CHARACTERIZATION AND ANTIFUNGAL DRUG SUSCEPTIBILITY OF CLINICAL ISOLATES OF *CANDIDA* SPECIES

VIOLET BOCHERE OOGA

MASTER OF SCIENCE

(Medical Mycology)

JOMO KENYATTA UNIVERSITY OF

AGRICULTURE AND TECHNOLOGY

2011

Characterization and antifungal drug susceptibility of clinical isolates of *Candida* species

Violet Bochere Ooga

A thesis submitted in partial fulfillment for the Degree of Master of Science in Medical Mycology in the Jomo Kenyatta University of Agriculture and Technology.

2011

DECLARATION

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

Signature……. Date……

Violet Bochere Ooga

This thesis has been submitted for examination with our approval as university supervisors.

Signature……..…… Date……

Dr. Christine Bii

KEMRI, Kenya

Signature…….…… Date…….

Dr. J. K. Gikunju

JKUAT, Kenya

DEDICATION

I dedicate this work to my husband, Joseph Nyakundi and son, Darwin Mogaka.

ACKNOWLEDGEMENTS

I acknowledge Professor Ng'ang'a, the Director Institute of Tropical Medicine and Infectious Diseases- Jomo Kenyatta University of Agriculture and Technology (ITROMID- JKUAT) and Dr. Mpoke, the Director Institute of Tropical Medicine and Infectious Diseases-Kenya Medical Research Institute (ITROMID –KEMRI) for their guidance during the study period.

Secondly, I acknowledge my supervisors; Dr. Christine Bii, Kenya Medical Research Institute for her guidance right from proposal development to the thesis writing and Dr. Gikunju, Jomo Kenyatta University of Agriculture and Technology for his constant academic support and advice.

Thirdly, I acknowledge Centre for Microbiology Research-KEMRI staff for their time and technical support while I was undertaking laboratory work.

Lastly, I thank my parents and all persons who provided financial, moral and technical support to this study.

TABLE OF CONTENTS

LIST OF TABLES

/

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

ABBREVIATIONS AND ACRONYMS

AIDS	Acquired Immune Deficiency Syndrome
ATCC	American Type Culture Collection
NAC	Non *albicans Candida*
CHROM	Chromogenic agar
CLO	Clotrimazole
CLSI	Clinical Laboratory Standards Institute
CMR	Centre for Microbiology Research
E-TEST	Epsilometer test
FLU	Fluconazole
HIV	Human Immunodeficiency Virus
I	Intermediate
ITC	Itraconazole
KEMRI	Kenya Medical Research Institute
MIC	Minimum Inhibitory Concentration
NCCLS	National Committee for Clinical Laboratory Standards
POS	Posaconazole
API	Analytical Profile Index
R	Resistant
S	Susceptible
SDA	Sabouraud's Dextrose Agar
TPN	Total Parenteral Nutrition

KOH	Potassium Hydroxide
Fig	Figure
Spp.	Species
MFC	Minimum Fungicidal Concentration

ABSTRACT

Candida species are responsible for a wide spectrum of infections in man. They can be isolated from most sites of a human body. These mycoses are most common in immunocompromised patients as opportunistic infections. Azoles have been used in treatment, prophylaxis and currently as maintenance therapy for candidiasis in these patients. The aim of the study was to characterize *Candida* species from clinical sources and determine their susceptibilities to azoles in Kenya. The study was conducted in 2009 in a mycology laboratory at Kenya Medical Research Institute (KEMRI). A total of 50 isolates of *Candida* were characterized and correctly identified to species level by germ tube test, Pal's agar, Chromogenic agar Candida, corn meal agar and Analytical Profile Index (API 20C AUX). 45 isolates were identified as *Candida albicans,* 1 as *Candida glabrata,* 1 as *Candida famata* and 3 as *Candida parapsilosis*. Susceptibilities of *Candida* species to fluconazole, posaconazole, itraconazole and clotrimazole were determined using Epsilometer-test and disc diffusion method. Their Minimum Inhibitory Concentrations (MIC's) were correlated. In Epsilometer test, 78% of *Candida* species were susceptible to clotrimazole and posaconazole, 60% to fluconazole and 50% to itraconazole. In disc diffusion method, 92% *Candida* species were susceptible to clotrimazole, 74% to itraconazole, 78% to posaconazole and 46% to fluconazole. There were no significant differences in susceptibility between E-test and disc diffusion methods for clotrimazole, itraconazole and posaconazole which had low significance levels ($p<0.002$). Fluconazole had the greatest difference between the two methods ($p=0.002$) and a kappa value of 0.329. There is emerging fungal resistance to

fluconazole and itraconazole therefore further investigations on fungal resistance and rational use of antifungal drugs is necessary.

CHAPTER ONE

INTRODUCTION

1.1 Background information

With the rising frequency and spectrum of fungal infections, as well as increasing reports of resistance to antifungal agents, it calls for adequate identification of fungi and constant antifungal susceptibility testing in clinical set ups (Barry *et al*., 2000).

Candida albicans once accounted for most serious nosocomial candidal infections.

Over the last decade, *Candida* infections, specifically candidemia, due to *C. albicans* have declined. Other non-*albicans Candida* species are now responsible for about half of candidemias and other deep *Candida* infections. Most non-*albicans Candida* infections are caused by *C. glabrata*, *C. parapsilosis*, or *C. tropicalis* (Krcmery and Barnes, 2002).

Rapid, reliable identification of *Candida* to species level is now needed more than ever in susceptibility testing. *In vitro* testing has revealed that there are clear differences among the various *Candida* species in their susceptibility to specific drugs.

In 1997, the National Committee for Clinical Laboratory Standards (NCCLS) published standard guidelines for antifungal susceptibility testing of *Candida* spp. to amphotericin B, flucytosine, fluconazole, itraconazole, and ketoconazole. Although the methods were standard, they were time consuming, difficult to interpret, and appropriate only for testing limited organisms and drugs. Modifications to the methods and alternative approaches have been proposed to make the tests more convenient and efficient, applicable to a greater number of species, and appropriate for performing in the clinical laboratory (Hoffman and Pfaller, 2001).

Due to an increasing array of systemic antifungal agents, there is need for accurate, reproducible and predictive susceptibility testing of fungal isolates in order to help the clinician in decision making in terms of choice of antifungal treatment (Barry *et al*., 2000).

1.2. Statement of the problem

Invasive fungal infections are an important cause of morbidity and mortality, and the number of these cases has been increasing. For example, *Candida* spp. is now the fourth leading cause of nosocomial infections (Pfaller, 2000). The rise may be attributed to advances in medical practice that have enabled the survival of critically ill patients such as the use of intravascular catheters, total parenteral nutrition (TPN), broad spectrum antimicrobials, and dialysis.

The spectrum of patients at risk of infection with opportunistic fungi, such as *Aspergillus* and *Candida* is also expanding. These include cases like solid organ and bone marrow transplants, acquired immune deficiency syndrome (AIDS), and intensive chemotherapy regimens. It is therefore imperative that the armamentarium of antifungals expands to treat these infections.

The ideal antifungal agent should: be fungicidal, have a novel mechanism of action, have a broad spectrum of activity, including resistant strains, and be well tolerated (Rex *et al*., 2001). The increased use of antifungal agents in recent years has resulted in the development of resistance to these drugs. The significant clinical implication of

resistance has led to heightened interest in the study of identification and antifungal resistance of fungi from different angles (Rex *et al*., 2001).

Isolation of *Candida* species less susceptible to commonly used anti-fungal drugs and recovery of increasingly resistant isolates during antifungal therapy is a growing problem (Pfaller *et al.*, 1998). For instance, *Candida albicans* and even *non- albicans candida* pathogenic for man have become resistant to antifungal agents, in particular triazole compounds.

The clinical consequences of antifungal resistance can be seen in treatment failures in patients and in changes in the prevalences of *Candida* species causing diseases (Dominique and Frank, 2001).For instance, prophylactic fluconazole only reduces the incidence of colonization by invasive *Candida* infection in neonates with very low birth weight but adults still get serious attacks, especially the immunocompromised and those with debilitating conditions (Vendettuoli *et al*., 2009). This is therefore a significant risk factor for emerging azoles resistance in HIV/AIDS patients.

Irrational use of antibiotics and prophylaxis on the other hand are fuelling antimicrobial resistance including resistance to antifungal drugs (Maenza *et al.*, 1998). Evidence of emergence azole resistance exists especially in patients empirically treated with fluconazole (Bii *et al*., 2002).

New generation triazoles have therefore been developed to meet the increasing need for new antifungals, and address the rising incidence of invasive fungal infections and the

emergence of fungal resistance (McGinnis and Rinaldi, 1996).Therefore susceptibility testing of new generation triazoles need to be determined to serve as baseline data for future studies. Following the trend of resistance to commonly used antifungals, susceptibility testing on new generation triazoles will thus determine if resistance has emerged to these drugs.

Several antifungal susceptibility testing methods with well defined test parameter have been devised. Most of the tests are reproducible with reliable intra- and inter-laboratory agreement particularly standardized methods for the susceptibility testing of yeast (Espinel *et al.*, 1998). They include Epsilometer test and disc diffusion methods which are based on broth micro dilution principle. It has been noted that the application of these standardized approaches has been used to produce susceptibility results that are comparable between laboratories and allow epidemiological analysis at both national and even international level to be done (Galgiani *et al.*, 1987).

1.3. Purpose of the study

The purpose of the study was to differentiate *Candida* spp. and determine the antifungal activities of the commonly used antifungals; fluconazole, clotrimazole and the new generation triazoles, namely, posaconazole and itraconazole against these isolates. The susceptibility results shall be applied in defining the spectrum of activities of the commonly used antifungal drugs compared to the new generation ones.

Differentiation of *Candida* to species level is also important because different strains exhibit different susceptibilities to azoles. This is because their virulence characteristics

and levels are quite different. *In vitro* susceptibility testing of an organism to an antifungal agent selected for therapy is one of the several reasons that influence decision making by the clinician on the drug of choice for treatment (Pfaller *et al*., 2006).

1.4 Significance of the study

To appreciate the value of characterization of fungi and susceptibility testing, it's essential to review the overall predictive utility of antibacterial susceptibility testing. Isolation and identification of fungi are preliminary steps to susceptibility testing. Hence differentiation of fungi and antifungal susceptibility testing are now increasingly and appropriately used as routine adjuncts to the treatment of fungal infections (NCCLS, 1997).

The prevalence and treatment of candidiasis is set to improve since the susceptibilities of strains of *Candida* to azoles have been established. Choice on antifungal therapy is clearer than before as every strain of *Candida* shall be treated individually (Hospenthal *et al.,* 2004).

The National Committee for Clinical Laboratory Standards (NCCLS) published its guidelines for standardized methods for *in vitro* testing of antifungal susceptibilities essential for clinical practice (NCCLS, 1997). Agar diffusion methods are often used as qualitative methods to determine whether a fungus is resistant, intermediately resistant or susceptible. Agar diffusion method can be used for determination of minimum

inhibitory concentration (MIC) values provided the necessary reference curves for conversion of inhibition zones into MIC values are available (Espinel *et al.,* 1998).

1.5. Justification of the study

Invasive fungal infections have increased over the last two decades causing formidable morbidity and mortality among the immuno-compromised. Individuals with HIV/AIDS, burns, malignancies, cancer, conditions like pregnancy and those who have undergone organ transplants procedures are particularly prone to fungal infections. Due to life threatening nature of fungal infections particularly candidiasis and reports of drug resistance, susceptibility testing of yeast pathogens has become very important.

The number of antifungal agents available continues to increase in the setting of a shift of candidal infections from *Candida albicans* to those caused by non-*albicans* species. Because of this, identification of *Candida* to species level and increased use of susceptibility testing has become necessary to appropriately select which agent to use (Hospenthal, 2004).

Routine susceptibility tests on major pathogens are useful as epidemiological tools as they accumulate epidemiological information on the resistance of fungi of public health importance. They are essential for informing the physician of the emergence of resistant strain to commonly used antifungal drugs and hence the need to modify standard treatment regimes. It also represents means of predicting therapeutic concentrations of antifungal drugs used to treat a variety of fungal infections (Espinel *et al*., 1998*)***.** This guides the clinician in selecting the best antifungal drug for an individual patient.

The MIC determinant is an important indicator of emerging resistance and changes in susceptibility patterns. Periodic surveillance of susceptibility testing is an excellent source of information on the emerging resistant strains. Resistance to commonly used azoles has been reported at 12% however, susceptibility to new generation triazoles has not been done in Kenya. The E-test and disc diffusion methods can be a valuable tool for resistance surveillance as they are reproducible with CLSI broth micro dilution method (Espinel-Ingroff *et al.*, 1998).

1.6 Research questions

1. Which is the most prevalent strain of *Candida* species from clinical sources?

2. Are the *Candida* species identified from clinical sources resistant to commonly used antifungal drugs?

3. Is there resistance to new generation triazoles among these *Candida* species from clinical sources?

1.7. Hypothesis

1.7.1. Alternative hypothesis

Candida albicans is the most common opportunistic pathogen in a clinical set up and that there is emerging resistance to commonly used antifungal drugs compared to new generation triazoles in Kenya.

1.7.2. Null hypothesis

Candida albicans is not a common opportunistic pathogen in a clinical setting and there is no emerging resistance to commonly used antifungal drugs compared to new generation triazoles in Kenya.

1.8. Objectives

1.8.1. General objective

To characterize and compare antifungal drug susceptibility of *Candida spp.* from clinical sources in Kenya.

1.8.2 Specific objectives

1. To characterize *Candida* species from clinical sources in Kenya.
2. To determine the antifungal susceptibilities of the isolates to commonly used antifungal drugs and new generation triazoles.
3. To compare the susceptibility results.

CHAPTER TWO

LITERATURE REVIEW

2.1. Introduction

Fungal infections are emerging significant pathogens and are the leading causes of morbidity and mortality in immunocompromised patients (Pfaller and Diekema, 2004). A dramatic change in the epidemiology of infectious diseases has taken place with the advent of new chemotherapeutic agents, new immunosuppressive agents, organ or bone marrow transplantation, parenteral alimentation, broad-spectrum antibiotics, and advanced surgical techniques. Fungal infections have emerged as a critical issue in the compromised host since *Candida* species are the most common fungal pathogens (Bodey,1988).

Human Immunodeficiency Virus /Acquired Immune Deficiency Syndrome (HIV/AIDS) is among the most important predisposing factors of fungal infections particularly candidiasis. In addition, hospital-related infections in patients not previously considered at risk such as those in an intensive care unit have become a cause of major health concern (Pappas, 2006).

2.2. Background information on candidiasis

Candidiasis, commonly called yeast infection or thrush, is a fungal infection (mycosis) of any of the *Candida* species, of which *Candida albicans* is the most common (Walsh and Dixon, 1996). Candidiasis thereby encompasses infections that range from superficial, such as oral thrush and vaginitis, to systemic and potentially life-threatening diseases. *Candida* infections of the latter category are also referred to as candidemia and are usually confined to severely immunocompromised persons, such as cancer, transplant, and AIDS patients, whereas superficial infections of skin and mucosal membranes by *Candida* causing local inflammation and discomfort is common in many human populations (Kourkoumpetis, 2010).

2.3. Diagnosis of candidiasis

There are two primary methods to diagnose yeast infections: microscopic examination and culturing.

For the microscope method, a scraping or swab of the affected area is placed on a microscope slide. A single drop of 10% potassium hydroxide (KOH) solution is then also placed on the slide. The KOH dissolves the skin cells but leaves the *Candida* untouched, so that when the slide is viewed under a microscope, the hyphae and pseudo spores of *Candida* are visible. Their presence in large numbers strongly suggests a yeast infection (Vazquez, 2003).

For the culturing method, a sterile swab is rubbed on the infected skin surface. The swab is then rubbed across a culture medium like sabouraudi's dextrose agar (SDA). The medium is incubated for 4 days at 37^0C, during which time colonies of *Candida* spp. develop. The characteristics of the colonies provide a presumptive diagnosis of candidiasis (Vazquez, 2003).

2.4. Treatment of candidiasis

In clinical settings, candidiasis is commonly treated with antimycotics. The antifungal drugs commonly used to treat candidiasis are topical clotrimazole, topical nystatin, fluconazole and topical ketoconazole (Pappas, 2004). In severe infections (generally in hospitalized patients), amphotericin B, caspofungin, or voriconazole may be used. Local treatment may include vaginal suppositories or medicated douches (Moosa, 2004).

Gentian violet can be used for treating breastfeeding thrush, but pediatrician recommends using it sparingly, since in large quantities it can cause mouth and throat ulcerations in nursing babies, and has been linked to mouth cancer in humans and to cancer in the digestive tract of other animals (Craigmill, 1991).

2.5. *Candida* species

These are ubiquitous fungi that represent the most common fungal pathogens that affect humans. They can be found in soil, inanimate objects, food, and hospital environments. However, it is rare for *Candida* spp. to produce contamination in laboratory settings.

They are normal commensals of man and can be recovered from many sources in normal and ill individuals. Candidiasis is caused by infection with species of the genus *Candida,* predominantly with *Candida albicans. Candida* spp. are also competing with the bacteria as one of the leading causes of nosocomial infections (Banerjee *et al.,*1991) and are true opportunistic pathogens that exploit recent technological advances to gain access to the circulation and deep tissues (Edmond *et al.,* 1999). However, they can also produce a wide variety of infections; and distinguishing between colonization and infection can sometimes be a challenge.

In addition, the ability of *Candida* spp. to produce oropharyngeal candidiasis in patients with HIV-AIDS has made it the leading fungal infection in immunosuppressed population (Sangeorzan *et al.,* 1994). Even though diseases related to *Candida* species have been known for centuries, the importance of these conditions has assumed increased relevance during the last two decades (Vazquez *et al.,* 2003).

There are 154 strains of *Candida* species but only a few are common and can cause life threatening conditions and these include:

2.5.1. *Candida albicans*

Referred to as monilia, is the most common type of *Candida*. They are budding and diploid fungi (a form of yeast). Like other types of *Candida,* it is an opportunistic causal agent for candidiasis in human, a type of fungal yeast infection

In fact, according to Clinical Microbiology Reviews (CMR), *Candida albicans* is also the most common causal agent of candidiasis. *Candida albicans* are commensals and

normal flora, in the human oral and gastrointestinal tract. Under normal circumstances, they colonize 80% of the human population with no harmful effects, although sometimes overgrowth occurs resulting in candidiasis (Pfaller and Diekema, 2007).

To be pathogenic, the usual unicellular yeast-like form of *Candida albicans* reacts to environmental cues and switches into an invasive, multi-cellular filamentous forms (Vendettuoli *et al.,* 2009). Infection is often observed in immunocompromised individuals such as HIV/AIDS patients. These yeast infections include thrush, which is an oral infection; vaginitis, an infection of the vagina; candida onchomycosis, a fungal nail infection; and diaper rash. *Candida* septicemia can also occur in the blood and in the genital tract in severely immunocompromised individuals.

2.5.2. *Candida krusei*

A rare type of *Candida* that is mainly found in the immunocompromised and those with hematological conditions (Hautala, 2007). *Candida Krusei* is also a nosocomial pathogen; its infections have been associated with high mortality rate than those of *Candida albicans*, particularly among hospitalized patients. Its prevalence in hospital environments has been associated with the prophylactic use of antifungals. It is also often found in patients who have had prior fluconazole exposure. Like *Candida parapsilosis*, *Candida krusei* has a high natural resistance to standard anti-fungal treatments (Pfaller, 2008).

2.5.3. *Candida glabrata*

Previously referred to as Torulopsis glabrata, is a type of *Candida* that is becoming a leading cause of oral candidiasis. It has recently emerged as a significant systemic pathogen, and even vaginal infections are being reported. It is particularly prevalent among people who have weakened or compromised immune systems, such as the elderly, cancer patients who are undergoing radiation treatment, patients undergoing bone marrow transplant and people who are HIV positive (Lockhart *et al,* 1999)**.** This epidemiological change may be due to the extensive use of fluconazole in severely immunocompromised patients (Marr *et al.,* 2000; Marr *et al.,* 1997).

2.5.4. *Candida parapsilosis*

A type of *Candida* that is of particular concern to hospitals. It has become a significant causal agent for sepsis and tissue infections in immuno-compromised patients (Levy *et al.,* 1999). According to the Sanger Institute; the increasing prevalence of this type of *Candida* in hospitals is particularly evident in Europe, where it is associated with almost 25 percent of all *Candida* infections in European hospitals (Weems, 1992). *Candida parapsilosi*s has an almost total, natural resistance to regular anti-fungal drugs.

2.5.5. *Candida tropicalis*

A relatively more benign type of *Candida*. It most commonly occurs in the digestive tract and can also be found on the skin. When it grows unchecked, it can develop into a

fungal infection. *Candida tropicalis* is sometimes used in the production of bio-diesel from the olive tree.

2.6. Prevalence of Candidiasis

There has been an increase in infections due to *Candida* species over the past decades particularly in hospitalized patients (Pappas *et al.,* 2003). This is usually accompanied by a significant increase in mortality and a prolonged length of stay in the hospital. In Kenya the *Candida* species remains one of the most common opportunistic pathogen, accounting for over 50% of all hospital-acquired opportunistic infections (Bii *et al.,* 2002).

However nosocomial transmission or 'cross-infection' and the recognition of resistance to antifungal agents pose a new and significant challenge. Studies indicate that *Candida* species may be isolated from the hands of 15-54% of health care workers in the intensive care unit (Sangeorzan *et al.,* 1994). It has also been demonstrated that the strain of *Candida* carried on the hands is shared by infected patients. These studies have been facilitated by molecular typing and careful epidemiological investigation which suggests that cross-infection is an important and preventable feature of candidiasis.

Both endogenous and exogenous sources of infection are now well-documented and such information is essential for instituting direct preventive measures to prevent infections in high risk individuals (Pfaller, 2000). Globally the prevalence for oral candidiasis is estimated at 7.8% however this is expected to be higher in Kenya and in sub-Saharan Africa where the rate of HIV/AIDS is high (Pfaller, 2000).

2.7. Antifungal susceptibility testing

Susceptibility testing refers to subjecting a drug to a certain fungi and observing its activity. It is based on a minimal concentration of drug that appears likely to inhibit (minimum inhibitory concentration, MIC, µg/ml) or kill (minimum fungicidal concentration, MFC, µg/ml) the fungus. If this level is low enough, then the drug may work against an infection and if it is high, the drug may not be effective (Hoffman and Pfaller, 2001).

At the beginning of 1990, the National Committee for Clinical Laboratory Standards (NCCLS) began the process of developing standardized methods for antifungal susceptibility testing. Initially eyes were focused on broth-based testing of yeasts especially the M27 Method which was entitled; reference method for broth micro dilution antifungal susceptibility testing of yeasts (NCCLS, 1997). However, it is rather cumbersome and not practically applicable in a routine clinical set-up. Thus, variants on this method are widely used.

Early reports of resistance were questioned on the grounds that different methods had been used to assess pre- and post-treatment isolates, and in the late 1980s, testing methods were not standardized and unsurprisingly inter- and even intra-laboratory reproducibility was poor (Galgian and Brass, 1990).

Numerous subsequent papers documented the importance of standardized test conditions and the impact on MIC determinations of test variables including, but not limited to,

inoculum size, test medium composition and pH, test format, temperature, duration of incubation and endpoint determination (Espinel-Ingroff *et al.,* 1998). One of the study detailing the effect of procedural variables on MIC's is that on examination of multiple combinations of 10 test variables and documentation on their effect on itraconazole susceptibility of a series of test isolates including *Candida* spp (Rambali *et al.,* 2001).

The same criteria also apply to the antifungal drugs in the study. It was clear that by modifying test conditions, isolates could be made to appear susceptible or resistant to a certain antifungal drug, thus highlighting the crucial importance of establishing a set of standardized testing criteria while acknowledging that any non standard method will represent a compromise rather than an ideal system (Rambali *et al.,* 2001).

In vitro susceptibility testing can predict outcome in selected clinical situations. The clearest data are from the fluconazole-treated AIDS patients with oropharyngeal candidiasis (Magaldi *et al.,* 2001). In this setting, the homogeneity of the underlying immune defect, combined with the ease of identification and monitoring of the infection, creates a near-perfect test situation.

The importance of host factors in the correlation of the MIC with outcome cannot be over emphasized. Examples of these parameters include patient status (underlying disease, the presence of intravascular catheters, and CD4+ T-cell number), drug pharmacokinetics (absorption and distribution), patient compliance, and drug-drug interactions (Pappas, 2006). Identification of relevant factors can substantially improve

the degree of the MIC-outcome correlation and thus improve the clinical utility of in vitro testing. An important feature in this entire process is the role of standardized susceptibility.

2.7.1. Epsilometer-test

The E-test is one of the commercially available tests for the detection of antifungal resistance. It is a simple method to perform and its methodology is similar to that used in bacteriology, that is, the standard method (NCCLS) however it is not cumbersome and labor intensive like the standard method (Matar *et al.,* 2003).

Individual antifungal may be tested and there is always reasonable correlation with the National Committee for Clinical Laboratory Standard (NCCLS). This method is suitable for yeast and mould isolates, and if done correctly, it is a reliable and reproducible method that produces results that correlate well with the NCCLS methodology. As a constraint, E-test is very expensive (NCCLS, 1997).

In E-test, if resistance is detected in a species in which it is not expected, it should be confirmed by a second method until the operatives have become proficient at differentiating true resistance from background intra-zonal growth. Minimum inhibitory concentration value of a given agent is defined by the experimental conditions used. Thus the MIC obtained under different *in vitro* conditions cannot be used interchangeably unless shown to be equivalent to the reference method (NCCLS, 1997).

2.7.1.1. Principle of Epsilometer-test

The E-test is basically an agar diffusion method. It utilizes a rectangular strip that has been impregnated with the drug to be studied. A test fungus is inoculated onto the surface of an agar plate and the E-test strip is laid on top; the drug diffuses out into the agar, producing an exponential gradient of the drug to be tested. There is an exponential scale printed on the strip. After 24 hours of incubation, an elliptical zone of inhibition is produced and the point at which the ellipse meets the strip gives a reading for the minimum inhibitory concentration (MIC) of the drug (NCCLS, 2000).

2.7.2. Disc diffusion method

Disc diffusion is the most attractive alternative method to standard method. It is not labor intensive, easy to carry out, reproducible and suitable for routine use in mycology laboratory. However it involves more scale to interpret. It is suitable for water-soluble agents such as fluconazole and it is an NCCLS method for *in vitro* susceptibility testing of *Candida* species. This provides a zone of inhibition, the measurement of which can be correlated with the MIC value (NCCLS, 2000).

2.7.2.1. Principle of disc diffusion method

When a filter paper disc impregnated with an antifungal drug is placed on agar the antifungal drug will diffuse from the disc into the agar. This diffusion will place the antifungal drug in the agar only around the disc. The solubility of the antifungal drug

and its molecular size will determine the size of the area of chemical infiltration around the disc. If a test fungus is placed on the agar it will not grow in the area around the disc if it is susceptible to the chemical (NCCLS, 2000).

2.7.3. Minimum Inhibitory Concentration

Minimum Inhibitory Concentration is the least amount of antifungal drug required to inhibit fungal growth. It is usually read in numerical. MIC_{90} is the concentration of a drug at which 90% of the population of micro-organisms are inhibited from growth. Successful if >90% of population show inhibition. Show emergence of resistance if 10% of population is resistant. MIC_{50} is the concentration of a drug at which 50% of the population of micro-organisms are inhibited from growth. It is actually the median for a series of MIC's (NCCLS, 1997).

Minimum Inhibitory Concentration value of a given agent is defined by the experimental conditions used. Thus the MIC obtained under different in vitro conditions can't be used interchangeably unless shown to be equivalent to the reference method (Hoffman and Pfaller, 2001).

2.8. Antifungal drugs

An azole is a class of five-membered nitrogen heterocyclic ring compounds containing at least one other non-carbon atom of either nitrogen, sulfur, or oxygen. They are commonly used as antifungal drugs (Eicher and Hauptmann, 2003). Triazole refers to

either one of a pair of isomeric chemical compounds with molecular formula C2H3N3, having a five-membered ring of two carbon atoms and three nitrogen atoms (Eicher and Hauptmann, 2003).

Commonly used antifungal drugs include clotrimazole, fluconazole, amphotericin B econazole, miconazole, nystatin, oxiconazole and terconazole. Newer generation antifungal drugs include ketoconazole, itraconazole and posaconazole. Fluconazole and itraconazole have proved to be safer than both amphotericin B and ketoconazole (Sheehan *et al.*, 1999).

Major developments in research into the azoles class of antifungal agents during the 1990s have provided expanded options for the treatment of many opportunistic and endemic fungal infections. The study of resistance to antifungal agents has since lagged behind that of antibacterial resistance for several reasons. Perhaps most importantly, fungal diseases were not recognized as important pathogens in the past (Anaissie and Bodey, 1989).

For example, the annual death rate due to candidiasis was steady between 1950 and about 1970. Since 1970, this rate increased significantly in association with several changes in medical practice, including more widespread use of therapies that depress the immune system, the frequent and often indiscriminate use of broad-spectrum antibacterial agents, the common use of in-dwelling intravenous devices and the advent of chronic immunosuppressive viral infections such as AIDS (Beck-Sagué and Jarvis,

1993). These developments and the associated increase in fungal infections intensified the search for new, safer, and more efficacious agents to combat serious fungal infections.

2.8.1. First generation triazoles

2.8.1.1. Fluconazole

Fluconazole is a triazole antifungal drug related to clotrimazole (Lotrimin), ketoconazole (Nizoral), itraconazole (Sporanox), and miconazole (Micatin, Monistat) (Bailey *et al.*, 1990). It is a white crystalline powder which is slightly soluble in water and alcohol. It is commonly marketed under the trade name Diflucan or Trican. Like other imidazole- and triazole-class antifungals, fluconazole inhibits the fungal cytochrome P450 enzyme 14α-demethylase. This inhibition prevents the conversion of lanosterol to ergosterol, an essential component of the fungal cytoplasmic membrane and subsequent accumulation of 14α-methyl sterols (Eicher and Hauptmann, 2003).

Fluconazole is primarily fungistatic, however may be fungicidal against certain organisms in a dose-dependent manner. It is active against most *Candida* species, with the absolute exception of *Candida krusei* and partial exception of *Candida glabrata* and a small number of isolates of *Candida albicans*, *Candida tropicalis*, *Candida parapsilosis* and other rare species (Kirkpatrick *et al.*, 1998).

Fluconazole is used in the treatment and prevention of superficial and systemic fungal infections. It has been used extensively to treat fungal infections in up to16 million patients, including over 300,000 AIDS cases, in the United States alone since the launch of this drug (Maenza et al., 1996).

Concomitant with this widespread use, there have been increasing reports of antifungal resistance. Emergence of *Candida* strains resistant to azole antifungal drugs owing to prolonged fluconazole treatment is now a recognized problem. This is rampant in AIDS patients particularly those with opportunistic infections. The appearance of secondary azole resistance to this date has been demonstrated most commonly in HIV-positive patients who previously received fluconazole (Rex *et al.,* 2001).

Typical rates of resistance, in *Candida albicans* in a general hospital are 3-6%, in *Candida albicans* in AIDS 10-15%, in *Candida krusei* 100%, in *Candida glabrata* ~50-70%, in *Candida tropicalis* 10-30% and in other *Candida* species less than 5%. Long-term fluconazole drug use maybe an important factor in the development of resistance (Dupont *et al.,* 1996).

2.8.1.2. Clotrimazole

Clotrimazole is an imidazole antifungal agent. It inhibits 14-α-demethylation of lanosterol in fungi by binding to one of the cytochrome P-450 enzymes. This leads to the accumulation of 14-α methyl sterols and reduced concentrations of ergosterol, a sterol essential for a normal fungal cytoplasmic membrane. The methyl sterols may affect the

electron transport system due to decreased fungal cell wall integrity thereby inhibiting growth of fungi (Sud and Feingold, 1981).

Clotrimazole is an antifungal medication commonly used in the treatment of fungal infections of both humans and animals such as vaginal yeast infections and ringworm. It is also used to treat athlete's foot and jock itch. Women who suffer from vaginal yeast infections, such as Candidiasis, can buy clotrimazole at a pharmacy (Spiekermann and Young, 1976).

Clotrimazole is commonly available as an over-the-counter drug in various dosage forms, such as a cream, lotion powders and with other antibiotic combination. In the latter case, it typically includes an antibiotic such as Marbofloxacin, and is applied in liquid form. Side effects include: occasional localized irritation of the skin with a mild burning sensation, redness and itching (M cGinnis and Rinaldi, 1996).

Most reports of resistance to antifungal agents among human pathogenic fungi indicate that naturally-occurring resistance is very rare. Strains of *candida* having a natural resistance to clotrimazole are also rare. Only *Candida guilliermondi* has been reported to have primary resistance to clotrimazole. Resistance to clotrimazole is highly associated with clinically overt failure of antifungal azole therapy (Holt, 1972).

Patients with AIDS present quite a different venue for clotrimazole. Chronic and repetitive use of antifungal azoles to treat protracted opportunistic infections in HIV/AIDS patients predisposes them to the development of resistance (Holt, 1972).

2.8.2. New generation triazoles

The rising incidence of invasive fungal infections and the emergence of broader fungal resistance have led to the need for novel antifungal agents. Use of the currently available azoles in combination with other antifungal agents with different mechanisms of action is likely to provide enhanced efficacy (Sheehan *et al.,* 1999).

2.8.2.1. Posaconazole

A new member of the triazoles class of antifungals with broad spectrum antifungal activity. Its mechanism of action includes inhibiting ergosterol synthesis hence affecting the integrity of the fungal cell membrane. It is available as an oral suspension and has a favorable toxicity profile (Kauffman, 2006).

Posaconazole has demonstrated clinical efficacy in the treatment of oropharyngeal candidiasis. In addition, data from randomized controlled studies support its efficacy for use in prophylaxis of invasive fungal infections in patients who are severely immune-compromised (Kauffman, 2006). The wide spectrum activity of posaconazole in *in-vitro* studies, animal models and preliminary clinical studies suggest that posaconazole represents an important addition to the antifungal armamentarium (Oliveira *et al.,* 2005).

The fungi whose growths are inhibited by posaconazole include *Aspergillus, Candida, Coccidioides, Histoplasma, Paracoccidioides, Blastomyces, Cryptococcus, Sporothrix*, various species of *Mucorales* (causing *Zygomyetes*). Majority of *Candida* species are

killed by posaconazole at clinically relevant concentrations. Acquired resistance to posaconazole does occur in *Candida* species and is otherwise rare in most fungal infections. In conclusion, posaconazole has been documented has the most potent azole derivative used in medicine (DuPont *et al.,* 1996).

2.8.2.2. Itraconazole

Itraconazole has a broader spectrum of activity than fluconazole but not as broad as voriconazole or posaconazole (Rambali *et al.,* 2001). It binds to the fungal p450 enzymes and stops the cells making ergosterol, the main component of the cell wall. Its major advantages over fluconazole are its activity particularly against most *Aspergillus* isolates and a subset of fluconazole-resistant *Candida* strains (Bailey *et al.*, 1990).

It is used to treat serious fungal or yeast infections, such as oropharyngeal candidiasis (oral thrush), and esophageal candidiasis (candida esophagitis) (McGinnis and Rinaldi, 1996). Itraconazole is active in general against various types of fungi, including yeasts, moulds and dimorphic fungi. These include *Aspergillus, Blastomyces, Candida, Coccidioides, Cryptoccocus, Histoplasma, Paracoccidioides, Scedosporium apiospermum* and *Sporothrix schenkii* (Walsh and Dixon, 1996).

Resistance to itraconazole has however been described in *Candida* and *Aspergillus* species. Typical resistance rates in *Candida albicans* in a general hospital are 4%, in *Candida albicans* in AIDS 12%, in *Candida krusei* >80%, in *Candida glabrata 65*%, in

Candida tropicalis 10% and in other *Candida* species less than 5 % (Dupont *et al.*, 1996). Resistance rates are much lower than those of fluconazole.

CHAPTER THREE

MATERIALS AND METHODS

3.1. Study Design

The study was laboratory based carried out on archived isolates of *Candida* species of clinical source in Nairobi area. It was conducted between May and December, 2009. All isolates of *Candida* were processed, identified, confirmed and their susceptibilities determined at a mycology laboratory, Center for Microbiology Research (CMR), KEMRI. The research protocol was reviewed and approved by KEMRI.

3.2. Study sites

The archived isolates of *Candida* were originally isolated in various clinical set ups in Nairobi area in a cross sectional study. Mycological investigations were carried out at a Mycology laboratory at the Centre for Microbiology Research, KEMRI.

3.3. Study population

50 archived isolates of *Candida* of clinical source originally obtained from clinical set ups in Nairobi area between May and December, 2009 were used in this study.

3.3.1. Inclusion criteria

- The specimen must be yeast positive.
- The specimen must be obtained from a clinical set up in Nairobi area.

3.3.2. Exclusion criteria

- All specimens that are yeast negative.
- All specimens that are not from a clinical set up in Nairobi area.

3.4. Procedures

3.4.1. Collection

According to the cross sectional study, specimens were obtained from throat, chest cavity, necrotic sites, vagina and bladder of patients suffering from candidiasis at the clinical set ups. Specimens were collected depending on the nature of candidiasis the patient was suffering from. For those who had pulmonary candidiasis, they were instructed to collect an early morning sputum sample in a clean small plastic sterile container; those who had urinary tract candidiasis, midstream urine was collected into clean sterile bottles; those who had oropharyngeal candidiasis were given sterile swabs which they rolled upon areas in their entire mouth that had oral thrush and those who had necrotic areas and lesions as evidenced in ocular candidiasis had these sites also rolled upon using sterile swabs. The swabs were transported in air tight containers**.** All

the specimens were transported in a cool box to the laboratory immediately for processing.

3.4.2. Storage of specimen and reagents

Specimens were stored at 4^0C if short delays before processing were anticipated. These include; pus, sputum, urine and necrotic material. Reagents were stored at -20^0 C while any open packages of the reagents was stored in separate air tight containers containing active desiccant and stored at -20^0C.

3.4.3. Safety

All procedures were carried out in accordance to the general laboratory bio-safety and waste disposal guidelines. These included working under a level two bio safety cabinet, wearing of safety apparel and careful handling of glassware and other tools while working with isolates.

3.5. Experimental procedures

3.5.1. Gram staining

Gram staining is a bacteriological laboratory technique used as a tool for the differentiation of Gram-positive and Gram-negative bacteria. It is a preliminary step in determining the identity of a particular bacterial or yeast sample. The Gram stain is not an infallible tool for diagnosis, identification, or phylogeny. Gram staining involves the

application of a series of dyes that leaves some bacteria purple (Gram +) and others pink (Gram -). Yeasts usually stain purple as the gram positive bacteria.

Smears of the swabs were made on heat sterilized glass slides. The slides were then flooded with crystal violet solution for up to one minute, washed off briefly with tap water (5 seconds) and drained; flooded with Gram's Iodine solution, and allowed to act (as a mordant) for about one minute, washed off with tap water and drained; excess water from slides was removed and blotted, slides were then flooded with 95% alcohol for 10 seconds and washed off with tap water (Smears that are excessively thick required longer decolorization, the slides were drained thereafter; flooded with safranin solution and allowed to counter stain for 30 seconds, washed off with tap water, drained and blotted dry with bibulous paper; examined under the oil immersion lens at x100 (David-Rollins and Joseph, 2000). Hyphal branching and budding yeasts were regarded as positive for fungi.

3.5.2. Culture and isolation

Specimens were cultured on Sabouraud's Dextrose Agar (SDA) supplemented with gentamicin and chloramphenicol to inhibit bacterial contamination and incubated at 35^0 C for 72 hours. The soft, moist white / cream colonies on the plates in 24 to 48 hours were considered positive.

3.5.3. Germ tube test- for observation of germ tubes.

To identify the yeasts, the germ tube test was carried out. A pure culture of the test fungus obtained from SDA was used; 3 drops of serum were introduced into a clean tube using a pipette; a colony of the test fungus was touched and gently emulsified in the serum; the tube was incubated at 35^0 C for 3 hours; after which, a drop of the incubated suspension was transferred onto a slide for microscopic examination at x40. *Candida albicans*, and the rarer oral pathogen *C. dubliniensis*, are the only species of the genus *Candida* that produce a short one piece germ tube under these conditions (Elmer *et al.*, 1992).

Yeasts that were germ tube-negative were tested on Chromogenic agar Candida and Pal's agar for further identification

3.5.4. Identification- Chromogenic agar Candida and Pal's agar.

Chromogenic agar Candida (CHROMagar) is a new differential culture medium that allows selective isolation of yeasts and simultaneously identifies colonies of *Candida albicans, C.dubliniensis C. tropicalis,* and *C. krusei* (Louwagie *et al.,* 1995). It is a primary medium for differentiating between various *Candida* species based on their colony colour and morphology (rough/smooth). Pal's agar was considered as a secondary medium for differentiation (Odds and Bernaerts, 1994). Colonies of *Candida albicans* are typically green while those of *C.dubliniensis* are usually atypically green on CHROMagar.

Pal's agar was prepared freshly with unsalted sunflower seeds and used within 5 days to ensure consistent results. First, an aqueous extract of sunflower seeds was prepared by pulverizing 50 g of seeds in a blender for 5 min and then adding the ground seeds to 1 liter of distilled water, followed by boiling for 30 min. Next, the seed extract was cooled and filtered, and the following ingredients were added: glucose (1 g), KH_2PO_4 (1 g), and creatinine (1 g). The pH was adjusted to 5.5, the volume was readjusted to 1 liter, and 15 g of agar was added before the mixture was autoclaved at 125°C for 2 hours.

For each isolate included in the study, part of a single colony grown on SDA at 37°C for 48 h was streaked onto Pal's agar and incubated at 30°C. Colony morphology was examined visually every 24 h for up to 10 days for the presence of a hyphal fringe and the data were recorded. Production of a hyphal fringe around colonies grown on Pal's agar in these conditions provides a simple means of discriminating between isolates of *C. dubliniensis* and *C. albicans* with 100% accuracy (Sahand *et al.,* 2005). Colonies of *C.dubliniensis* and *Candida krusei* produce a hyphal fringe on Pal's agar whereas those of *Candida albicans* do not (Al *et al.,* 2003).

At 48 to 72 h, colonies from CHROMagar and Pal's agar were examined microscopically for the presence or absence of chlamydospores: 10 to 20 well-separated single colonies were stained with 1 drop of lacto phenol cotton blue stain (which stains chlamydospores preferentially), allowed to stain for 5 min, and then covered with sterile glass cover slips and examined microscopically. Chlamydospores-negative isolates continued to be examined at 24-h intervals for up to 10 days. Colonies of *C dubliniensis*

produce abundant chlamydospores in clusters on Pal's agar, casein agar and CHROMagar while *Candida albicans* do not.

3.5.5. Dalmau plate culture-Corn Meal Agar

Dalmau plate culture is a morphological test used for the identification of germ tube negative yeasts. This test provides a simple and cost-effective tool for the presumptive differentiation of *Candida* species and is particularly suited for clinical microbiology laboratories where biochemical or molecular methods for the differentiation of these two species are not available. Corn meal agar is a well established mycological medium which is a suitable substrate for chlamydospores production by *Candida albicans* and the maintenance of fungal stock cultures.

Corn meal agar was prepared by suspending 17g of the powder in 1 liter of distilled water. It was brought to boil to dissolve completely and sterilized by autoclaving at 121°C for 2 hours.

2-3 colonies obtained from primary isolation were suspended in distilled water to make a light inoculum; a flame sterilized straight wire was dipped into the inoculum and scratched on the surface of cornmeal agar lightly; flamed cover slips were placed on the agar surface covering the scratches; Dalmau plate was then incubated at 26°C for 5 days and examined in situ microscopically at x40.They were examined daily for up to five days for the presence of chlamydospores.

Candida albicans produces good growth; white colonies and chlamydospores whereas *C .tropicalis* and *C. parapsilosis* produce good growth; white / cream colonies without the chlamydospores. Most strains of *C. albicans* and *C. stellatoidea* form typical chlamydospores after 24-48 hours incubation. *C. dubliniensis* will also form chlamydospores, but in clusters, rather than singles as with the *C. albicans.*

3.5.6. Analytical Profile Index (API 20C AUX-Biomeriux)

This was used as a confirmatory test for all the *Candida spp.*; it is based on fermentation and utilization of substrates such as glucose, maltose, sucrose and lactose and to observe fermentation of glucose and maltose and absence in sucrose. The basal medium in the ampoules were thawed by placing them in a boiling water bath for two minutes and allowed to cool; an incubation tray was prepared and 20 ml of water dispensed and strip placed into the incubation tray; the ampoules were opened by breaking the glass tip inside its tip, the molten medium was inoculated with two colonies (>2 mm diameter) of the test yeast fungus; the inoculated media was introduced onto the strip (20 cupules; approximately 0.2 ml each) following the manufacturer's directions; the lid was replaced on the tray and incubated at 28 to 30^0 C for 72 hours.

Results were read and recorded after 24, 48, and 72 hours of incubation. Growth in each cupule was compared to cupule 0 which was used as negative control. A cupule more turbid than the control was recorded as a positive reaction. Identification was obtained with numerical profile. On the result sheet, the test was separated into groups of three

and a number 1, 2 and 4 was indicated for each group sequentially. The numbers corresponding to positive reactions within each group were added to obtain a number which constituted the numerical profile.

3.5.7. Validity and reliability of test

Quality control fungal isolates of *Candida albicans* ATCC 90028 were used (Barry *et al.,* 2000). The strains were periodically tested for viability and validation to ensure accuracy.

3.6. Epsilometer test for drug susceptibility testing

3.6.1. Preparation of the test inoculum

The inoculums were obtained from fresh cultures of *Candida spp* grown on SDA at 35^0 C; 3-4 colonies were touched and transferred to a tube of sterile distilled water, emulsified inside the tube to obtain a yeast suspension. Turbidity of the inoculum was compared to that of 0.5 Mc Farland standards and adjusted visually to match it (NCCLS, 2000).

3.6.2. Inoculation of the plate with test strains

Each solidified medium of Mueller Hinton was inoculated by dipping a nontoxic (latex-free) sterile swab into the respective inoculum suspension and evenly streaking it in

three directions over the entire surface of a 90-mm Petri plate containing 25ml of medium The swab was dipped into the inoculum suspension and pulled out slightly each time that the agar surface was streaked. The swab was rotated several times against the inside of the tube above the fluid level to remove excess fluid; the agar surface was allowed to dry for 15 min and any excess moisture was absorbed before the strips are applied (Rex *et al.,* 2001). *Candida albicans* ATCC 90028 were also processed each time a set of isolates were evaluated

3.6.3. Application of the Epsilometer-test strips

The E-test package had to be thawed for at least 30 minutes prior to application. Strips were applied to agar surface using sterile forceps with the 'E' end at the edge of the plate and with the scale visible. The strips were parted gently with the back of the inoculating wire to ensure contact with the agar surface; the lids were replaced and the plates were incubated at 35°C. MICs were determined following incubation for 24 to 48 hours. *Candida albicans* ATCC 90028 were tested each time a set of isolates were evaluated.

MIC was read at a point where the ellipse intersected the scale at complete or reduced growth of all colonies including isolated ones. If it intersected on either side of the strip MIC was read as the greater value. Growth at the edge of the strip was ignored. The MIC of isolates *Candida spp.* was categorized as susceptible, resistant or intermediate based on the CLSI break points.

3.7. Agar diffusion method using discs

3.7.1. Antifungal agent

Commercially available discs were ordered from Liofilchem Company in Italy and Sweden. They contained clotrimazole, fluconazole, posaconazole and itraconazole.

3.7.2. Preparation of the inoculum

Test strains were emulsified in a sterile tube of sterile distilled water to make a suspension. Turbidity was compared to that of the 0.5 McFarland standards (Barry and Brown, 1996).

3.7.3. Inoculation of the plate

Each solidified medium of Mueller Hinton was inoculated by dipping a nontoxic (latex-free) sterile swab into the respective inoculum suspension and evenly streaking it in three directions over the entire surface of a 90-mm Petri plate containing 25 ml of medium; the procedures were done as previously described (Lozano-Chiu, 1999). *Candida albicans* ATCC 90028 were also processed each time a set of isolates were evaluated.

3.7.4. Application of the discs

Discs containing the antifungal drugs were placed on the agar surface using a pair of sterile forceps. The discs were slightly touched with sterile inoculating loop to ensure that they were in good contact with the agar surface. The plates were incubated upside down at 35°C, and the MICs were determined following incubation for 24 h to 48 hours (Lozano-Chiu, 1999).

3.7.5. Minimum inhibitory concentration (MIC) determination

A metric ruler was placed across zone of inhibition at the widest diameter and measured from one edge to another while holding the plate up to light. Zone diameter was recorded in millimeters, matched up with the breakpoints and reported as susceptible, resistant or intermediate (Meis *et al.,* 2000) (Plate 4.2). However large colonies growing within a clear zone of inhibition were sub cultured, re-identified and retested (Kirkpatrick *et al.,* 1998).

CHAPTER FOUR

RESULTS

4.1 Distribution of *Candida* species from various sources

During the period of the study, 50 isolates of *Candida* species were obtained. From the isolates, 4 % (n=2) were from necrotic swabs, 22% from pus swabs (n=11), 6% (n=3) from throat swabs, 6% (n=3) from High Vaginal Swab, 20 % (n=10) from sputum and 42% (n=21) from urine (Fig 4.1).

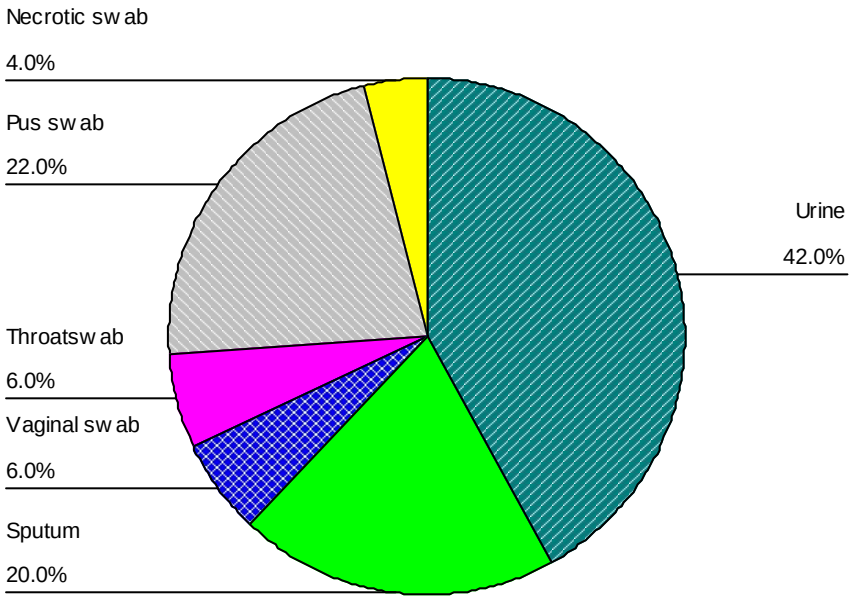

Fig 4.1; Distribution of *Candida* species from various sources

The isolates of *Candida* were gram stained to differentiate fungal elements from bacterial. Branching hyphae and budding yeasts that were purple in color were detected (Plate 4.1, Plate 4.2). The yeast positive isolates were isolated on SDA to obtain pure cultures. Cream-colored, circular and convex colonies with large, spherical cells were obtained.

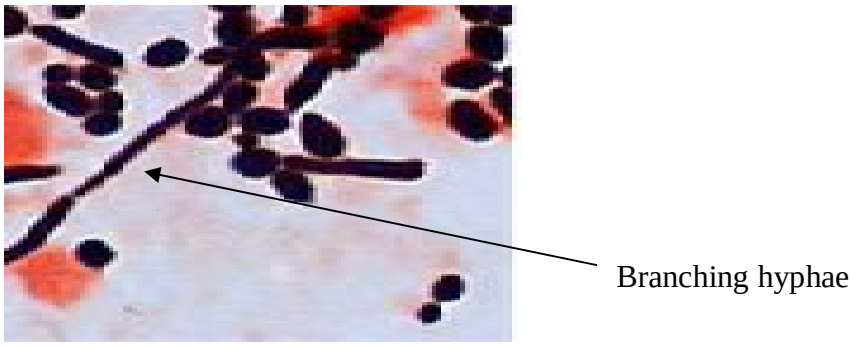

Plate 4.1: Appearance of *Candida* species showing branching hyphae in Gram stain

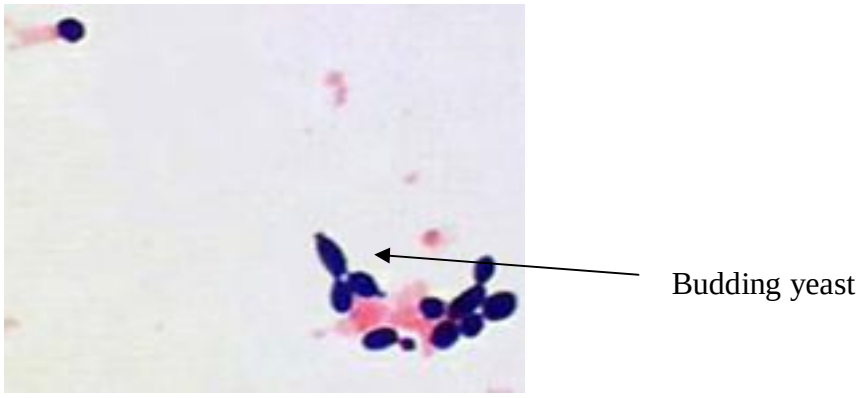

Plate 4.2: Appearance of *Candida* species showing budding yeast in Gram stain

Candida albicans produced short one piece germ tubes (Plate 4.3) and this discriminated them from *non albicans Candida* as they produced germ tubes with constrictions. However the time of germ tube formation varied from 30minutes to 60 minutes.

Based on the colony colour developed on CHROM agar Candida, 44 isolates of *Candida* were differentiated as: *C. albicans, C. krusei* (1), *C.parapsilosis (3)* and *C. glabrata* (1). One isolate showed an intermittent green-coloured colony on CHROM agar, thereby making it difficult to differentiate between *C.dubliniensis* and *C. albicans*. However, this isolate was reported as *C. albicans* as it grew on SDA. Colonies of *Candida albicans* appeared green while those of *Candida parapsilosis* were pink (Plate 4.4).

All the 44 isolates identified as *C. albicans* on CHROMagar Candida failed to exhibit the hyphal fringe on Pal's agar. The single *Candida* isolate that showed an intermittent green-coloured colony on CHROMagar did not produce a hyphal fringe either and thus was reported as *C. albicans*. They instead appeared as smooth colonies (Plate 4.6). However the only isolate of *C. krusei* exhibited a hyphal fringe (Plate 4.7).

In addition, *Candida albicans* produced few chlamydospores and only in singles (Plate 4-5) unlike the NAC species which exhibited them abundantly. All the four *Candida* species utilized glucose, galactose, sucrose and maltose but *C. albicans* utilized raffinose too (Plate 4.5).

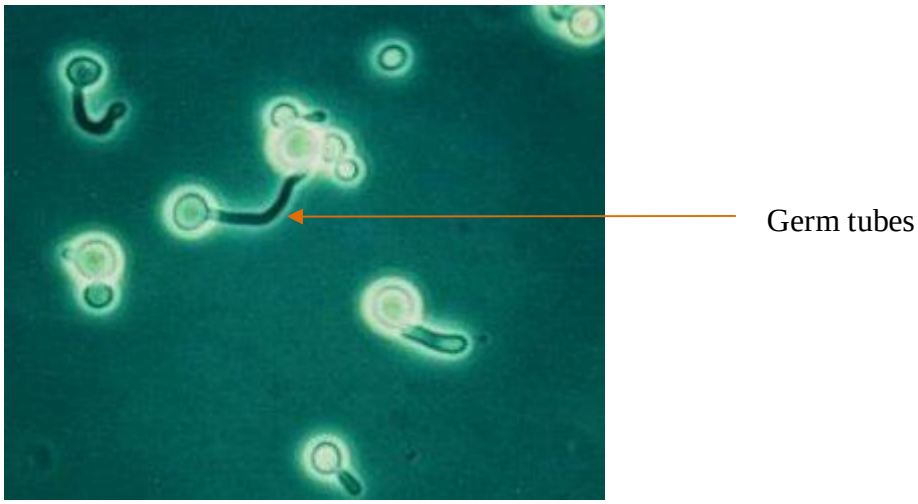

Plate 4.3: Appearance of *Candida albicans* showing germ tubes in serum

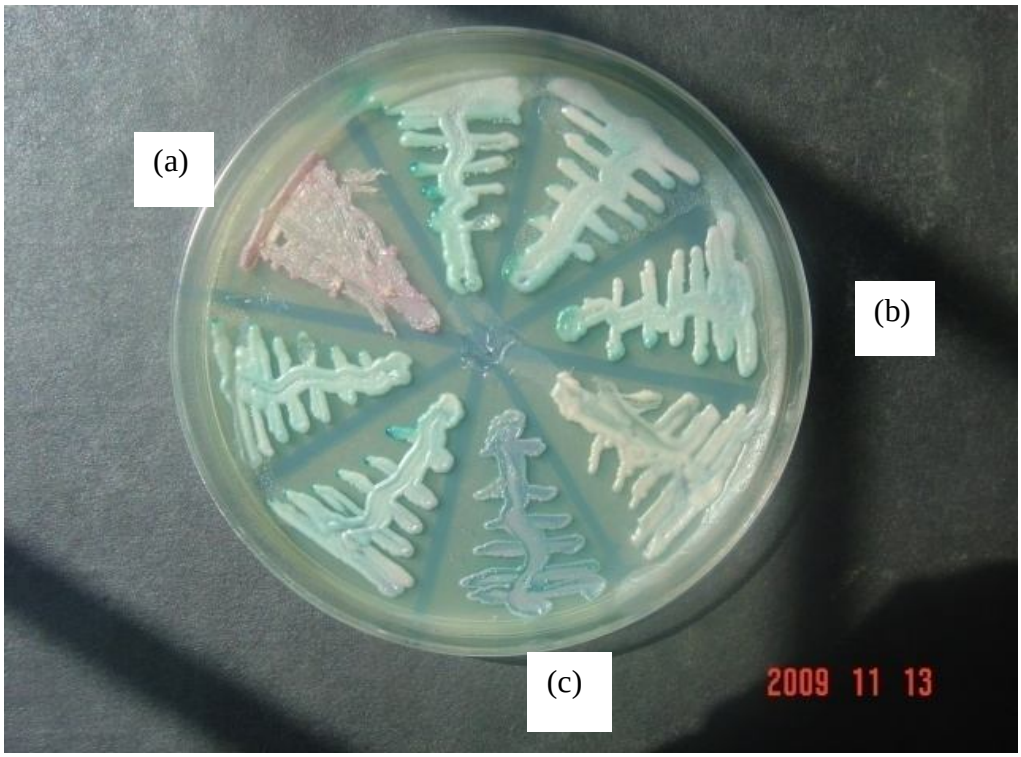

Plate 4.4: Chromogenic agar Candida plate showing colonies of (a) *Candida parapsilosis, (b) Candida albicans and (c) Candida tropicalis*

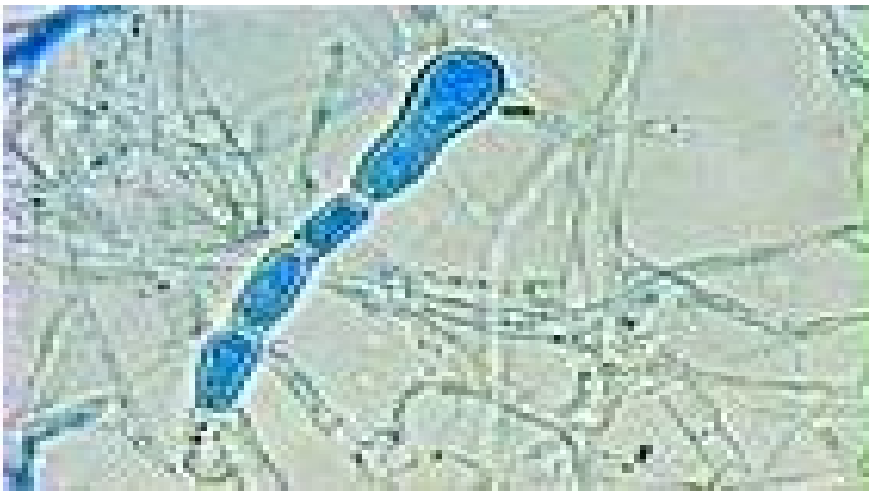

Plate 4.5: Microscopic appearance of chlamydospores in singles in Chromogenic agar Candida by *Candida albicans*

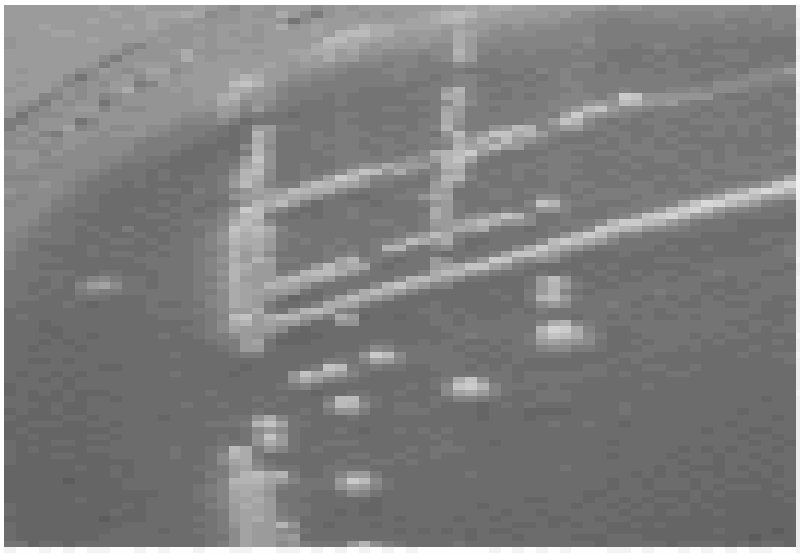

Plate 4.6: Appearance of smooth colonies of *Candida albicans* on Pal's agar

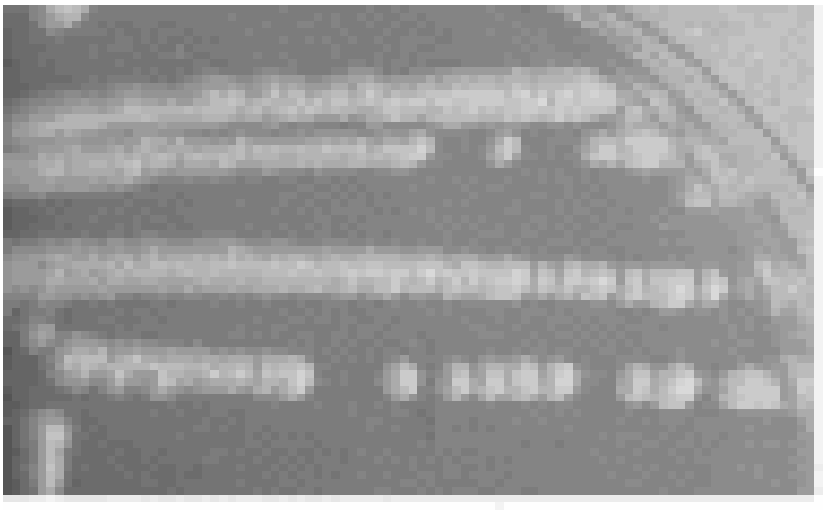

Plate 4.7: Appearance of rough colonies with a hyphal fringe of *Candida krusei* on Pal's agar

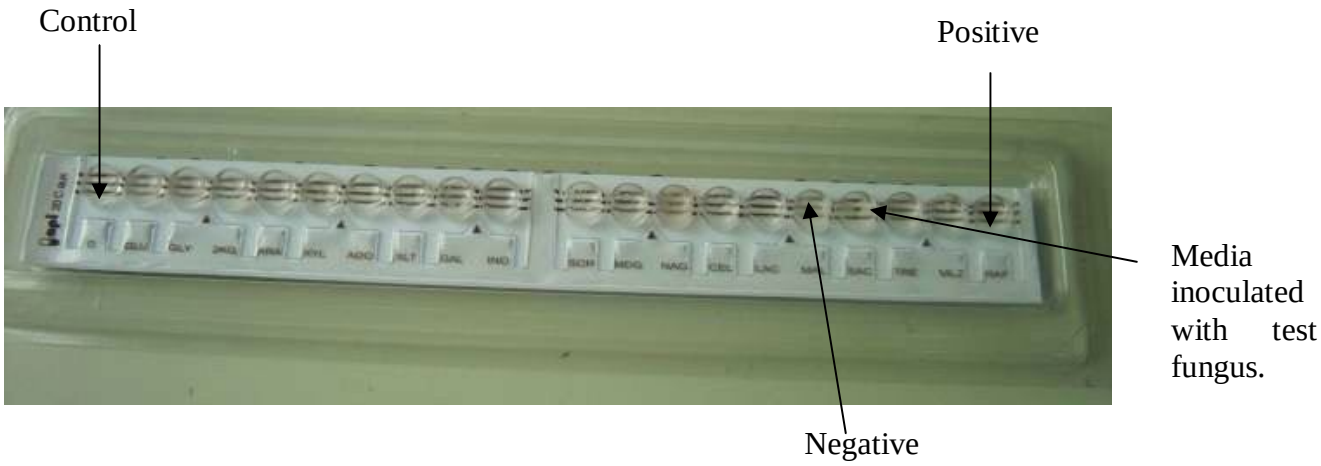

Plate 4.8: Analytical profile index (API 20C AUX) test kit containing media inoculated with test fungus

(Table 4.1) most of the isolates were identified as *Candida albicans*. They accounted for 90% of all the isolates with *C. glabrata, C. famata* and *C. parapsilosis* accounting for

2% and 6% respectively. This is a clear manifestation that most *Candida* conditions in the study were due to *C. albicans.*

Table 4.1: Distribution of *Candida* species isolated.

	Number of isolates	Percent	Cumulative Percent
C. albicans	45	90.0	90.0
C. glabrata	1	2.0	92.0
C. parapsilosis	3	6.0	96.0
C. famata	1	2.0	100.0
Total	50	100.0	

4.2 Antifungal susceptibility tests

To determine the performance of the new generation and the old generation drugs *in vitro*. In both the disc diffusion and E-test test, isolates were classified as being susceptible, intermediate or resistant.

4.2.1 Disc diffusion susceptibility tests for first generation antifungal drugs

In the disc test for clotrimazole there was resistance in only 1 isolate, 3 isolates were classified as intermediate while 46 were susceptible (Table 4.2). In the case of

fluconazole, there was resistance in 48% of the isolates, 6% were intermediate while 46% were susceptible (Table 4.3).

Table 4.2: Susceptibility of *Candida* isolates to clotrimazole using the disc diffusion method

	Number of isolates	Percent	Cumulative Percent
Susceptible	46	92.0	92.0
Intermediate	3	6.0	98.0
Resistant	1	2.0	100.0
Total	50	100.0	

Table 4.3: Susceptibility of *Candida* isolates to fluconazole using the disc diffusion method

	Number of isolates	Percent	Cumulative Percent
Susceptible	23	46.0	46.0
Intermediate	3	6.0	52.0
Resistant	24	48.0	100.0
Total	50	100.0	

4.2.2 Disc diffusion susceptibility tests for new generation drugs

Out of the 50 isolates 74% (n=37) were susceptible, 22% (n=11) were resistant while 4% (n=2) were classified as intermediate when itraconazole was used (Table 4.4). For the case of posaconazole 78% (n=39) were susceptible, 10% (n=5) were resistant while 12% (n=6) intermediate (Table 4.5).

Table 4.4: Susceptibility of *Candida* isolates to Itraconazole using the disc diffusion method

	Number of isolates	Percent	Cumulative Percent
Susceptible	37	74.0	74.0
Intermediate	2	4.0	78.0
Resistant	11	22.0	100.0
Total	50	100.0	

Table 4.5: Susceptibility of *Candida* isolates to posaconazole using the disc diffusion method

	Number of isolates	Percent	Cumulative Percent
Susceptible	39	78.0	78.0
Intermediate	6	12.0	90.0
Resistant	5	10.0	100.0
Total	50	100.0	

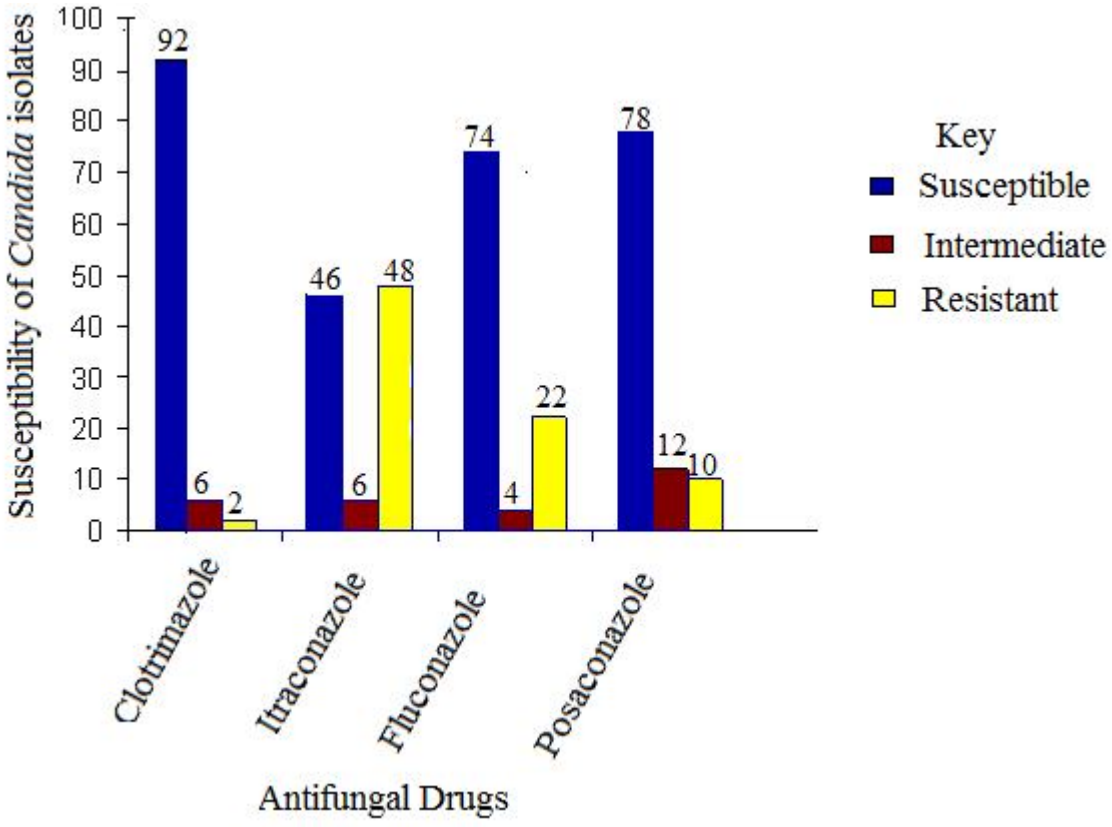

Fig 4.2: Susceptibility of *Candida* isolates to azoles using Disc Diffusion Method

4.2.3 Epsilometer susceptibility tests for first generation antifungal drugs.

In the E-test for clotrimazole, there was resistance in 4 %(n=2) of the isolates, 18% (n=9) were classified as intermediate while 39 were susceptible (Table 4.6). In fluconazole, there was resistance in 26 %(n=13) of the isolates, 14% (n=7) were intermediate while 60 %(n=30) were susceptible (Table 4.7).

Table 4.6: Susceptibility of *Candida* isolates to clotrimazole using Epsilometer-test

	Number of isolates	Percent	Cumulative Percent
Susceptible	39	78.0	78.0
Intermediate	9	18.0	96.0
Resistant	2	4.0	100.0
Total	50	100.0	

Table 4.7: Susceptibility of *Candida* isolates to fluconazole using Epsilometer-test

	Number of isolates	Percent	Cumulative Percent
Susceptible	30	60.0	60.0
Intermediate	7	14.0	74.0
Resistant	13	26.0	100.0
Total	50	100.0	

4.2.4 Epsilometer susceptibility tests for new generation azoles

Of the 50 *Candida* isolates tested, 56% (n=28) were susceptible, 32% (n=16) were resistant while 12% (n=6) were classified as intermediary susceptible to itraconazole (Table 4.8). For posaconazole, 78% (n=39) were susceptible, 6% (n=3) were resistant while 16% (n=8) were intermediary susceptible (Table 4.9).

Table 4.8: Susceptibility of *Candida* isolates to itraconazole using Epsilometer-test

	Number of isolates	Percent	Cumulative Percent
Susceptible	28	56.0	56.0
Intermediate	6	12.0	68.0
Resistant	16	32.0	100.0
Total	50	100.0	

Table 4.9: Susceptibility of *Candida* isolates to posaconazole using Epsilometer-test

	Number of isolates	Percent	Cumulative Percent
Susceptible	39	78.0	78.0
Intermediate	8	16.0	94.0
Resistant	3	6.0	100.0
Total	50	100.0	

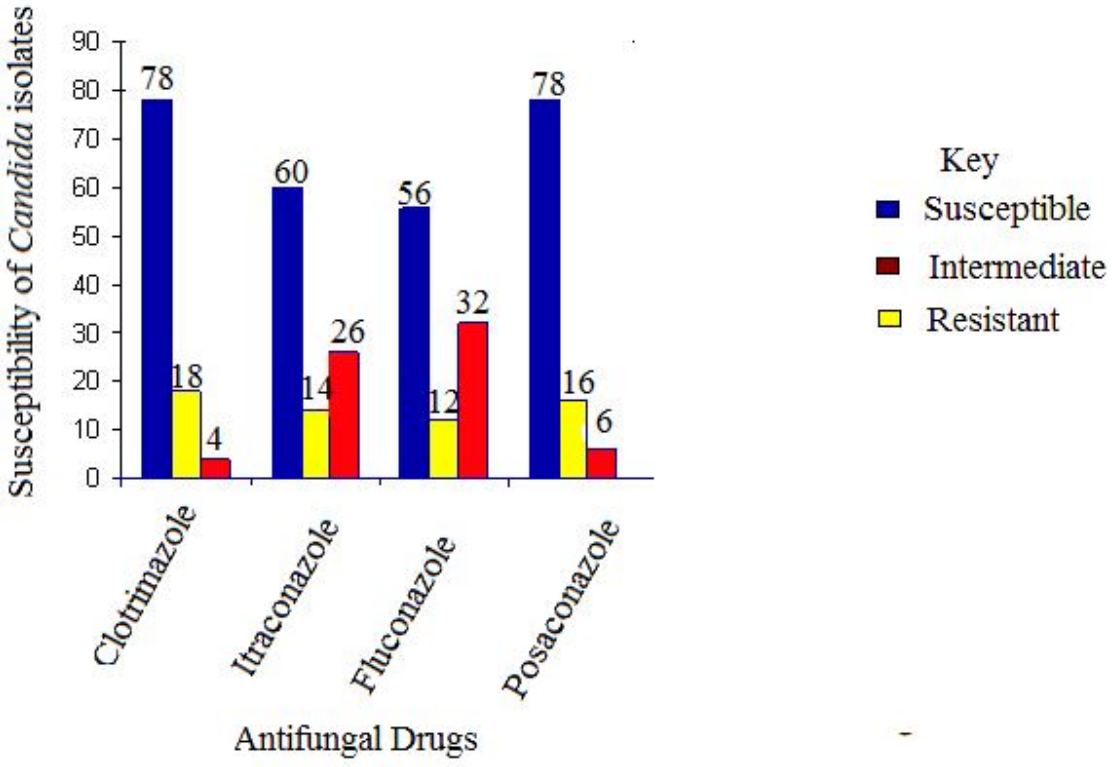

Fig 4.3: Susceptibility of *Candida* isolates to azoles using Epsilometer-test

4.3 Minimum inhibitory concentration levels (MIC_{90} and MIC_{50}) for *Candida* isolates

The MICs at which 50% of isolates were inhibited (MIC_{50}s), and the MIC_{90}s (MICs at which 90% of isolates of *Candida* species were inhibited) are presented in (Figures 4.4-4.7). MIC_{90} and MIC_{50} for posaconazole were 0.600µg/ml and 0.007µg/ml respectively (Fig.4.4). The MIC_{90} and MIC_{50} for fluconazole were higher (140µg/ml and 0.750µg/ml respectively) (Fig.4.5). Those of itraconazole were also high recording MIC_{90} of 15.00 µg/ml and MIC_{50} of 0.064µg/ml (Fig. 4.6). The MIC_{90} value for clotrimazole was 28

µg/ml which was low compared to the break point value for the drug which is 64 µg/ml (Fig 4.7).

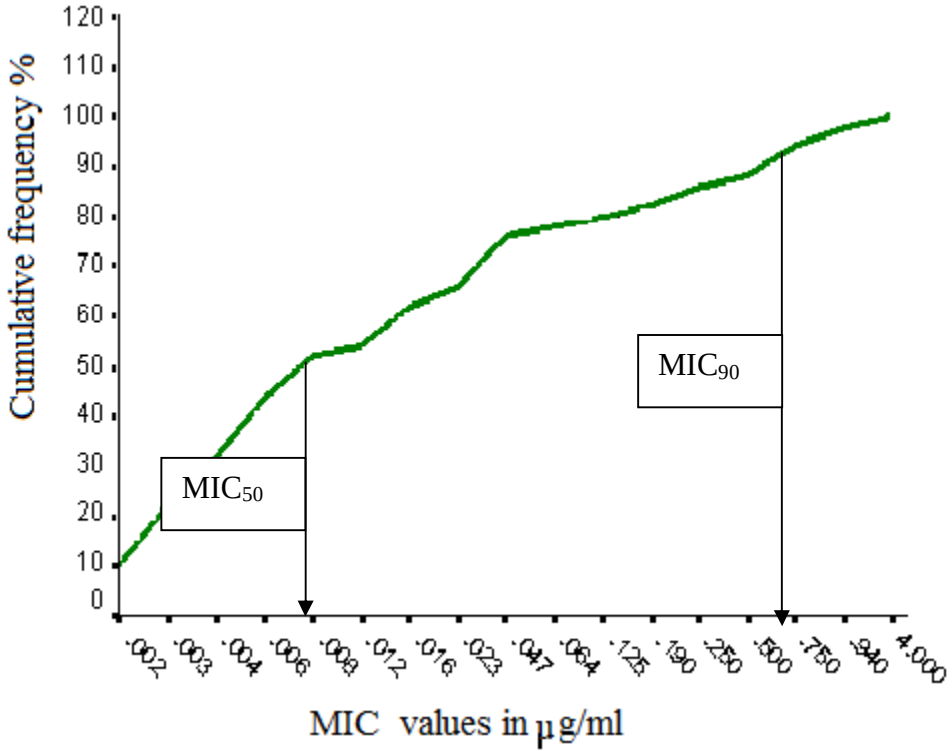

Fig 4.4: Minimum inhibitory concentration levels of posaconazole for *Candida* isolates

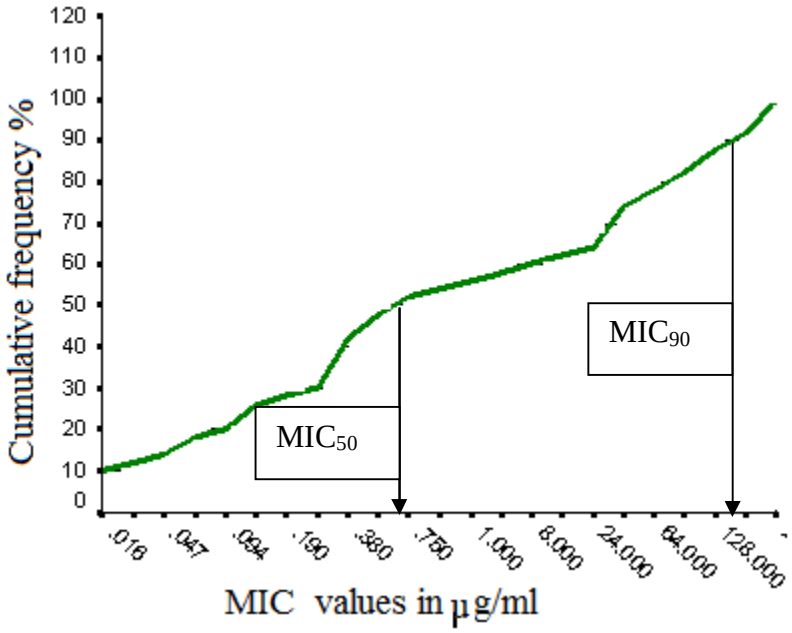

Fig 4.5: Minimum inhibitory concentration levels of fluconazole for *Candida* isolates

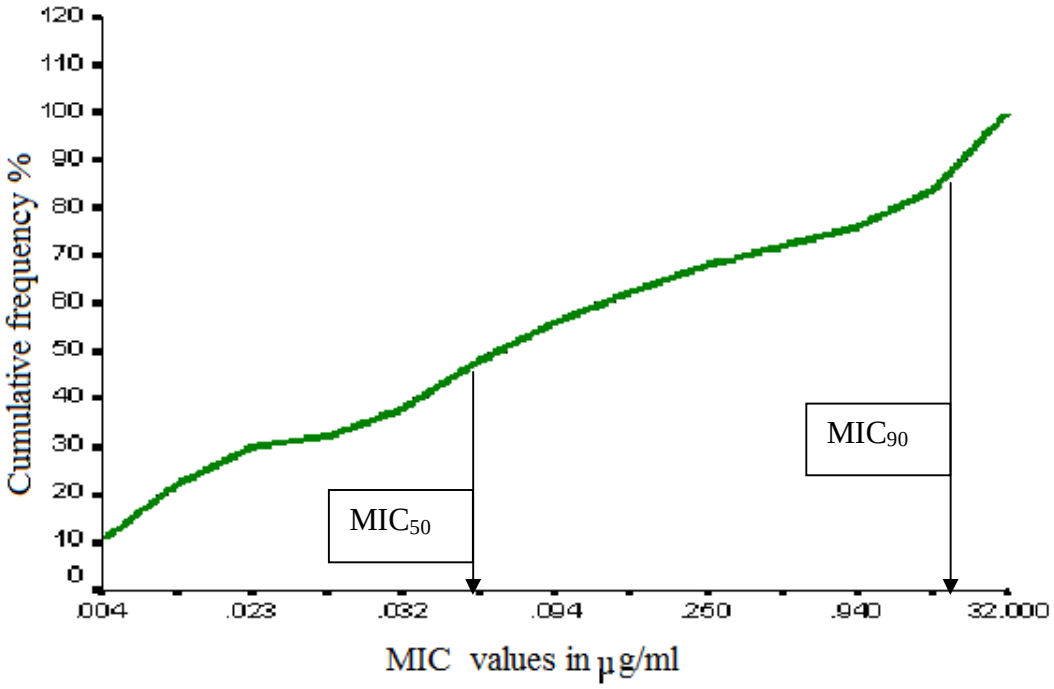

Fig 4.6: Minimum inhibitory concentration levels of itraconazole for *Candida* isolates

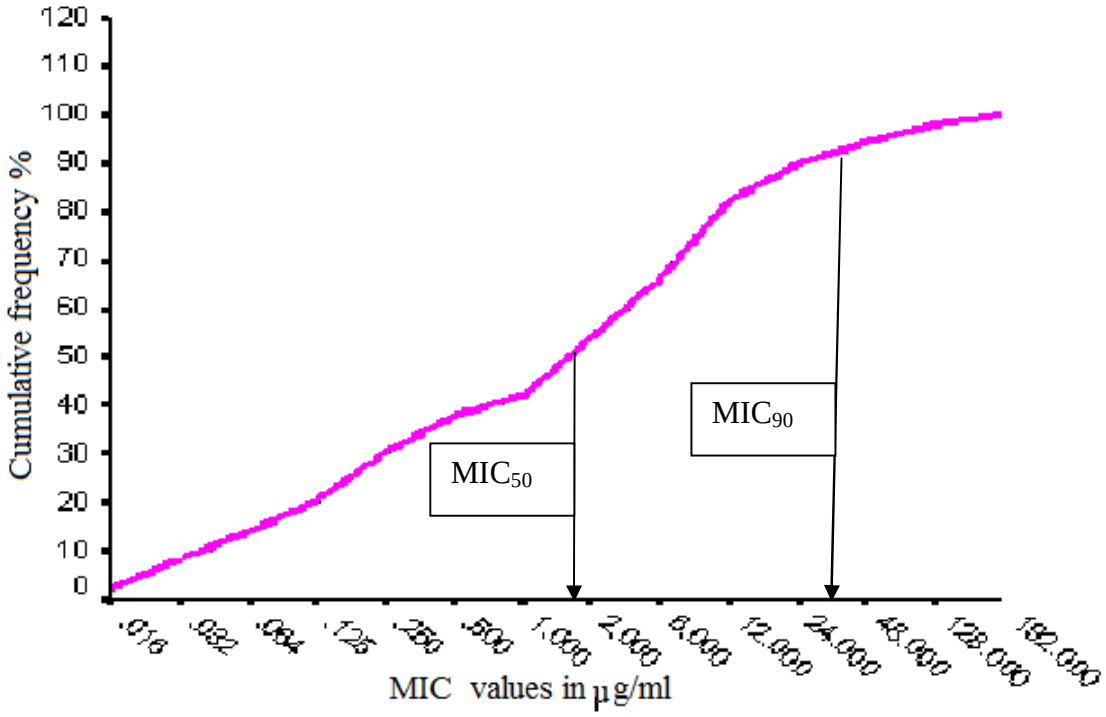

Fig 4.7: Minimum inhibitory concentration levels of clotrimazole for *Candida* isolates

4.4 Kappa tests

Kappa tests were done in order to quantify the degree of agreement between the E-test and disc diffusion method. A kappa value very close to zero indicated a lot of agreement between the two tests while a value that is tending towards one showed total disagreement consequently a *p* value that was very small compared to 0.05 showed a high statistical significance and a value close to 0.05 indicated a low statistical significance.

4.4.1 Kappa test for fluconazole

For fluconazole, 19 isolates were classified as susceptible in both the E-test and disc method, while 10 were classified as resistant in both cases. The biggest difference was in the 11 isolates which were classified as susceptible in E-test while in disc test they were resistant. The kappa value in this case was 0.329 while the p value was 0.002 (Table 4.10a and b).

Table 4.10a: Kappa test for fluconazole

		Fluconazole E-Test Performance			Total
		Susceptible	Intermediate	Resistant	
Fluconazole-Disc-Performance	Susceptible	19	2	2	23
	Intermediate	0	2	1	3
	Resistant	11	3	10	24
Total		30	7	13	50

Table 4.10b: Fluconazole p-values

		Value	Asymp. Std. Error(a)	Approx. T(b)	Approx. Sig. (P value)
Measure of Agreement	Kappa	.329	.106	3.043	.002
Number of isolates		50			

a) Not assuming the null hypothesis.

b) Using the asymptotic standard error assuming the null hypothesis.

4.4.2 Kappa test in clotrimazole

In clotrimazole, there was a lot of agreement with 38 of the samples being classified as susceptible in both E-test and disc method. The kappa value was 0.261while the p value was given as 0.011.There was more agreement in the clotrimazole tests than those in fluconazole test (table 4.11a and b).

Table 4.11a: Kappa test in clotrimazole

		Clotrimazole E test Performance			Total
		Susceptible	Intermediate	Resistant	
Clotrimazole-Disc-Performance	Susceptble	38	8	0	46
	Intermediate	1	1	1	3
	Resistant	0	0	1	1
Total		39	9	2	50

Table 4.11b: Clotrimazole p-values

		Value	Asymp. Std. Error(a)	Approx. T(b)	Approx. Sig. (P value)
Measure of Agreement	Kappa	.261	.148	2.556	.011
Number of isolates		50			

a) Not assuming the null hypothesis.

b) Using the asymptotic standard error assuming the null hypothesis.

4.4.3 Kappa test in itraconazole

There was disagreement too in itraconazole with kappa value of 0.216 and the p value was 0.045 (Table 4.12a and b). 25 isolates were susceptible in both disc and E-test. However, 3 isolates that were susceptible in disc were intermediate in E-test and 9 isolates that were susceptible in disc were resistant in E-test.

Table 4.12a: Kappa test in itraconazole

		Itraconazole E -Test Performance			Total
		Susceptible	Intermediate	Resistant	
Itraconazole-Disc-Performance	Susceptible	25	3	9	37
	Intermediate	0	0	2	2
	Resistant	3	3	5	11
Total		28	6	16	50

Table 4.12b: Itraconazole p-value

		Value	Asymp. Std. Error(a)	Approx. T(b)	Approx. Sig. (P value)
Measure of Agreement	Kappa	.216	.102	2.004	.045
Number of isolates		50			

a) Not assuming the null hypothesis.

b) Using the asymptotic standard error assuming the null hypothesis.

4.4.4 Kappa test in posaconazole

In posaconazole there were 33 isolates that were susceptible in both E-test and disc test. The kappa value was 0.290 while the p value was 0.008 (Table 4.13a and b).

Table 4.13a: Kappa test in posaconazole

		Posaconazole E Test -Performance			Total
		Susceptible	Intermediate	Resistant	
Posaconazole-Disc-Performance	Susceptible	33	5	1	39
	Intermediate	4	2	0	6
	Resistant	2	1	2	5
Total		39	8	3	50

Table 4.13b: Posaconazole p-values

		Value	Asymp. Std. Error(a)	Approx. T(b)	Approx. Sig. (P value)
Measure of Agreement	Kappa	.290	.143	2.667	.008
Number of isolates		50			

a) Not assuming the null hypothesis.

b) Using the asymptotic standard error assuming the null hypothesis.

4.5 Effectiveness of drug on individual *Candida* species

To conclusively measure the effectiveness of the drugs, they were investigated on individual *Candida* species. Cross tabulation technique yielded the following results.

4.5.1 Itraconazole performance on individual *Candida* species

From Table 4.14a, all the resistance to itraconazole occurred in *C. albicans* whereas C *.famata, C. parapsilosis and C. glabrata* were susceptible to the drug. The two isolates that fell under intermediate were also *C. albicans.* There were similar results for the E-test with all the non *Candida albicans* being susceptible to the drug (Plate 4.9) There was resistance in 14 of *Candida albicans* while 6 were classified as intermediate (Table 4.14.b). This is a clear indication that itraconazole is a very effective drug on other species like *C.famata, C.parapsilosis* and *C. glabrata* but has low effectiveness on *Candida albicans.*

Table 4.14a: Itraconazole performance on individual *Candida* species using disc diffusion method

Species	Susceptible	Intermediate	Resistant	Total
C. albicans	32	2	11	45
C. glabrata	1	0	0	1
C. parapsilosis	2	0	0	2
C. famata	2	0	0	2
Total	37	2	11	50

Table 4.14.b: Itraconazole performance on individual *Candida* species using Epsilometer-test

Candida Species	Susceptible	Intermediate	Resistant	Total
C .albicans	25	6	14	45
C. glabrata	0	0	1	1
C. parapsilosis	1	0	1	2
C. famata	2	0	0	2
Total	28	6	16	50

4.5.2 Fluconazole sensitivity

Most of the non *C. albicans* were resistant to fluconazole in E-test in addition to the 21 *C. albicans* which were also resistant under the disc test. Under disc method 1 of the *C. parapsilosis* was classified under intermediate while the other one was resistant. Similar results were obtained in *C. famata* (Plate 4.10). All the non *C. albicans* isolates were resistant to fluconazole (Table 4.15a). For the E-test, apart from the 12 *C. albicans* that were resistant, only *C. glabrata* was resistant while *C. famata, C. parapsilosis* were all susceptible (Table 4.15b).

Table 4.15a: Fluconazole performance on individual *Candida* species using disc diffusion method

Candida Species	Susceptible	Intermediate	Resistant	Total
C.albicans	22	2	21	45
C. glabrata	0	0	1	1
C.parapsilosis	0	1	1	2
C.famata	0	1	1	2
Total	22	4	24	50

Table 4.15b: Fluconazole performance on individual *Candida* species using Epsilometer test

Candida species	Susceptible	Intermediate	Resistant	Total
C.albicans	27	6	12	45
C. glabrata	0	0	1	1
C.parapsilosis	1	1	0	2
C.famata	2	0	0	2
Total	29	7	13	50

4.5.3 Posaconazole performance

In disc diffusion method there were 4 resistant isolates of *C.albicans* and only *C.parapsilosis* was resistant among other species. With E-test, there was no resistance in the non *C. albicans* (Plate 4.11).

Table 4.16a: Posaconazole performance on individual *Candida* species using disc diffusion method

C*andida* species	Susceptible	Intermediate	Resistant	Total
C. albicans	35	6	4	45
C. glabrata	1	0	0	1
C.parapsilosis	1	0	1	2
C. famata	2	0	0	2
Total	39	6	5	50

Table 4.16b: Posaconazole performance on individual *Candida* species using Epsilometer-test

Candida species	Susceptible	Intermediate	Resistant	Total
C. albicans	35	7	3	45
C. glabrata	1	0	0	1
C.parapsilosis	1	1	0	2
C. famata	2	0	0	2
Total	39	8	3	50

4.5.4 Clotrimazole sensitivity

From the table 4.17a none of the *C.albicans* was resistant to clotrimazole (plate 4.12). However *C.parapsilosis* isolates were resistant in disc method and E-test method (table 4.17a and b).

Table 4.17a: Clotrimazole performance on individual *Candida* species using disc diffusion method

Candida species	Susceptible	Intermediate	Resistant	Total
C. albicans	42	3	0	45
C. glabrata	1	0	0	1
C.parapsilosis	1	0	1	2
C. famata	1	1	0	2
Total	45	4	1	50

Table 4.17.b: Clotrimazole performance on individual *Candida* species using Epsilometer-test

Candida species	Susceptible	Intermediate	Resistant	Total
C. albicans	36	8	1	45
C. glabrata	1	0	0	1
C.parapsilosis	0	1	1	2
C. famata	1	1	0	2
Total	38	10	2	50

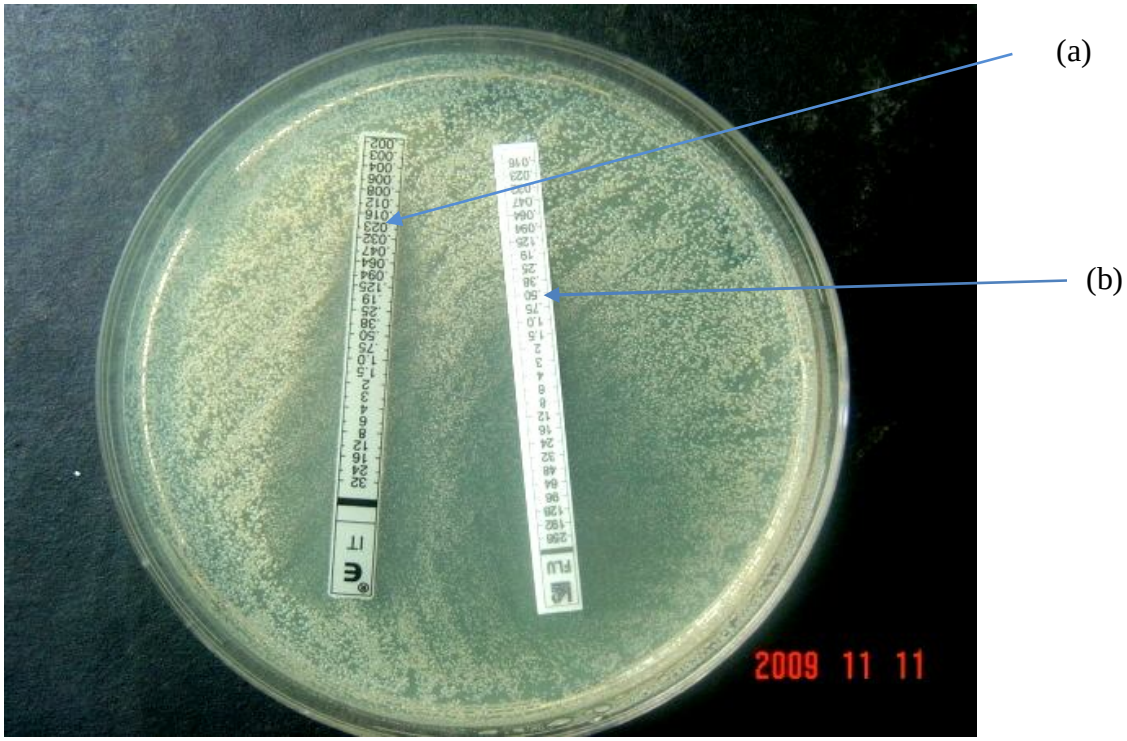

Plate 4.9: Mueller Hinton Agar plate showing susceptibility of *Candida species* to fluconazole and itraconazole by Epsilometer-test

KEY

(a)-Minimum Inhibitory Concentration level to itraconazole on E-Test strip

(b)- Minimum Inhibitory Concentration level to fluconazole on E-Test strip

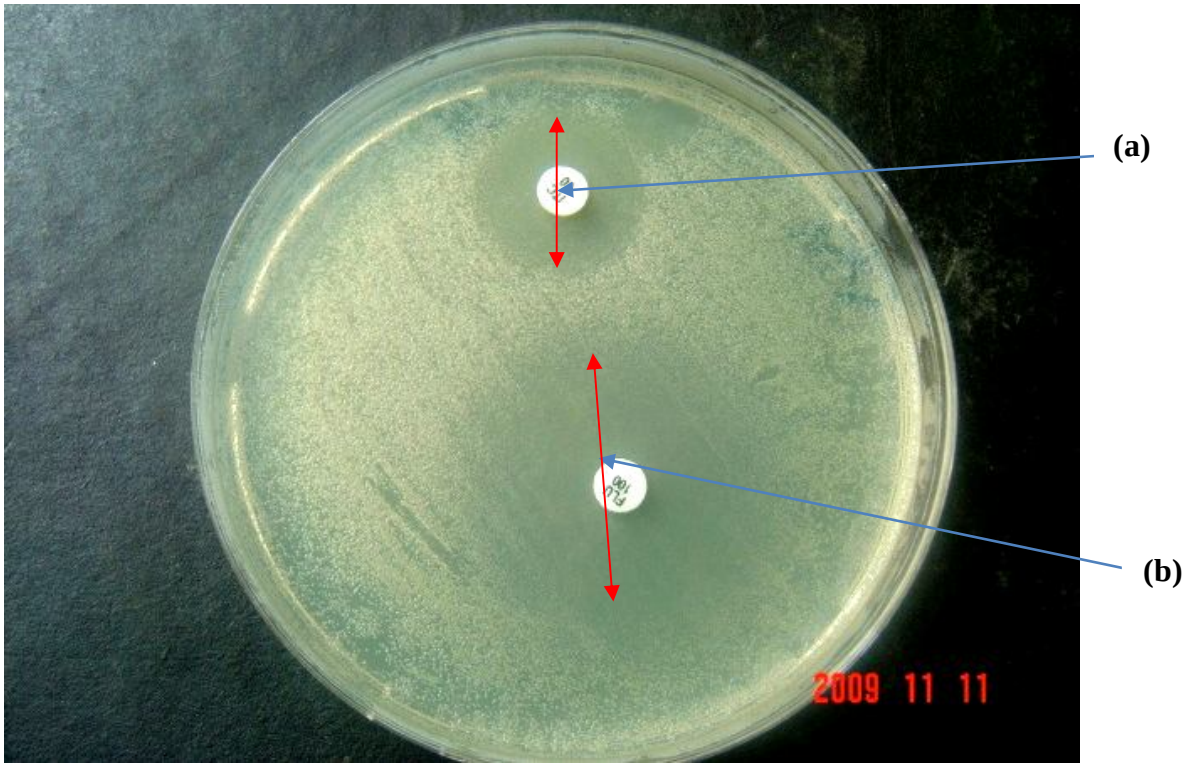

Plate 4.10: Mueller Hinton Agar plate showing susceptibilities of *Candida* species to itraconazole and fluconazole using disc diffusion method

KEY

(a)-Zone diameter of susceptibility to itraconazole

(b)-Zone diameter of susceptibility to fluconazole

Plate 4.11: Mueller Hinton Agar plate showing susceptibilities of *Candida* species to posaconazole and clotrimazole using the Epsilometer test method

KEY

(a)-Posaconazole E-Test strip showing Minimum Inhibitory Concentration level

(b)-Clotrimazole E-Test strip showing Minimum Inhibitory Concentration level

Plate 4.12: Mueller Hinton Agar plate showing susceptibility of *Candida* species to clotrimazole using the disc diffusion method

KEY

(a)-Clotrimazole disc showing zones of inhibition

(b)- Colonies of *Candida* species

CHAPTER FIVE

DISCUSSION

Candida species can be recovered from almost any site in a human body including chest cavity, bladder, vagina, wounds, cervix and even urethra (Walsh and Dixon, 1996). In this research study, origins of strains of *Candida* species studied were those of sputum, urine, High Vaginal Swabs (HVS), pus swabs, throat swabs and necrotic wounds of clinical sources. Most isolates of *Candida* were recovered from urine (42%) probably because the method of collection is not invasive and urinalysis is affordable in many clinical set ups. Unlike urine, collecting swabs from throat, the high vagina and necrotic wounds is a bit complicated and costly; hence low turnout of stock specimens.

As *Candida* species differ in their antifungal susceptibility, it is essential to identify and differentiate them. *Candida albicans* once accounted for most serious nosocomial candidal infections (Pfaller, 2000). Over the last decade *Candida* infections, specifically candidemia, due to *C. albicans* have declined. Other non-*albicans Candida* species are now responsible for about half of candidemias and other deep *Candida* infections (Walsh and Dixon, 1996). In this study, *C. albicans* was found to be the most prevalent strain (90%) of *Candida* species. Out of the 50 isolates of *Candida* that were used in this study, 45 were identified as *Candida albicans,* 1 as *Candida glabrata,* 1 as *Candida famata* and 3 as *Candida parapsilosis*. This indicates that most infections of candidiasis were due to *Candida albicans* (>90%). Infections due to non *albicans candida* were less significant (<10%).

Gram staining technique was used as a preliminary test prior to identification just to ensure that the archived *Candida* species were still viable and free of contamination. Gram negative isolates were regarded as contaminated with bacteria as all yeasts are gram positive.

Culture on SDA was done to obtain pure colonies that could be used for identification and later susceptibility testing. In this study, morphocultural characterization of the isolated *Candida* species showed cream-colored, circular and convex colonies with large, spherical cells with sizes ranging from 2.5-12.5 μm. The difference in colony sizes could have been attributed to the individual characteristics of the different species of *Candida.* However their sufficient growth indicated a proper adaptation of *Candida* species to the contents of the medium.

Candida albicans produced short one piece germ tubes in serum in varied times (30-60 minutes). This happened because the different subtypes of *C.albicans* absorbed essential growth nutrients required for germ tube formation at unequal rates and proportions (Richardson *et al.,* 1981).

CHROMagar *Candida* was used as a primary medium for differentiating between various *Candida* species based on their colony colour and morphology (rough/smooth) (Pfaller *et al.,* 1996). Pal's agar was considered as a secondary medium for differentiation of *Candida* species. In this study, colonies of *C.krusei* produced a hyphal fringe on Pal's agar. Ability to produce hyphal fringe on Pal's agar is attributed to pseudohyphae production by *C.krusei.*

Most fungi play an important role in the utilization of various substrates including hydrocarbons, nuceic acids, carbohydrates and uric acid **(**Buesching *et al.,* 1979)**.** *Candida* species utilise these substrates for carbon sources like glucose, galactose, maltose, sucrose, trehalose, xylose, sorbitol, N-acetyl-glucosamine, 2-keto-gluconate, glycerol, arabinose, cellobiose, raffinose, xylitol, inositol, and lactose (Buesching *et al,* 1979)**.** The API 20C AUX (bio Merieux) system was used in this study and was able to provide the correct identification for all the isolates tested after 48 h of incubation, because it also incorporated the results of a morphological assessment. In this study, all *Candida* species utilized glucose, galactose, sucrose and maltose. All the non *albicans candida* except *Candida famata* utilized starch. *Candida albicans* utilized raffinose in addition to the four sugars in common.

Many laboratories now use commercial identification systems to determine the physiological profiles of yeast isolates. Some of these systems offer extensive databases; capable of identifying a wide range of taxa, but others are much more limited in scope. In either case, a morphological assessment of isolates remains essential to avoid errors in the identification of organisms with identical biochemical profiles. However, small but worrisome number of incorrect identifications may be obtained with morphological assessment (Buchaille *et al.,* 1998). For instance, in this research study one chlamydospore-forming *Candida* spp. was misidentified as *C. famata* during this evaluation but later confirmed with API AUX (Bio Meriux).

Imidazoles have been effective in the treatment of mycotic infections over the past years but emergence of resistance is continually increasing. Thus, there is a big challenge in

the future treatment of fungal infections which makes it necessary for constant monitoring of antifungal drugs to detect further emergency of resistance.

However, patients with AIDS present quite a different venue for these imidazoles. Chronic and repetitive use of antifungal azoles to treat protracted opportunistic infections in HIV infected patients predisposes to the development of resistance (Law *et al.,* 1994). The appearance of secondary azole resistance to this date has been demonstrated most commonly in HIV positive patients who previously received fluconazole (Millon *et al*., 1994).

Clotrimazole is a tritylimidazole derivative [bis-phenyl-(2chlorophenyl)-1 imidazolylmethane] that was discovered in 1968. The use of clotrimazole has grown substantially since its discovery in 1968. Far from its initial limited use as a systemic antifungal agent, clotrimazole is now utilized extensively as a front-line topical antifungal for prevention or treatment of mucosal candidiasis in immunocompromised patients (Plempel *et al.,* 1969).

While many studies were performed assessing the efficacy of clotrimazole for different uses over the past 3 decades (Holt, 1972), clotrimazole was examined in the contemporary context of emerging azole resistance. It has been shown to inhibit all the major fungi causing systemic infections at a concentration of 1 ug /ml particularly *Candida, Histoplasma* and *Aspergillus* spp. Consequently most isolates of *Candida* spp. show susceptibility to clotrimazole in E-test and disc diffusion method. From the findings it can be concluded that clotrimazole is still an effective antifungal agent. This

is because there was only 2% resistance in the *Candida* species tested on disc method. Likewise to other studies, only 4% of *Candida* species were resistant in E-test method. As much as the drug is a first generation azole, there is a clear indication that it is still strong and effective against *Candida* infections. Although resistance has been reputed in the previous studies (Bii *et al*., 2002), the MIC range to clotrimazole has been shown to be <1ug/ml in this study. However due to introduction of newer azoles, clotrimazole is now not commonly used.

Fluconazole's susceptibility testing revealed that there is emerging resistance to the drug. In this study, isolates of *C. albicans, C. parapsilosis*, and *C. tropicalis* were all highly resistant to fluconazole (48 to 90% at > or =8 microg/ml). In the disc test 48% of the isolates were resistant to the drug while 46% were susceptible. However, some appreciable disagreement was noted between the disc and E-test where there was 46% susceptibility and 50% resistance and 60% susceptibility and 26% resistance, respectively.

Modifications to the NCCLS method in terms of drug characteristics such as solubility, incubation time, buffer and glucose concentration, pH, inoculum size and endpoint determination has been shown to affect susceptibility results (NCCLS, 1997). However as much as there were discrepancies in the tests it indicates that there is high resistance to the drug. The MIC_{90} value was >140 µg/ml which is far above the breakpoint (64.000 µg/ml). According to the results of this study, there is an indication that there is emerging resistance and the drug may be ineffective especially in the treatment of

mycoses due to *Candida albicans* as these accounted for >90% of all species investigated. Fluconazole being a first generation azole, the resistance could be due to the over exposure of the *Candida spp*. to the drug associated with HIV/AIDS pandemic, irrational use of antibiotics and prophylactic use of the drug (Maenza *et al*., 1996).

Isolation of *Candida species* less susceptible to commonly used anti-fungal drugs and recovery of increasingly resistant isolates during antifungal therapy is a growing concern (Pfaller *et al*., 1998). For instance, prophylactic fluconazole only reduces the incidence of colonization by invasive *Candida* infection in neonates with very low birth weight but adults still get serious attacks, especially the immunocompromised and those with debilitating conditions (Vendettuoli *et al.,* 2009). This is therefore a significant risk factor for emerging azoles resistance in HIV/AIDS patients.

Posaconazole is a new member of the triazoles class of antifungals with broad spectrum antifungal activity. Based on the findings of this study; it could be predicted that posaconazole would be active against the agents responsible for the majority of moderate and severe mycoses isolated in clinical set ups in Nairobi, Kenya. In this study, 78% of the *Candida* species were susceptible in both E-test and disc methods. Its low MIC_{90} of 0.600 µg/ml is a clear indication that the drug is still very effective in the treatment of infections due to *Candida* species. Likewise, 97 to 100% of *Candida* species were inhibited by < or =1 microg/ml of posaconazole in the study.

It has demonstrated clinical efficacy in the treatment of oropharyngeal candidiasis (Kauffman, 2006). In addition, data from randomized controlled studies support its

efficacy for use in prophylaxis of invasive fungal infections in patients who are severely immune- compromised (Kauffman, 2006). The wide spectrum activity of posaconazole in *in-vitro* studies, animal models and preliminary clinical studies suggest that posaconazole represents an important addition to the antifungal armamentarium (Oliveira *et al.*, 2005).

In this study, the majority of the fluconazole-resistant strains were susceptible to posaconazole**.** Other species of *Candida* that is, *C. parapsilosis* and *C. famata* were also highly susceptible to posaconazole. In disc diffusion method there were only 4 resistant isolates of *C.albicans.* Only *C. parapsilosis* was resistant among the non- *albicans candida*. Under the E-test there was no resistance in non *C. albicans*. Hence posaconazole is a very strong universal antifungal being a new generation drug.

Itraconazole has a broader spectrum of activity than fluconazole (Rambali *et al.*, 2001). Its major advantages over fluconazole are its activity particularly against most *Aspergillus* isolates and a subset of fluconazole-resistant *Candida* strains (Bailey *et al.*, 1990). However in the study, itraconazole was active on only one case of the fluconazole-resistant *Candida albicans*. Itraconazole has been shown to be active against serious fungal or yeast infections, such as oropharyngeal candidiasis (oral thrush), and esophageal candidiasis (*candida* esophagitis) (McGinnis and Rinaldi, 1996). Performance of itraconazole also indicated considerable resistance. 22% of *candida* species were resistant in disc method while that of E-test was 16%. In disc method, resistance to the drug occurred mainly in *Candida albicans* whereas *C. famata, C.*

parapsilosis and C. glabrata were susceptible to the drug. The two isolates that fell under intermediate were also *Candida albicans.*

There were similar results for the E-test with all the non *Candida albicans* being susceptible to the drug. There was resistance in 14 of *Candida albicans* while 6 were classified as intermediate. Given that 90% of the species were *C. albicans* there could be a possibility that the drug is effective on other *Candida* species like *C. famata, C. parapsilosis* and *C. glabrata* and less on *C. albicans*. The MIC_{90} of 15.00µg/ml for this drug is far beyond the breakpoint of the drug. Since itraconazole is a new generation drug, it is expected to be more effective. Therefore further investigation needs to be done on the performance of the drug on other *Candida* species especially those not included in the present study.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The research study characterized archived *Candida* species that originated from various specimens. The conventional morphocultural methods (germ tube test, CHROMagar Candida, slide culture and Pal's agar) re-identified the *Candida* spp. The biochemical tests (API 20C AUX) further confirmed the isolates as *C. albicans, C. famata, C. parapsilosis* and *C. glabrata* in agreement with the identification based on their morphocultural characteristics. One isolate, initially identified as *C. albicans* using the germ tube test was later identified as *C.famata* using the API 20C AUX profile. The combined application of morphocultural and biochemical methods proved useful in the identification and confirmation of *Candida* species.

Susceptibility results show that there is emerging fungal resistance to azoles particularly fluconazole and new generation triazoles particularly itraconazole. However clotrimazole and posaconazole are still effective in the treatment of *Candida* infections.

6.2 Recommendations

- Further investigations on fungal resistance against various new generation tri-azoles using various susceptibility methods.
- Rational use of antifungal and antibiotics drugs to curb emerging resistance.

- Development of new drugs with different mechanisms of action from those of the azoles.
- Expansion of the spectrum of activities of the current antifungal drugs to cover a wide spectrum of *Candida* infections.

REFERENCES

Al M. A., Sullivan D. J. and Coleman D.C. (2003). Differentiation of *Candida dubliniensis* from *Candida albicans* on Pal's agar. *CLINICAL MICROBIOLOGY*; **41:**4787-4789.

Anaissie E. J. and Bodey G. P. (**1989)**. Nosocomial fungal infections—old problems and new challenges. *INFECTIOUS DISEASES*, **3:**867-882.

Bailey E. M., Krakovsky D. J. and Rybak M. J. (1990). The triazole antifungal agents: A review of itraconazole and fluconazole. *PHARMACOTHERAPY*, **10:**146-153.

Banerjee S. N., Emori T. G., Culver D. H., Gaynes R. P., Jarvis W. R., Horan T., Edwards J. R., Tolson J., Henderson T., Marone W. J. (1991). Secular trends in nosocomial primary bloodstream infections in the United States, 1980-1989. *MEDICAL MYCOLOGY*, **91:**86S-89S.

Barry A. L. and Brown S. D. (1996). Fluconazole disk diffusion procedure for determining susceptibility of *Candida* species. *CLINICAL MICROBIOLOGY*, **34:**2154-2157.

Barry A. L., Pfaller M. A., Brown S. D., Espinel-Ingroff A., Ghannoum M. A., Knapp C., Rennie R. P., Rex J. H. and Rinaldi M. G. (**2000).** Quality control limits for broth micro dilution susceptibility tests of ten antifungal agents. *JOURNAL OF CLINICAL MICROBIOLOGY*, **38:**3457-3459.

Beck-Sagué C. M. and Jarvis W.R. (1993). National nosocomial infections surveillance system**.** Secular trends in the epidemiology of nosocomial fungal infections in the United States. *JOURNAL OF INFECTIOUS DISEASES,* **167:**1247-1251.

Bii C.C., Ouko T.T., Amukoye E. and Githinji L.W. (2002). Antifungal drug susceptibility of Candida albicans. *EAST AFRICAN MEDICAL JOURNAL,* **79**:3.

Bodey, G. P. (1988). The emergence of fungi as major hospital pathogens. *HOSPITAL INFECTIONS,* **11:**411-426.

Buchaille L., Freydiere A. M., Guinet R. and Gille. Y. (1998). Evaluation of six commercial systems for the identification of medically important yeasts. *CLINICAL MICROBIOLOGY AND INFECTIOUS DISEASES,* **17:**479-488

Buesching W. J., Kurek K. and Roberts G. D. (1979). Evaluation of the modified API 20C system for identification of clinically important yeasts. *JOURNAL OF CLINICAL MICROBIOLOGY.* **9:**565-569.

Craigmill A. (1991). Gentian Violet Policy Withdrawn. *ENVIRONMENTAL TOXICOLOGY,* **11:** 5-7.

David R. M. and Joseph S.W. (2000). Pathogenic Microbiology-routine techniques in laboratory, **4:**125-126.

DuPont, B. F., F. Dromer, and L. Improvisi. (1996). The problem of azole resistance in Candida. *MEDICAL MYCOLOGY,* **6**:12-19.

Edmond M. B., Wallace S. E., McClish D. K., Pfaller M. A., Jones R. N. and Wenzel R. P. (1999). Nosocomial bloodstream infections in United States hospitals: A three-year analysis. *CLINICALLY INFECTIOUS DISEASES,* **29:**239-244.

Eicher T. and Hauptmann S., (2003).The chemistry of heterocycles: structure, reactions, synthesis and applications. *CLINICAL CHEMISTRY,* **5:**116-116.

Elmer W.K., Stephen D.A. and William M.J. (1992). Laboratory approach to the diagnosis of fungal infections. *CLINICAL MICROBIOLOGY,* **1:** 387 – 840.

Espinel-Ingroff A., Barchiesi F. K., Hazen.C., Martinez-Suarez J.V. and Scalise G. (**1998)**. Standardization of antifungal susceptibility testing and clinical relevance. *MEDICAL MYCOLOGY,* **36:**68-78.

Galgiani J. N. and Brass **C. (1990).** Susceptibility testing of fungi and current status of the standardization process. *ANTIMICROBIOAL AGENTS CHEMOTHERAPY,* **12:**2517-2521.

Galgiani J.N., Reiser J. and Brass C. (1987). Comparison of relative susceptibilities of *Candida* species to three antifungal agents as determined by un-standardized methods. *ANTIMICROBIOAL AGENTS CHEMOTHERAPY,* **31:**1343–1347.

Hautala T. (2007). A cluster of *Candida krusei* infections in a hematological unit. *BMC INFECTIOUS DISEASES,* **7**:97.

Hoffman H. L. and Pfaller M. A. (2001). *In vitro* antifungal susceptibility testing. *PHARMACOTHERAPY,* **21:**111–123.

Holt R. J. (1972). Laboratory assessment of the antimycotic drug clotrimazole. *CLINICAL PATHOLOGY,* **25:**1089–1097.

Hospenthal D.R., Murray C.K. and Rinaldi M.G. (2004). The role of antifungal susceptibility testing in the therapy of candidiasis. *DIAGNOSTIC MICROBIOLOGY OF INFECTIOUS DISEASES,* **48:**153-160.

Kauffman C.A. **(2006).** Clinical efficacy of new antifungal agents. *CURRENT OPINION IN MICROBIOLOGY,* **9:**483-488.

Kirkpatrick R., Turner T. M., Fothergill A. W., McCarthy D. I., Redding S. W., Rinaldi M. G. and Patterson T. F. (**1998).** Fluconazole disk diffusion susceptibility testing of *Candida* species. *CLINICAL MICROBIOLOGY,* **36:**3429-3432.

Kourkoumpetis T. (2010). Candida infection and colonization among non-trauma emergency surgery patients. *VIRULENCE,* **111:**14-17.

Krcmery V and Barnes A.J. (2002). Non-*albicans Candida* species causing fungaemia: pathogenicity and antifungal resistance. *JOURNAL OF INFECTIONS CONTROL,* **50:**243-260.

Law D., Moore C. B., Wardle H. M., Ganguli L. A. and Denning D. W. (1994). High prevalence of antifungal resistance in *Candida* spp. from patients with AIDS. *ANTIMICROBIAL CHEMOTHERAPY*, **34:**659-668.

Levy I., Rubin L.G., Vasishtha S., Tucci V and Sood S.K. (1999). Emergence of Candida parapsilosis as the predominant species causing candidemia in children. *CLINICALLY INFECTIOUS DISEASES*, **26:**1086–1088.

Lockhart S. R., Joly S., Vargas K., Swails-Venger J. and Soll D. R. (1999). Natural defenses against Candida colonization breakdown in the oral cavities of the elderly. *JOURNAL OF DENTAL RESEARCH*, **78:**857-868.

Louwagie B., Surmont I., Verhaegen J and Odds F. (1995). Differential and enrichment media for selective culture and recognition of yeast species from clinical material. *MICROBIOLOGY AND INFECTIOUS DISEASES* ,**14:**406–411.

Lozano-Chiu M., Nelson P. W., Paetznick V. L. and Rex J. H. (1999). Disk diffusion method for determining susceptibilities of *Candida* spp. to MK-0991. *CLINICAL MICROBIOLOGY*, **37:**1625-1627.

McGinnis M. and Rinaldi M.G. (**1998).** Antifungal drugs: mechanisms of action, drug resistance, susceptibility testing, and assays of activity in biological fluids. *ANTIBIOTICS IN LABORATORY MEDICINE*, **13:**176-211.

Maenza J. R., Keruly J. C., Moore R. D., Chaisson R. E., Merz W. G. and Gallant J. E. (1996). Risk factors for fluconazole-resistant candidiasis in human immunodeficiency virus-infected patients. *INFECTIOUS DISEASES*, **173:**219-225.

Magaldi S., Mata S., Hartung C., Verde G., Deibis L., Roldan Y. and Marcano C. (2001). *In vitro* susceptibility of 137 *Candida* spp. isolates from HIV positive patients to several antifungal drugs. *MYCOPATHOLOGY,* **149:**63-68.

Marr K. A., Seidel K., White T. C. and Bowden R. A. (2000). Candidemia in allogeneic blood and marrow transplant recipients: evolution of risk factors after the adoption of prophylactic fluconazole. *INFECTIOUS DISEASES*, **181:**309-316.

Marr K. A., White T. C., Van Burik J. A. and Bowden R. A. (1997). Development of fluconazole resistance in *Candida albicans* causing disseminated infection in a patient undergoing marrow transplantation. *CLINICALLY INFECTIOUS DISEASES*, **25:**908-910.

Matar M. J., Ostrosky-Zeichner L., Paetznick V. L., Rodriguez J. R., Chen E and Rex J. H. (2003). Correlation between E-Test, disk diffusion, and micro dilution methods for antifungal susceptibility testing of fluconazole and voriconazole. *ANTIMICROBIOAL AGENTS CHEMOTHERAPY,* **47:**1647-1651.

Meis J., Petrou M., Bille J., Ellis D. and Gibbs D. (2000). A global evaluation of the susceptibility of Candida species to fluconazole by disk diffusion. *DIAGNOSTIC MICROBIOLOGY AND INFECTIOUS DISEASES,* **36:**215-223.

Millon L., Manteaux A., Reboux G., Drobacheff C., Monod M., Barale T.and Michel B. Y. (1994). Fluconazole-resistant recurrent oral candidiasis in human immunodeficiency virus-positive patients: persistence of *Candida albicans* strains with the same genotype. *CLINICAL MICROBIOLOGY JOURNAL,* **32:**1115–1118.

Moosa M.Y, Sobel J.D, Elhalis H, Du W. and Akins R.A. (2004). Fungicidal activity of fluconazole against *Candida albicans* in a synthetic vagina-simulative medium. *ANTIMICROBIAL AGENTS AND CHEMOTHERAPY.* **48:**161–167.

National Committee for Clinical Laboratory Standards. (1997). Reference method for broth dilution antifungal susceptibility testing of yeasts: approved standard M27-A. *NCCLS,* **11:**514-712.

National Committee for Clinical Laboratory Standards. (2000). Performance standards for antimicrobial disc susceptibility tests, approved standard M2-A7. *NCCLS,* **7:**1-133.

Odds F.C. and Bernaerts R. (1994). CHROMagar Candida, a new differential isolation medium for presumptive identification of clinically important Candida species. *CLINICAL MICROBIOLOGY* **, 32:**1923–1929.

Oliveira E. R., Fothergill A. W., Kirkpatrick W. R., Coco B. J., Patterson T. F and Redding S. W. (2005). *In vitro* interaction of posaconazole and caspofungin against clinical isolates of *Candida glabrata*. *ANTIMICROBIOAL AGENTS CHEMOTHERAPY,* **49:**3544-3545)

Pappas P.G. (2006). Invasive Candidiasis. *INFECTIOUS DISEASES*, **3:**485-506.

Pappas P.G., Rex J.H. and Lee. (2003). A prospective observational study of candidemia: epidemiology, therapy and influences on mortality in hospitalized adult and pediatric patients. *CLINICALLY INFECTIOUS DISEASES*, **37:**634-43.

Pappas P.G., Rex J.H., Sobel J.D., Filler S.G., Dismukes W.E., Walsh T.J. and Edwards J.E. (2004). Guidelines for treatment of candidiasis. *CLINICALLY INFECTIOUS DISEASES,* **38:**161-189.

Pfaller M. A and Diekema D. J. (2007). Epidemiology of invasive candidiasis: a persistent public health problem. *CLINICAL MICROBIOLOGY* , **20:**133-163.

Pfaller M. A. and Diekema D. J. (2004). Rare and emerging opportunistic fungal pathogens: Concern for resistance beyond *Candida albicans* and *Aspergillus fumigatus. CLINICAL MICROBIOLOGY,* **42:**4419-4431.

Pfaller M. A., Diekema D. J. and Sheehan D. J. (2006). Interpretive breakpoints for fluconazole and *Candida* revisited: a blueprint for the future of antifungal susceptibility testing. *CLINICAL MICROBIOLOGY* , **17:**435–447.

Pfaller M. A., Messer S. A. and Houston A. (1998). National epidemiology of mycoses survey: a multicenter study of strain variation and antifungal susceptibility among isolates of *Candida* species. *DIAGNOSTIC MICROBIOLOGY IN INFECTIOUS DISEASES,* **31:**289-296.

Pfaller M. A., Houston A. and Coffmann S. (1996): Application of CHROMagar Candida for rapid screening of clinical specimens for *Candida albicans***,** *Candida tropicalis***,** *Candida krusei***,** and *Candida* **(***Torulopsis***)** *glabrata***.** *CLINICAL MICROBIOLOGY,* **34:**58-61.

Pfaller M.A. (2000). Epidemiology of nosocomial candidiasis and the importance of molecular typing. **53:**161–167.

Pfaller MA. (2008) *Candida krusei*, a multidrug-resistant opportunistic fungal pathogen: geographic and temporal trends from the ARTEMIS DISK Antifungal Surveillance Program, 2001 to 2005. *CLINICAL MICROBIOLOGY,* **46:**515-521.

Plempel M., Bartmann K., Bu¨che K. H. and Regel E. (1969). BAY b 5097,a new orally applicable antifungal substance with broad-spectrum activity. *ANTIMICROBIOLAGENTS AND CHEMOTHERAPY,***9:**271–274.

Rambali B., Fernandez J.A., Van Nuffel L., Woestenborghs F., Baert L., Massart D. L. and Odds F. C. (2001). Susceptibility testing of pathogenic fungi with itraconazole: a process analysis of test variables. *ANTIMICROBIOL CHEMOTHERAPY,* **48:**163–177.

Rex J. H., Pfaller M. A., Walsh T. J., Chaturvedi V., Espinel-Ingroff A., Ghannoum M. A., Gosey L. L., Odds F. C., Rinaldi M. G., Sheehan D. J. and Warnock D. W. (2001). Antifungal susceptibility testing: Practical aspects and current challenges. *CLINICAL MICROBIOLOGY,* **14:**643-658.

Richardson M. D. and Smith H. (1981). Production of germ tubes by virulent and attenuated strains of Candida albicans. *JOURNAL OF INFECTIOUS DISEASES,* **144:**565 – 569.

Sahand I., Moragues H., Eraso M. D., Villar-Vidal E., Quindos M., Ponton G. (2005). Supplementation of CHROMagar Candida Medium with Pal's Medium for Rapid Identification of Candida dubliniensis. *CLINICAL MICROBIOLOGY* ,**43:**5768-5770 .

Sangeorzan J. A., Bradley S. F., He X., Zarins L. T., Ridenour G. L., Tiballi R. N. and Kauffman C. A. (1994). Epidemiology of oral Candidiasis in HIV-infected patients: Colonization, infection, treatment, and emergence of fluconazole resistance. *MEDICAL MYCOLOGY,* **97:**339-346.

Sanglard D. and Odds F.C. (2001). Resistance of *Candida* species to antifungal agents: molecular mechanisms and clinical consequences. *MICROBIOLOGY,* **20:**14-15.

Sheehan D. J., Hitchcock C. A. and Sibley C. M. (1999). Current and emerging azole antifungal agents**.** *CLINICAL MICROBIOLOGY,* **12:**40-79.

Spiekermann P. H. and Young M. D. (1976). Clinical evaluation of clotrimazole: A broad-spectrum antifungal agent, *ARCHIVES OF DERMATOLOGY,* **112:**350-352.

Sud I. J. and Feingold D.S. **(1981).** Mechanisms of action of the antimycotic imidazoles. *INVESTIGATIVE DERMATOLOGY,* **76:**438–441.

Vazquez J.A., Sobel J.D., Dismukes W.E., Pappas P.G and Sobel J.D. (2003). Candidiasis. *CLINICAL MYCOLOGY***, 3:**143-87.

Vendettuoli V., Vento G., Tirone C., Posteraro B. and Romagnoli C. (2009). Antifungal prophylaxis: identification of preterm neonates at highest risk for invasive fungal infection. **123:**368-369.

Walsh T.J. and Dixon D.M. (1996). Deep Mycoses. *BARON'S MEDICAL MICROBIOLOGY*. **4:**239-241.

Weems J.J. Jr. (**1992).** *Candida parapsilosis*: epidemiology, pathogenicity, clinical manifestations, and antimicrobial susceptibility. *CLINICALLY INFECTIOUS DISEASES*, **4:**756–766.

APPENDICES

Appendix 1: Different species of *Candida* used and their information.

Isolate code	Source of isolate	Patient sex	Identified strain of *Candida* isolate
2	Sputum	M	*C.A*
32	Sputum	F	*C.A*
3	Urine	M	*C.A*
10	HVS	M	*C.A*
30	Urine	F	*C.A*
31	Urine	M	*C.A*
78	Sputum	M	*C.A*
21	Urine	F	*C.A*
18	Sputum	M	*C.A*
27	Urine	F	*C.A*
33	Sputum	M	*C.A*
26	HVS	F	*C.A*
51	Urine	F	*C.F*
50	Throat swab	F	*C.A*
108	HVS	F	*C.A*
83	Pus swab	F	*C.A*
100	Pus swab	F	*C.A*
53	Urine	M	*C.A*
87	Sputum	M	*C.A*
48	Pus swab	F	*C.P*
62	Necrotic swab	F	*C.A*
4	Pus swab	F	*C.A*
28	Urine	F	*C.A*
66	Urine	F	*C.A*
39	Urine	F	*C.A*
123	Sputum	M	*C.P*
130	Sputum	F	*C.A*
67	Urine	F	*C.A*
46	Pus swab	F	*C.A*
63	Pus swab	F	*C.A*
23	Urine	F	*C.A*
17	Throat swab	M	*C.A*
47	Urine	F	*C.A*

85	Throat swab	M	*C.A*
22	Sputum	M	*C.A*
16	Pus swab	F	*C.A*
19	Sputum	M	*C.A*
24	Urine	M	*C.A*
5	Pus swab	F	*C.A*
20	Urine	M	*C.G*
25	Urine	F	*C.A*
29	Urine	F	*C.P*
11	Pus swab	F	*C.A*
9	Necrotic swab	F	*C.A*
6	Urine	F	*C.A*
14	Urine	F	*C.A*
12	Pus swab	F	*C.A*
8	Pus swab	F	*C.A*
7	Urine	F	*C.A*
106	Urine	F	*C.A*

KEY

HVS High vaginal swab

F Female

M Male

C.A *Candida albicans*

C.F *Candida famata*

C.P *Candida parapsilosis*

C.G *Candida glabrata*

Appendix 2: Interpretive standards of antifungal agents in disc diffusion method.

Antifungal agent	*Antifungal Content*	*Susceptible*	*Intermediate*	*Resistant*
ITC	8 µg	>16mm	10-15mm	<10mm
FLU	25 µg	>19mm	15-18mm	<14mm
CLO	25 µg	>19mm	15-18mm	<14mm
POS	8 µg	>16mm	10-15mm	<10mm

KEY

ITC Itraconazole

FLU Fluconazole

CLO Clotrimazole

POS Posaconazole

mm Millimeter

µg Microgram

Appendix 3: Interpretive standards of antifungal agents in Episilometer test method.

Antifungal agent	*Range of MIC profile*	*Susceptible*	*Intermediate*	*Resistant*
ITC	0.002-32	<0.12 µg/ml	0.25-0.50 µg/ml	>1.0ug/ml
FLU	0.016-256	<8 µg/ml	12-48 µg/ml	>64ug/ml
CLO	0.016-256	<8 µg/ml	12-48 µg/ml	>64ug/ml
POS	0.002-32	<0.12 µg/ml	0.25-0.50 µg/ml	>0.90ug/ml

KEY

ITC Itraconazole

FLU Fluconazole

CLO Clotrimazole

POS Posaconazole

µg/ml Microgram per mililitre

MIC Minimum inhibitory concentration

Appendix 4: Zone diameter in millimeters in disc diffusion method

		ITC		FLU			CLO			POS		
CANDIDA	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**
C.A	25			42			30			39		
C.A	26					6	35			32		
C.A	18					12	30			28		
C.A	20			23			36			34		
C.A	21					6	30			23		
C.A	20			35			42			42		
C.A			6			6	42			19		
C.A	29					6	30			36		
C.A	28			36			30			40		
C.A			6			6	33				13	
C.A	25					6	30			44		
C.A	23			43			16			33		
C.A			6			6	26				15	
C.A	19			43			39			35		
C.A	21			48			40					7
C.A			6			6	28			22		
C.A	22			37			43			39		
C.A	26			42			35			41		
C.A		14				12	36				14	
C.P	25			32			42			34		
C.A	16			25			22			33		
C.A	21					6	28			26		
C.A	28			22			32			39		
C.A		14				6	22			34		
C.A	20			25			19			44		
C.P			6			12		18		36		
C.A			6			6	36			42		
C.A	23			40			41			33		
C.A	24			36			21				15	
C.A	30			47			20			26		
C.A			6			6	30			17		
C.A	26			24			33			24		
C.A	32			36			44			31		
C.A	18			22			36			29		

C.A	25			25			42			42		
C.A	20				18		26			44		
C.A			6			6		18			14	
C.A	17					12	38			18		
C.A	19					6			13			9
C.G	16					14	39			23		
C.A	19					6	29			18		
C.P	22					8		15		24		
C.A	17					12	27			23		
C.A	35			36			40			32		
C.A	24			24			30					6
C.A			6		18		28					6
C.A	20					6	26			29		
C.A			8			6	31				14	
C.A	31			32			36			40		
C.A			6		18		40					8

KEY

S Susceptible

I Intermediate

R Resistant

ITC Itraconazole

FLU Fluconazole

CLO Clotrimazole

POS Posaconazole

C.A *Candida albicans*

C.F *Candida famata*

C.P *Candida parapsilosis*

C.G *Candida glabrata*

Appendix 5: Minimum inhibitory concentration profile in Epsilometer-test

			ITC			FLU		CLO			POS	
CANDIDA	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**
C.A	0.094			0.75			8			0.004		
C.A		0.25				256	4			0.003		
C.A	0.094					192	8			0.023		
C.A	0.004			0.016			2			0.008		
C.A			32	0.064			8			0.006		
C.A		0.25		0.016			0.25			0.004		
C.A		0.19				128	0.25			0.008		
C.A	0.004			0.016			8			0.002		
C.A	0.004			0.38				12		0.008		
C.A	0.094			0.016			0.5				0.125	
C.A			32			256	6			0.002		
C.A	0.064			0.064				48		0.003		
C.F		0.19		2				24		0.004		
C.A	0.064			1			0.19			0.004		
C.A	0.094			0.125			0.125					4
C.A			32			192	1.5			0.006		
C.A			32			48	0.38			0.003		
C.A	0.016			0.38			4			0.003		
C.A			32			256	4			0.006		
C.P	0.016			0.38			0.094			0.002		
C.A		0.25		0.5				12		0.047		
C.A			0.94	0.19			8			0.006		
C.A	0.023			0.125			0.75			0.012		
C.A			1.5	0.094			1.5			0.016		
C.A	0.023			0.5				24		0.023		
C.P			1.5		48				192	0.047		
C.A			32			256	0.047			0.003		
C.A	0.004			0.38			0.032			0.016		
C.A	0.064			0.38			1.5				0.25	
C.A	0.016			0.25			1				0.75	
C.A			0.5			256	0.38				0.5	

C.A	0.064			0.75			0.25			0.003		
C.A	0.023			0.032			0.023			0.002		
C.A		0.19			24		0.125				0.75	
C.A	0.032			8			0.023			0.004		
C.A			1.5		48			16			0.25	
C.A	0.016			0.047				24		0.047		
C.A	0.064				12		0.047			0.006		
C.A	0.004			0.016					128	0.006		
C. G	0.016			0.94			0.016			0.002		
C.A	0.023				48		6			0.016		
C.P			0.5			64			96		0.75	
C.A	0.032			0.38			1.5			0.008		
C.A			1.5			64	0.064			0.047		
C.A			32			96	1.5				0.19	
C.A			0.94			128		12				0.94
C.A			32			96		32				0.94
C.A	0.016			0.125			4			0.047		
C.A	0.032			0.5			0.38			0.064		
C.A		0.25			48		0.25			0.016		

KEY

S Susceptible

I Intermediate

R Resistant

ITC Itraconazole

FLU Fluconazole

CLO Clotrimazole

POS Posaconazole

C.A *Candida albicans*

C.F *Candida famata*

C.P *Candida parapsilosis*

C.G *Candida glabrata*