Analysis

Data collected were documented in Microsoft Excel (Microsoft Corp, USA) before transferring to SPSS version 20 (IBM, USA) for analysis. Descriptive Statis-

tics was done to determine the frequencies of the species distribution. Cross Tabulation was used to determine the Prevalence of *Candida* species in relation to age, ward, and gender. Chi-square was used for the level of significance. T-test was used to calculate the p-value association between HDU and ICU based on age, gender, and prevalence. Minimum and maximum susceptibility values were calculated for all the drugs tested to obtain the MIC range.

3. Results

3.1. Characteristics of Study Population

A total of 378 patients were tested from January 2019 to January 2020. The study population was patients admitted to ICU and HDU of the tertiary care hospital. Out of 378 patients, 203 were from ICU and 105 from HDU. The median age for ICU was 55 years (2 mon-95 years) and 61 years (16 - 101 years) in HDU with a p-value of 0.0534 (Table 1). The majority of the patients were males at 158 (57.9%) and 60 (57.1%), while females were 115 (42.1%) and 45 (42.9%) in ICU and HDU respectively. The prevalence of *Candida* infections was more prominent in ICU with a total of 24 (77.4%) positive cases, with HDU having a lower prevalence of 7 (22.6%) positive cases.

3.2. Prevalence and Distribution of *Candida* Species

Out of the 378 patients tested thirty-one (31) patients were found to have candidemia comprising **8.2%** of the total patients. The highest prevalence was *Candida auris at* 29.03% and the lowest prevalence in *Candida duobushaemolumonii* and *Candida lusitaniae*, at 3.23% each (Figure 1).

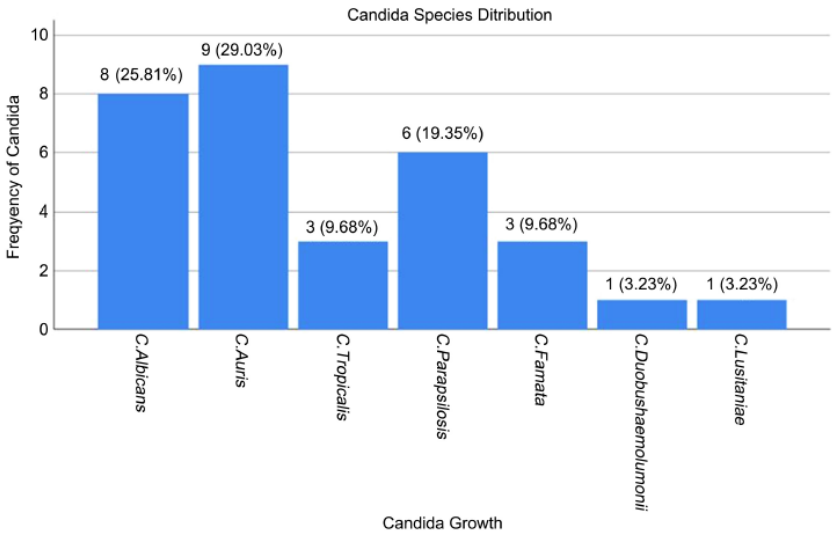

Figure 1. Distribution of *Candida* species from the API 20C AUX result.

An overall higher prevalence was observed in ICU compared with HDU. In patients under the age of 20 years, there were two positive cases in male patients of HDU. In patients between the age of 20 - 40, there were four positive cases, three being among the male patients and 1 in the female patient of ICU. A total of seven patients (male and female) were positive for *Candida* species in ICU in the age group of 41 - 60 while only two male patients were positive in HDU. The most noteworthy prevalence was seen in patients above the age of 60 with females comprising higher incidences of candidemia at 29.2% (7) while male patients had lesser prevalence at 25% (6) in ICU. In contrast, males had a higher occurrence at 28.5% (2) than females at 14.3% (1) in HDU (Table 2). A chi-square test on both sides showed there was no significant association of the prevalence results with age, gender, and ward giving a p-value of 0.543, 0.401 and 0.436, respectively.

Table 1. Characteristics of the study population.

		ICU	HDU	P-VALUE
Age				
Median (Range)		55 years (2 months - 95 years)	61 years (16 years - 101 years)	0.0534
Gender	**M**	158	60	0.908
	F	115	45	
Prevalence (%)		24 (77.4%)	7 (22.6%)	0.319

Table 2. Distribution of *Candida* species based on age, ward, and gender.

ICU								
Age Group (Years)	**<20**		**20 - 40**		**41 - 60**		**>60**	
Gender	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Organisms								
Candida auris	0 (0%)	0 (0%)	1 (4.2%)	1 (4.2%)	0 (0%)	0 (0%)	0 (0%)	4 (16.6%)
Candida albicans	0 (0%)	0 (0%)	1 (4.2%)	0 (0%)	0 (0%)	2 (8.3%)	2 (8.3%)	2 (8.3%)
Candida parapsilosis	0 (0%)	0 (0%)	1 (4.2%)	0 (0%)	2 (8.3%)	0 (0%)	2 (8.3%)	0 (0%)
Candida tropicalis	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (4.2%)	1 (4.2%)
Candida famata	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2 (8.3%)	0 (0%)	1 (4.2%)	0 (0%)
Candida duobushaemolumonii	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Candida lusitaniae	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (4.2%)	0 (0%)	0 (0%)
HDU								
Age Group (Years)	**<20**		**20 - 40**		**41 - 60**		**>60**	
Gender	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Organisms								
Candida auris	1 (14.3%)	0 (0%)	0 (0%)	0 (0%)	1 (14.3%)	0 (0%)	1 (14.3%)	0 (0%)
Candida albicans	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (14.3%)	0 (0%)
Candida parapsilosis	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1(14.3%)
Candida tropicalis	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (14.3%)	0 (0%)	0 (0%)	0 (0%)
Candida famata	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Candida duobushaemolumonii	1 (14.3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Candida lusitaniae	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

3.3. Antifungal Susceptibility of Isolated *Candida* Species

Antifungal Drug Susceptibility tests were accomplished for the 31 isolates against Fluconazole, Voriconazole, Micafungin, Caspofungin, Amphotericin B, and Flucytosine covering the three-drug classes of anti-fungal drugs. All *Candida* isolates in the study were sensitive to Micafungin, Caspofungin, Voriconazole, and Amphotericin B. However, *Candida auris* and *Candida parapsilosis* demonstrated complete resistance to Fluconazole whereas *Candida tropicalis* strains were resistant to Flucytosine (Table 3). The lowest MIC value was observed in Echinocandins, Micafungin at 0.023 µg/mL for *Candida famata*. Meanwhile, the highest MIC value obtained was in fluconazole resistance at 256 µg/mL and was observed in *Candida auris* and *Candida parapsilosis* strains. The test was conducted utilizing the Liofilchem MIC test strip and was interpreted based on the classification of CLSI breakpoints [23].

4. Discussion

This study documents the prevalence of candidemia among patients admitted to the critical care unit of a tertiary care hospital in Nairobi, Kenya. The results showed a higher prevalence of 8.2% compared to a study previously conducted in Kenya showing a rate of 5 cases of candidemia per 100,000 patients [15]. The increased candidemia could be attributed to the number of invasive measures taken in hospitalized patients when handling their medical care and the immunocompromised state of the patients also plays a role in the severity of the infection. This ascent in prevalence demonstrates a public health challenge, especially to those who are hospitalized in ICU and HDU and have relatively severe illnesses.

Existing publications of *Candida* infections in Kenya states that the most frequently isolated *Candida* species was *Candida albicans* [14] [16], however, this study highlights the onset of the new species, *Candida auris* in high prevalence. *Candida auris* was first isolated in 2009 and recorded cases are portrayed by elevated levels of total mortality [2] [24] [25] and high antifungal resistance rates [26]. Of note, the vast majority of the infections reported involved patients that are critically ill [24] [27]. Furthermore, identification has been difficult by microbiological techniques [2] [28] [29] notwithstanding *Candida auris* being one of the most prevalent species which increases the risk of misidentification and hence improper treatment. *Candida auris*'s high virulence [30] [31], a profile of multi-drug resistance [32], and rapid outbreak and global propagation marks it as a worldwide threat [2].

The antifungal susceptibility tests performed showed a susceptible profile of all isolates towards Echinocandins and Amphotericin B and Voriconazole. A study conducted in a tertiary care hospital in Italy, however, showed a resistance pattern of *Candida albicans* towards Voriconazole [33]. Besides, as evidenced in certain reports *Candida* species are usually susceptible to Amphotericin-B as an antifungal drug [33], and the resistance to Echinocandins is in very low percentages

Table 3. Anti-fungal susceptibility results and minimum inhibitory concentration (MIC) range against six antifungal drugs as evaluated by the Liofilchem MIC Test strip and distribution of isolates of *Candida* spp.

Candida Species	No. (%) Sensitivity Profile			
	Total (%)	MIC Range (µg/mL)	Sensitive (%)	Resistant (%)
Candida auris	**9 (29.03%)**			
Fluconazole		>64 - 256	0	9 (100%)
Voriconazole		0.19 - 1.5	9 (100%)	0
Caspofungin		0.19 - 1	9 (100%)	0
Micafungin		0.06 - 1	9 (100%)	0
Amphotericin-B		0.064 - 0.75	9 (100%)	0
Flucytosine		0.50 - 1	9 (100%)	0
Candida albicans	**8 (25.81%)**			
Fluconazole		0.5 - 4	8 (100%)	0
Voriconazole		0.125 - 1	8 (100%)	0
Caspofungin		0.012 - 0.94	8 (100%)	0
Micafungin		0.06 - 1	8 (100%)	0
Amphotericin-B		0.25 - 0.50	8 (100%)	0
Flucytosine		1 - 1.5	8 (100%)	0
Candida tropicalis	**3 (9.68%)**			
Fluconazole		1 - 1	3 (100%)	0
Voriconazole		0.125 - 0.75	3 (100%)	0
Caspofungin		0.016 - 0.25	3 (100%)	0
Micafungin		0.50 - 0.75	3 (100%)	0
Amphotericin-B		0.38 - 0.50	3 (100%)	0
Flucytosine		>32	0	3 (100%)
Candida famata	**3 (9.68%)**			
Fluconazole		0.50 - 1	3 (100%)	0
Voriconazole		0.094 - 1	3 (100%)	0
Caspofungin		0.25 - 1	3 (100%)	0
Micafungin		0.023 - 0.38	3 (100%)	0
Amphotericin-B		0.047 - 0.5	3 (100%)	0
Flucytosine		0.38 - 1	3 (100%)	0
Candida parapsilosis	**6 (19.35%)**			
Fluconazole		>64 - 256	0	6 (100%)
Voriconazole		0.047 - 1	6 (100%)	0
Caspofungin		0.50 - 1	6 (100%)	0
Micafungin		0.25 - 1	6 (100%)	0
Amphotericin-B		0.25 - 1	6 (100%)	0
Flucytosine		0.75 - 1	6 (100%)	0

Continued

Candida Lusitaniae	**1 (3.23%)**			
Fluconazole		-	1 (100%)	0
Voriconazole		-	1 (100%)	0
Caspofungin		-	1 (100%)	0
Micafungin		-	1 (100%)	0
Amphotericin-B		-	1 (100%)	0
Flucytosine		-	1 (100%)	0
Candida Duobushaemolumonii	**1 (3.23%)**			
Fluconazole		-	1 (100%)	0
Voriconazole		-	1 (100%)	0
Caspofungin		-	1 (100%)	0
Micafungin		-	1 (100%)	0
Amphotericin-B		-	1 (100%)	0
Flucytosine		-	1 (100%)	0

Classification of sensitive and resistant according to the rules of the Clinical and Laboratory Standards Institute M27-A3 [23]: Fluconazole (S: 8 μg/mL; R: 64 μg/ml); Voriconazole (S: 1 μg/mL; R: 4 μg/mL); Flucytosine (S: 4 μg/mL; R: 32 μg/mL); Amphotericin B (S < 1 μg/mL; R > 2 μg/mL); Echinocandins (S < 2 μg/ml, sensitive breakpoint only). S, sensitive; R, resistant.

to *Candida albicans* and non-albicans except for *Candida glabrata [34]*. Moreover, a study conducted in Kenya showed a resistance pattern of *Candida albicans* towards Fluconazole at a rate of 26% [16]. Interestingly, there was no isolate of *Candida albicans* showing resistance to any of the drug classes used in this study. Aware of the latter assertion, the increased susceptibility of *Candida albicans* might indicate the enhancement of drug regime towards this species as research was done massively targeting it in Kenya [14] [15] [35].

Furthermore, an increased resistance pattern in three species was observed in this study. *Candida auris* was seen to have a high resistance pattern up to 256 μg/ml for fluconazole indicating no susceptible isolate from all the 9 isolates tested against fluconazole. The power of *Candida auris* to establish uniform resistance to fluconazole might increase the mortality rates of immunocompromised patients [2] [36]. This study additionally acquired a drug resistance pattern towards Flucytosine by *Candida tropicalis* with 3 (100%) isolates showing increased MIC values as was classified resistant by CLSI breakpoints. A study conducted in Paris indicates the increased resistant activity of *Candida tropicalis* towards Flucytosine [37], however, this has not been documented in Kenya previously. In spite of certain reports expressing *Candida parapsilosis* as frequently susceptible to azoles [38] [39], several pieces of research have indicated its increased resistance towards Fluconazole [23] [40] [41]. In this study, all isolated *Candida parapsilosis* were discovered resistant to Fluconazole.

The drug susceptibility results exhibited a resistance pattern in the entirety of the isolated species of *Candida parapsilosis*, *Candida auris*, and *Candida tropica-*

lis. Critically ill patients face the threat of morbidity and mortality as a result of the wide use of broad-spectrum anti-fungal drugs where there is a development of resistance by *Candida* species [2]. Inherent causes of resistance are also noted on top of the usual acquired pattern of drug resistance after antifungal drugs are administered to patients [2] [42]. This intrinsic nature of resistance is usually seen in *non-albicans*, as is documented in this study. Despite the fact that the onset of drug resistance happens in several cases the pattern of complete resistance in all three species might show transmission of the *Candida* species among the patients. Infection control in patients with *Candida* has never been a challenge previously since it was comprehended that *Candida* infections derive essentially from the translocation of host flora to usually sterile locations, such as the bloodstream. Similar patterns of resistance, however, may suggest that the same strain circulates across the patients attributed to hospitalized patients' transmission as is confirmed by numerous studies [43] [44] [45]. The increased resistance of the *Candida* species inclines a high risk to hospitalized patients and might attribute to increased death rate particularly in critically ill patients.

5. Conclusion

There are increased candidemia cases in the critical care units of the health facility and dependent on the outcomes acquired, the study determined an exceptionally pervasive occurrence of new *Candida* species: *Candida auris*, and its drug-resistant property as a high threat to the public. Thus, measures ought to be taken to control the infection and transmission to diminish the mortality and morbidity of critically ill patients. Additionally, Echinocandins showed high potency as antifungal drugs against *albicans* and non-*albicans* whereas Fluconazole and Flucytosine were proved to be less effective drugs in the conspicuously seen species of *Candida auris*, *Candida parapsilosis*, *and Candida tropicalis*. Consequently, for an appropriate drug regime, physicians should be alert of the drug susceptibility patterns of *Candida* species in the locality before prescribing any of the anti-fungal drugs. All in all, the onset of new species and increased drug resistance patterns warrant constant drug monitoring. As this study was limited to Nairobi, researches of *Candida* blood stream infections in critical care units should be done in hospitals in other parts of Kenya for a comprehensive and updated prevalence study and to monitor the spread of *Candida auris* in the country. Moreover, the molecular basis of resistance should also be established in future studies for enhanced treatment.

Acknowledgements

We would like to acknowledge AFRICA-ai-JAPAN Project for funding this research. We would also like to convey our gratitude to Nairobi Hospital, especially the Microbiology Department and Mrs. Felister Musyoki, and One health, HORN for their material and physical support. Our appreciation goes to Mr. Pawlos Semere Tsegay for his continuous support throughout the research and

Mr. James Gachugia for his assistance in editing the manuscript.

Funding

This project is funded by AFRICA-ai-JAPAN Project for materials and reagents used in the research.

Authors Contribution

DAS, AKN, AK, CWN conceived and designed the study. DAS performed laboratory analysis and data analysis. DAS drafted the manuscript. All authors read, reviewed, and approved the final manuscript.

Ethical Approval

Ethical approval was obtained from Jomo Kenyatta University of Agriculture and Technology's Ethics Review Committee and the study site hospital's Ethics Review Committee. No patient was recruited solely for the study, but rather the study was part of the normal patient care process. Access to the data was strictly prohibited to maintain the confidential information of the patient. Patient Identification was done in a way that doesn't disclose the real information of the patient to avoid the leak of patient history.

Data Availability

The data can be provided upon request from the corresponding author.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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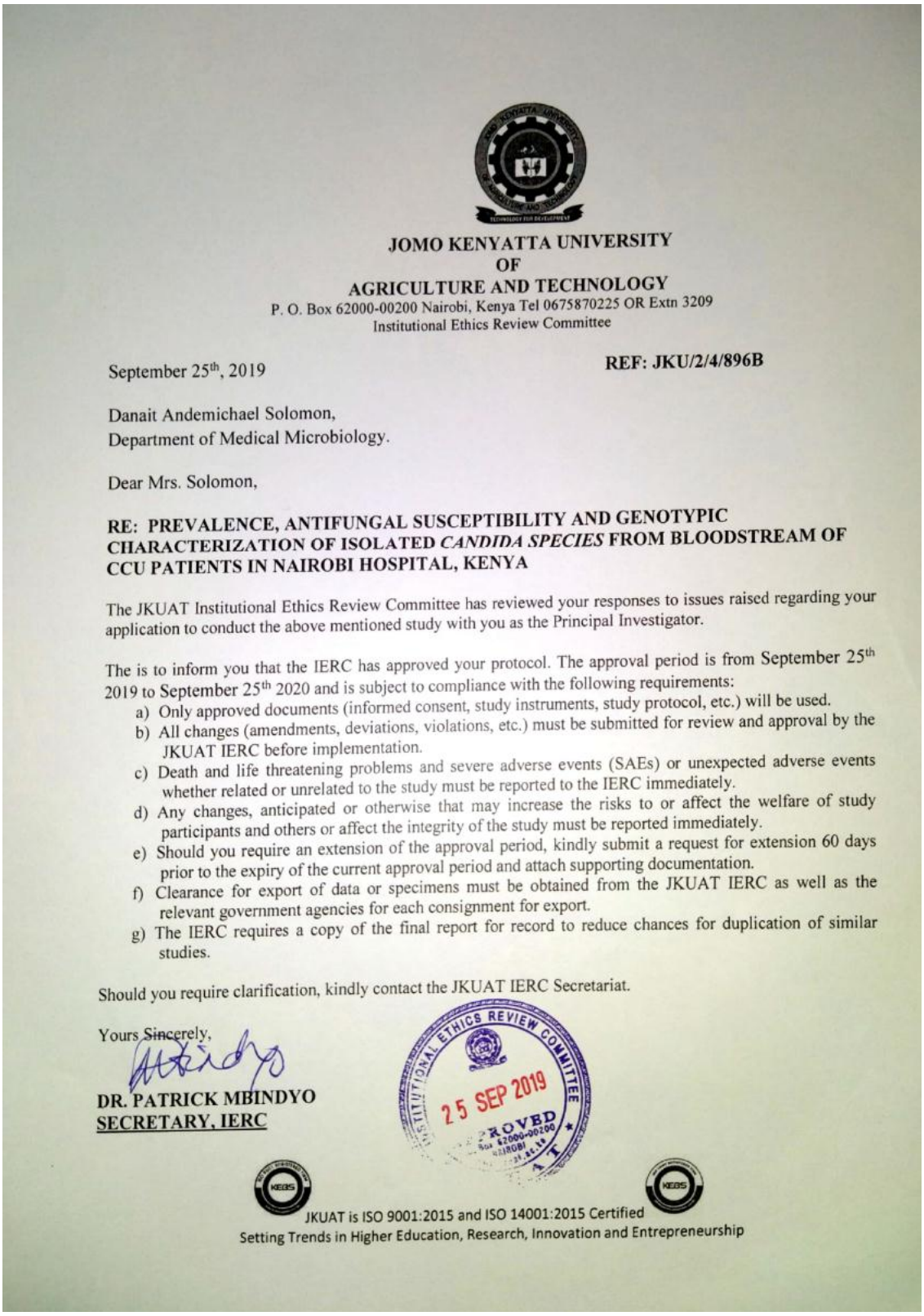
Appendix VI. Ethical Approval and Waiver of Informed Consent from JKUAT and Nairobi Hospital



JOMO KENYATTA UNIVERSITY
OF
AGRICULTURE AND TECHNOLOGY
P. O. Box 62000-00200 Nairobi, Kenya Tel 0675870225 OR Extn 3209
Institutional Ethics Review Committee

September 25th, 2019

REF: JKU/2/4/896B

Danait Andemichael Solomon,
Department of Medical Microbiology.

Dear Mrs. Solomon,

RE: PREVALENCE, ANTIFUNGAL SUSCEPTIBILITY AND GENOTYPIC CHARACTERIZATION OF ISOLATED *CANDIDA SPECIES* FROM BLOODSTREAM OF CCU PATIENTS IN NAIROBI HOSPITAL, KENYA

The JKUAT Institutional Ethics Review Committee has reviewed your responses to issues raised regarding your application to conduct the above mentioned study with you as the Principal Investigator.

The is to inform you that the IERC has approved your protocol. The approval period is from September 25th 2019 to September 25th 2020 and is subject to compliance with the following requirements:

a) Only approved documents (informed consent, study instruments, study protocol, etc.) will be used.
b) All changes (amendments, deviations, violations, etc.) must be submitted for review and approval by the JKUAT IERC 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 IERC immediately.
d) Any changes, anticipated or otherwise that may increase the risks to or affect the welfare of study participants and others or affect the integrity of the study must be reported immediately.
e) Should you require an extension of the approval period, kindly submit a request for extension 60 days prior to the expiry of the current approval period and attach supporting documentation.
f) Clearance for export of data or specimens must be obtained from the JKUAT IERC as well as the relevant government agencies for each consignment for export.
g) The IERC requires a copy of the final report for record to reduce chances for duplication of similar studies.

Should you require clarification, kindly contact the JKUAT IERC Secretariat.

Yours Sincerely,

DR. PATRICK MBINDYO
SECRETARY, IERC

INSTITUTIONAL ETHICS REVIEW COMMITTEE
25 SEP 2019
APPROVED
P.O. Box 62000-00200
NAIROBI

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JOMO KENYATTA UNIVERSITY
OF
AGRICULTURE AND TECHNOLOGY

P. O. Box 62000-00200 Nairobi, Kenya Tel 0675870225 OR Extn 3209
Institutional Ethics Review Committee

October 8th, 2019 **REF: JKU/2/4/896B**

Danait Andemichael Solomon,
Department of Medical Microbiology.

Dear Mrs. Solomon,

RE: PREVALENCE, ANTIFUNGAL SUSCEPTIBILITY AND GENOTYPIC CHARACTERIZATION OF ISOLATED *CANDIDA SPECIES* FROM BLOODSTREAM OF CCU PATIENTS IN NAIROBI HOSPITAL, KENYA

The is to inform you that the IERC has reviewed your request for waiver of informed consent to undertake the above titled study as the study utilizes isolates drawn from patients as part of routine care as authorized by the presiding doctor. There is minimal risk to patient and thus the waiver of informed consent will not adversely affect the rights and welfare of patients.

The request for waiver of informed consent is hereby granted. Kindly note that the approval period remains September 27th 2019 to September 27th 2020 and is subject to compliance with the requirements already issues.

Should you require clarification, kindly contact the JKUAT IERC Secretariat.

Yours Sincerely,

DR. PATRICK MBINDYO
SECRETARY, IERC

INSTITUTIONAL ETHICS REVIEW COMMITTEE
08 OCT 2019
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THE NAIROBI HOSPITAL

Our Ref. TNH/ADMIN/CEO/18/11/19

18 November 2019

Danait Andemichael Solomon
JKUAT Main Campus
P. O. Box 62000 - 00200
Juja

Dear Ms. Danait,

RE: PREVALENCE, ANTIFUNGAL SUSCEPTABILITY AND GENOTYPIC CHARACTERIZATION OF ISOLATED CANDIDA SPECIES FROM BLOODSTREAM OF CCU PATIENTS IN NAIROBI HOSPITAL

Reference is made to your request to carry out the above study at The Nairobi Hospital. We are pleased to advise that approval has been granted.

In line with the Research Projects Policy, you will be required to submit quarterly update reports of the study to the Committee. You are also required to submit a copy of the final findings for the Committee's records.

Do note that information/data collected and potential findings shall not be in conflict with the Hospital's Confidentiality Clause which states that "You will not without consent of the Association disclose any of its secrets or other confidential matters to anyone who is not authorized to receive them".

Please note that this approval is valid for the period November 2019 to November 2020, if an extension is required, a fresh application should be done before proceeding with the study.

Yours sincerely,
FOR: THE NAIROBI HOSPITAL

Christopher Abeid M.D.
AG. CHIEF EXECUTIVE OFFICER

C.c. Chairman - Bioethics & Research Committee
Laboratory Services Manager
Ag. Human Resources Director

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