CHARACTERIZATION OF *Candida* SPECIES FROM CLINICAL SOURCES IN NAIROBI, KENYA

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CHARACTERIZATION OF *Candida* SPECIES FROM CLINICAL SOURCES IN NAIROBI, KENYA

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DECLARATION

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

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

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DEDICATION

To my parents, brothers, sisters, friends and my daughter Stacey. Thank you for all the support.

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ABBREVIATIONS

AIDS	Acquired Immunodeficiency Disease Syndrome
AMB	Amphotericin B
CMA	Corn Meal Agar
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
HIV	Human Immunodeficiency Virus
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KEMRI	Kenya Medical Research Institute
MIC	Minimum Inhibitory Concentration
NCCLS	National committee for clinical laboratory standards.
rpm	Revolutions per minute
RPMI	Roswel Park Memorial Institute
SDA	Sabaraud’s Dextrose Agar
SrDNA	Single Stranded Ribosomal Deoxyribonucleic Acid
SDD	Susceptible Dose Dependent

ABSTRACT

Candida is a yeast of economic importance as it causes infections of the esophageal, oral, anorectal, vaginal mucosa; eyes, nails as well as life threatening fungemia.They are among emerging opportunistic pathogens especially due to HIV / AIDS. Emerging resistance to commonly used antifungal drugs has further complicated their management resulting in increased morbidity and mortality. The present study analyzed phenotypic and molecular characteristics of *Candida* strains from clinical sources in Nairobi. Drug susceptibility profiles of all the *Candida* isolates were also analyzed. A total of one hundred and fifty (150) *Candida* species isolated since 1997 at Kenya Medical Research Institute (KEMRI), Mycology laboratory were characterized. Preliminary identification was done using germ tube test, CHROMagar *Candida*, Corn meal agar, and confirmed using Analytical profile index (API 20 C aux). Genotypic analysis was done using primer pairs that span the site of the transposable intron in the 25S rDNA. Antifungal drug susceptibility to Fluconazole, Nystatin, Clotrimazole and Amphotericin B was performed using broth microdilution techniques.

The isolates were recovered from swabs 37.3 %, urine 33.3 %, sputum 16.7 %, aspirates 8 %, blood 3.3 %, CSF and others 0.7 %. Out of the 150 isolates 86.7 % were *Candida albicans* whereas 13.3 % were non *albicans Candida* as confirmed by API 20 C aux. Non *albicans Candida* included; *C. parapsilosis* 4 %, *C. tropicalis* 2.7 %, *C. krusei* 2.7 %, *C. guilliemondii* 1.3 %, *C. glabrata* 1.3 % and *C. famata* 1.3 %. Germ tube positive *C. albicans* were 96.1 % whereas only 3.8 %

were germ tube negative. All the 130 isolates identified as *C. albicans* formed chlamydospores and all grew at both 37 °C and 45 °C ruling out the possibility of *Candida dubliniensis.* Genotypic analysis indicated that most of the *C. albicans* were genotype A (60 %) with one band of 450 base pairs followed by genotype C (16 %) with two bands of 450 and 650 base pairs and B (8 %) with 1 band of 650 base pairs. In this study 4 % of the *C. albicans* isolates were categorized as genotype F that had one band of 550 base pairs.

The isolates were fairly susceptible to commonly used antifungal drugs. *C. albicans* susceptibility to Fluconazole (MIC ≤ 8 µg/ml) was 73.1 %, susceptible dose-dependent (MIC 16-32 µg / ml) 14.6 % and resistant (MIC ≥ 64 µg / ml) 12.3 %. The MIC_{50} and MIC_{90} to Fluconazole were 1 µg / ml and 64 µg / ml respectively. At 1 µg / ml of Amphotericin B, most of the isolates were inhibited with 90.3 % having an MIC of ≤ 1 µg / ml. The MIC_{50} and MIC_{90} to Amphotericin B were 0.25 µg / ml and 1.0 µg / ml respectively. Elevated MIC ≥ 4 µg / ml to Clotrimazole and Nystatin were demonstrated in 80.5 % and 90.5 % respectively. The MIC_{50} and MIC_{90} of Clotrimazole and Nystatin were 1.0 µg / ml, 0.29 µg / ml and 16 µg / ml, 18.5 µg / ml respectively. The rest (20) non-*albicans Candida* were fairly susceptible to all the four drugs with reduced susceptibility reported on very few isolates.

From the study *C. albicans* was the most prevalent and hence the most common cause of candidiasis. The result has demonstrated some evidence of emerging

resistance to commonly used antifungal drugs. For management of *Candida* infection, there is need to identify all the yeast from clinical sources as some have intrinsic resistance to commonly used antifungal drugs. There is also need to constantly carry out *in-vitro* antifungal susceptibility testing in order to establish any emerging resistance. This is essential in the management of *Candida* infections especially in HIV / AIDS where recurrent candidiasis is common.

CHAPTER ONE

1. INTRODUCTION

Candida is an endogenous yeast, which dwells predominantly in the gastrointestinal tract, and sometimes in the respiratory tract of healthy individuals. *C. albicans* is the most prevalent species, although other *Candida* species such as *C. tropicalis, C. krusei, C. glabrata* (also classified as *Torulopsis glabrata*), and *C. parapsilosis* can cause disease (Williams 1985 and 1996). When present in low numbers, *Candida* species form part of the normal mouth, intestinal and vaginal flora. They may also be found on the skin of healthy individuals. In most people, commensal bacteria control the overgrowth of *Candida* within the body (McCullough *et al.,* 1998). However as an opportunistic pathogen, due to the administration of antibiotics, immunosupression or the presence of an underlying medical condition, *Candida* can cause infection (Beneke and Rogers, 1996).

Fast identification of *Candida* species in the clinical laboratory is becoming increasingly important as the incidence of candidiasis continue to rise due to HIV/AIDS. Rapid species-specific identification is also necessary for timely, targeted interventions and to facilitate hospital infection control measures (Kwong - Chung *et al*., 1985). Determination of prevalence of different *Candida* species and their drug susceptibility profiles is essential for management of candidiasis.

The purpose of this study therefore was to identify and characterize *Candida* species from clinical sources and to examine the drug susceptibility profiles. The study was carried out in Mycology Laboratory, Center for Microbiology Research, Kenya Medical Research Institute (KEMRI). It entailed subjection of the *Candida* species to various identification schemes including morphology, growth characteristics, carbon source assimilation or fermentation as well as appearance on differential isolation media (Corn Meal Agar and CHROMagar). API 20 C AUX was used for confirmation. Genotypic analysis and drug susceptibility tests were carried out to determine the most prevalent genotype and the current level of antifungal drug resistance. This is important for understanding treatment failures and relapses in Candidiasis, which is essential in mounting appropriate management strategies especially in HIV-AIDS patients.

1.1 JUSTIFICATION

Candidiasis is a common cause of morbidity and mortality in HIV/AIDS. Earlier studies showed that *C. albicans* accounts for 80 % of candidiasis. However other *Candida* species have emerged significantly as opportunistic fungal pathogens. Studies that have been done in Kenya show increased level of resistance to commonly used antifungal drugs. Further these studies are limited and understanding antifungal resistance level, genotype diversity and differentiation of *Candida* species is essential for mounting appropriate clinical management strategies of candidiasis and especially for HIV / AIDS patients. In addition rapid and reliable identification to species level is necessary for clinicians to make accurate / appropriate treatment choices.

1.2 OBJECTIVES

1.2.1 Broad Objective

To characterize different *Candida* isolates from clinical sources in Nairobi, Kenya and to determine drug susceptibility profiles.

1.2.2 Specific Objectives

1. To characterize *Candida* species from clinical sources in Nairobi Kenya, using phenotypic and biochemical characteristics.
2. To perform molecular characterization of *Candida albicans* isolates from clinical sources in Nairobi, Kenya.
3. To determine drug susceptibility profiles of *Candida* species from clinical sources in Nairobi, Kenya.

CHAPTER TWO

2. LITERATURE REVIEW

2.1 *Candida* Species

The yeast *Candida* are unicellular organisms that generally reproduce by budding. If the buds (blastoconidia) elongate and remain attached to the parent cell they form chains known as pseudohyphae (Odds, 1988). Colonies are smooth and glabrous and may be moist or dry. They are usually white to cream colored, but some are tan, pinkish, or orangey.

Candida species are the most common fungi isolated in the clinical laboratory. The organisms are ubiquitous in the environment and also live as normal flora in human bodies, so it is often difficult to determine the clinical significance of an isolate. Implication of *Candida* species as etiological agents of infection often requires repeated recovery from the site and direct microscopic demonstration in the infected tissue.

2.2 Classification of Candida

2.2.1 Taxonomic classification

Candida belongs to the kingdom Fungi, phylum Ascomycota, class Ascomycetes within the family *Saccharomyceae*. The genus contains approximately 200 species (Odds, 1988). According to Rippon (1988), there are about 18 species of the genus *Candida* considered to be pathogenic, however among them only *C. tropicalis* is known to be more virulent than *C. albicans* (Wingard , 1995). Other *Candida*

species causing infection in humans include; *Candida glabrata*, *Candida krusei*, *Candida parapsilosis*, *Candida dubliensis* and *Candida lusitainiae*. The ability to produce pseudohyphae, true hyphae, and / or terminal chlamydospores and the shape and arrangement of blastoconidia are used along with other morphological characteristics and biochemical tests to identify the yeasts to genus and species level.

2.2.3 Description of *Candida* and its Natural Habitats

Candida is a yeast and the most common cause of opportunistic mycoses worldwide. It is also a frequent colonizer of human skin and mucous membranes. *Candida* is a member of normal flora of skin, mouth, vagina, and stool. As well as being a pathogen and a colonizer, it is found in the environment, particularly on leaves, flowers, water, and soil. While most of the *Candida* species are mitosporic, some have known teleomorphic state and produce sexual spores (Williams *et. al.*1995).

The genus *Candida* designation is used for asexual yeasts with any one of the following features: acetic acid production, red pigmentation, arthroconidia, unipolar or bipolar budding on a broad base, blastoconidia formed on sympodulae, buds formed on stalk, needle shaped terminal conidia, triangular cells, enteroplastic basipetal budding and the ability to grow on inositol as a sole carbon source and blastoconidia (Kreger-Van Rij, 1984). The overall carriage rate in

healthy individuals is estimated at 80 %. Teleomorphs of the genera have been demonstrated for different species of *Candida* (Hazen, 1995).

2.3 *Candida* Infection

Candida can cause infections of the esophageal, oral, anorectal, and vaginal mucosa, the eyes, skin and nails, as well as life-threatening fungemia (Benedict and Colagreco 1994, Krcmery and Barnes, 2002). Constant use of antibacterial agents encourages the overgrowth of fungal *Candida* species. This is because most of the bacteria are killed causing overgrowth of the yeasts (Bodey, 1988).

Thrush or oral candidiasis is the most common opportunistic infection in people with HIV/AIDS (Selik *et al.,* 1987, Dodd *et al.,* 1991). It occurs in approximately one third of people with HIV before progression to AIDS, and over 90 % of people with AIDS will develop oral thrush at some time during the course of their illness (McCarthy, 1992). Thrush is perceived to be a marker of impaired immune response in people with HIV, since about half of those with the condition develop an AIDS-defining illness within two years (Selik *et al.*, 1987, Dodd *et al.*, 1991).

Candida invades cells of the oral mucosa, forming several types of lesions. The most common are pseudomembranous lesions - raised creamy white patches that can be easily scraped off to reveal a bleeding red base upon the cheek, the palate or the dorsum of the tongue. Erythematous lesions are spotty red patches without plaques that may be easily overlooked. Hyperplastic lesions are similar to the

pseudomembranous lesions, except that the plaque cannot be removed. Painful fissuring and ulceration of the corner of the mouth indicates angular cheilitis (inflammation of the lips) (Pindborg *et al.,* 1986 and Brawner, 1992).

Esophageal candidiasis is an AIDS-defining illness that occurs mainly in people with AIDS (Reef and Mayer, 1995). It is associated with pain upon swallowing and retrosternal pain making it extremely difficult to eat.

Candidemia and invasive candidiasis are more common in cancer patients, but can occur in people with AIDS, especially those with an indwelling catheter or those who are receiving parenteral nutrition (Weems, 1992). It can disseminate to any organ of the body especially the liver, spleen, or lungs. There may also be skin involvement, which presents as a nodular rash that may hemorrhage.

The increased incidence of localized and systemic *Candida* infections caused by *Candida* species during the past decade has been well documented and the reason for this include the growing number of immunocompromised individuals in the population as a result of HIV (Human Immunodeficiency Virus) pandemic and increased use of immunosuppressive therapy in cancer and organ transplant patients. In addition, the widespread use of broad-spectrum antibacterial drugs and the increased use of invasive procedures including intubation, drains and catheters are likely to be important risk factors (Ozlem *et al*., 2000).

2.4 Identification of *Candida* isolates

2.4.1 Morphological Identification Methods

Yeasts easily grow in standard blood culture systems therefore diagnosis in those with candidaemia is straightforward. Yeast cells are easily seen under microscopy without staining although dark field or phase contrast microscopy is better. They are gram positive and grow well on blood or Sabouraud's dextrose agar at temperatures between 20 ^{o}C and 37 ^{o}C.

The observation of germ tube (GT) production as a method of presumptive identification of *C. albicans* has been in use for many years. It is based on the fact that *C. albicans* produces short slender, tube like structures (germ tubes) when incubated at 35° C to 37 ^{o} C in serum for 2 – 3 h. Although Germ Tube Test (GTT) is simple, up to 5 % of the strains of *C. albicans* are germ tube negative and other yeasts may produce pseudohyphae. However it is possible for a germ tube to be distinguished from pseudohyphae by an experienced mycologist. Although the recently identified species *C. dubliniensis* has been shown to produce both germ tubes and chlamydospores, efforts to differentiate this species from *C. albicans* have been made. According to Emmanuelle *et al.,* (1998), the two can be differentiated on the basis of growth in either Emmons modified Sabourauds Glucose agar and yeast peptone dextrose broth. *C. albicans* grows on both Emmons modified Sabourauds Glucose agar and yeast peptone dextrose broth whereas *C. dubliniensis* grows only on Emmons modified Sabourauds Glucose

agar. Moreover temperature tolerance has been shown to be useful in differentiating the two. *C. albicans* grows at temperatures up to 45 ^{o}C whereas *C. dubliniensis* does not.

Various *Candida* species have some degree of unique behaviour with respect to their colony texture, microscopic morphology on corn meal tween 80 agar at 25 ^{o}C (Dalmau method) and fermentation or assimilation profiles in biochemical test (Larone, 1995). Corn meal agar with Tween 80 is used in distinguishing the different genera of yeasts and the various species of *Candida* and can also be useful in slide cultures as it stimulates conidiation in many fungi. It suppresses vegetative growth of many fungi while stimulating sporulation. Corn Meal Agar allows *Candida albicans* to produce chlamydospore which is one of the best criterions for identification (Ozlem *et al*., 2000).

Candida guilliermondii colonies are flat, moist, smooth, and cream to yellow in color on Sabouraud dextrose agar. It does not grow on the surface when inoculated into Sabouraud broth. On cornmeal tween 80 agar and at 25°C after 72 h, it produces clusters of small blastospores along the pseudohyphae and particularly at septal points. Pseudohyphae are short and few in number (Larone, 1995).

Candida famata colonies on Sabouraud's dextrose agar are white to cream colored, smooth, glabrous yeast-like in appearance. Microscopic morphology shows

numerous ovoid, budding yeast-like cells or blastoconidia, 2.0 - 3.5 x 3.5 - 5.0 um in size. No are pseudohyphae produced (Kreger, 1984).

Candida tropicalis colonies on Sabouraud's dextrose agar are cream colored with a slightly mycelial border. It may produce a thin surface film and bubbles when grown in sabaraud broth. On cornmeal tween 80 agar and at 25 °C after 72 h, it produces oval blastospores which are located along the long pseudohyphae.The blastospores may appear singly or in clusters. The pseudohyphae branch abundantly. They may also produce true hyphae (Suton *et al*, 1998).

Candida parapsilosis colonies are white, creamy, shiny and smooth or wrinkled on SDA. It does not grow on the surface when inoculated into sabaraud broth. On cornmeal tween 80 agar and at 25 °C after 72 h, it produces blastospores which are located along the pseudohyphae. Typically the pseudohyphae may be curved and large mycelial (hyphal) elements which are called "giant cells" may be observed (Suton *et al*, 1998).

Candida krusei colonies on SDA are off-white, dull and smooth with margins ranging from smooth to lobed. The isolate grows at 42 °C but fail to grow on media containing cyclohexamide. On cornmeal tween 80 agar and at 25 °C after 72 h, abundant pseudohyphae are formed with moderate branching (Larone, 1995). *Candida glabrata* colonies on SDA are white, smooth and glistening. The isolate grows at 42 °C but fail to grow on media containing cyclohexamide cornmeal

tween 80 agar and at 25 °C after 72 h, only blastoconidia are observed. Yeast cells are quite small measuring 2.5 - 4.0 x 3.0 - 6.0 µm as compared to *Candida albicans* which measures 3.5- 6.0 x 4.0 – 8.0 µm (Kreger, 1984)

2.4.2 Biochemical Tests

Recently, rapid enzymatic test kits (non-microscopic methods) such as MUREX *C. albicans* (MC) (Murex Diagnostics), Albicans Sure (AS) (Clinical Standard Laboratories), and BactiCard *Candida* (BC) (Remel) have been developed as alternatives to the Germ tube test for the rapid screening of *C. albicans* (Ozlem *et al*., 2000). These enzymatic test kits detect the production of the enzymes L-proline aminopeptidase and β galactosaminidase by yeast isolates grown in culture, and fail to identify *Candida* species other than *C. albicans* because it produces both enzymes. The other species produces only one or none of these enzymes. To identify non-*albicans Candida* species, as well as to confirm their identification, commercial carbohydrate assimilation systems, such as API 20 C aux system, are widely available (Ozlem *et al*., 2000). The API 20 C AUX strip consists of 20 cupules containing dehydrated substrates which enable the performance of 19 assimilation tests. The cupules are inoculated with a semi-solid minimal medium and the yeasts will only grow if they are capable of utilizing each substrate as sole carbon source. The reactions are read by comparing them to growth controls and identification is obtained by referring to the Analytical Profile Index or using the identification software (Bergan and Vangdal, 1983).

A chromogenic media that contain chromogenic substrates, which react with enzymes secreted by the target microorganisms to yield colonies of varying colors, have been developed. CHROMagar *Candida* (CHROMagar Microbiology, Paris, France.) is a medium that can identify three *Candida* yeasts, *C. albicans* (green colonies), *C. tropicalis* (blue colonies), and *C. krusei* (fuzzy, rose colored colonies) after 48 hours of incubation at 30 - 37°C. Some reports have proposed that it can also reliably identify *C. dubliniensis* and *C. glabrata* (Tintelnot *et al.*, 2000).

Fatty acid analysis can also be used in characterization of *Candida* species. This uses gaschromatography of cellular fatty acids. A commercial identification system of fatty acids from yeasts has been developed by MIDI (Microbial Identification System, 1992).

2.4.3 Molecular Techniques

Identification of yeast pathogens by traditional methods requires several days and specific mycological media. Newer identification methods have emerged, such as genotyping methods, including restriction fragment length polymorphism (RFLP) and Pulse Field Gel Electrophoresis (PFGE). Techniques based on the use of PCR have been demonstrated to be faster and more sensitive in detecting specific sequences of DNA. Some of the currently used techniques include Randomly Amplified Polymorphic DNA (RAPD). Arbitrary primers are used to amplify

target DNA sequences by PCR and the amplified product is electrophoresed in agarose gels to form strain specific fingerprints (Juliana, 2004).

However most of these techniques are laborious, cumbersome and time-consuming and only a few techniques are actually able to differentiate between *Candida* species other than *C. albicans* (Chen *et al.*, 2000). Coding regions of the 18s, 5.8s and 28s nuclear rRNA genes evolve slowly, are relatively conserved among fungi, and provide a molecular basis of establishing phylogenetic relationship (Chen *et al.*, 2000). Between coding regions are the internal transcribed spacer one and two regions (ITS1 and ITS2 respectively) which evolve more rapidly and may therefore vary among different species within a genus. Thus PCR amplification may facilitate the identification of its region DNA sequences with sufficient polymorphism to be used for identifying fungal species especially *Candida* species. In addition, this region offers distinct advantages over other molecular targets including increased sensitivity due to the existence of approximately 100 copies per genome. This advantage is important in detecting fungal DNA in clinical samples. This region has been used in identification of some medically important fungi particularly *Candida* and *Aspergillus* species by the various approaches such as DNA probe and RFLP (Chen *et al.,* 2000 and Henry *et al.,* 2000).

Molecular typing methods have been used with increasing frequency in epidemiological investigations for the development of rational infection control measures (Pfaller, 1995). One of the earliest molecular methods for the differentiation of *C. albicans* strains used a simple technique analyzing the Restriction Fragment Length Polymorphism (RFLPs) of cellular DNA to divide isolates into two large groups on the basis of the position of a dimorphic band. Group A strains had a band of 3.7 kb and group B strains had a band of 4.2 kb (Scherer and Stevens, 1987).

Mercure *et al*., (1993) reported that *C. albicans* produces a well - characterized *ECORI* restriction fragment length polymorphism pattern whose patterns are intensively stained by ethidium bromide. Of these bands, the dimorphic (3.7 and 4.2 kb) fragments, which were shown to have originated from the rRNA encoding regions (rDNA), were used to classify *C. albicans* into two genotypes (genotypes A and B). Further studies confirmed that the presence or absence of group introns among *C. albicans* strains accounts for the differences in DNA band lengths of the rDNA fragment (3.7 or 4.2 kb) observed (Person and Lipman, 1998). Those studies led to the preparation by McCullough *et al,* 1997 of a PCR Primer pair group that can demonstrate the presence of group 1 introns in the 25S rDNA. *C. albicans* genotype B has been shown to have a transposable intron in the 25 S rDNA whereas genotype A isolates do not.

Restriction enzymes are endonucleases that cleave DNA in response to recognition site on the DNA. The recognition site (restriction site) consists of a specific sequence of nucleotides in the DNA duplex, typically 4 - 8 base pairs. Experiment with restriction enzymes is simple, relatively inexpensive and their result is reproducible. These enzymes have vast application in the molecular biology especially the diagnostic purposes. The pattern in restriction enzyme profile is important. When the restriction enzyme is applied for differentiation between some related organisms, it must be distinguishable according to the size and number of bands and in general produced patterns must be so clear that can identify the given organism. Williams *et al.* (1995) tried to delineate medically important *Candida* species by some restriction enzymes (*HaeIII, DdeI, BfaI*) after amplification of ITS - ITS4 region, but their bands could not match with known genotypes perhaps due to unavailability to related ITS1 - ITS4 sequences. In other studies, Morace *et al.*, (1997) targeted the amplification of a 300 - 350 bp segment in P-450 Ll AI gene and subsequent digestion by 3 enzymes (HincII, NsiI and *Sau* 3A) for identifying 8 *Candida* species.

2.5 Antifungal Drug Resistance

Several *Candida* species vary in their susceptibility to antifungal drugs. For example *C. krusei* and *C. lipolytica* are naturally (intrinsic) resistant to triazole fluconazole, while *C. lusitaniae* is resistant to amphotericin B (Rinaldi, 1996).

Antifungal drug dilutions are used to determine Minimum Inhibition Concentration (MIC) or Minimum Inhibitory Concentration for an isolate when grown in the presence of an antifungal agent. Serial dilutions of antifungal agents are dispensed into appropriately labeled tubes. Each tube is then inoculated with standardized nutrient broth suspension of the yeast being tested. The primary advantage of the broth dilution test is that it permits quantitative estimate of both the inhibitory and fungicidal activities of the antifungal agent (Weems, 1992).

Antifungal susceptibility is done (i) when the patient is failing therapy with an antifungal agent (s) that is known to be active against the infecting organism; (ii) to learn of potentially efficacious alternative drugs when the pathogenic yeast is one with well-known resistance to the drug of choice e.g., *Candida lusitaniae* and Amphotericin B (AMB). (iii) when one is treating with 5-Fluorocytosine (5-FC), an agent to which yeasts may be innately resistant or to which resistance rapidly develops; (iv) to ascertain antifungal activity with new agents for which no substantial or previously published database exists. (v) for yeasts recovered from severely immunocompromised hosts with systemic disease, e.g., those with neutropenia; and (vi) for prospective studies of *in vitro* or *in vivo* correlation (Reef, 1995).

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Antifungal susceptibility testing is normally performed by the reference broth micro dilution method as outlined in Clinical Laboratory Standard Institute (CLSI) document M27-A2 (2002).

This method recommends the use of RPMI-1640 medium (with glutamine and phenol red, without bicarbonate) supplemented with 0.2 % glucose and buffered to a pH of 7.0 with 0.165 mol/L Mops (3-[N-morpholino] propanesulfonic acid), inoculum standardized to 0.5 McFarland using a densitometer and incubation at

35 °C. Plates are incubated for 24 hours and 48 hours and microdilution wells visualized with the aid of a reading mirror and growth in each well compared with that of the growth control. A numerical score from 0 to 4 is given to each well using the following scale: 0 =optically clear, 1 = slightly hazy, 2 = prominent reduction in turbidity compared with that of the drug-free growth control, 3 = slight reduction in turbidity compared with that of the drug - free growth control, 4 = no reduction in turbidity compared with that of the drug-free growth control.

2.6 The mechanism of action of antifungal drugs

2.6.1 Mechanism of action of Polyenes

The polyenes (Amphotericin B (AMB) and Nystatin) have been proposed to act on a number of fungal cell targets, all involving the cellular membrane. The most documented target of AMB is ergosterol (Fig. 1), the fungal cell membrane sterol equivalent to cholesterol in mammalian cells. This “sterol hypothesis” is supported by three lines of evidence (Medoff *et al.*, 1983): firstly that the presence of

ergosterol in natural or artificial membranes makes them sensitive to AMB; secondly free sterols antagonize the effects of AMB on *Saccharomyces cerevisiae* and *C. albicans* and finally several physical-chemical methods demonstrate an AMB-sterol interaction in water or water-methanol mixtures. However AMB have been shown to induce sensitivity in sterol-free model membranes, suggesting that sterols may not be the only fungal cell membrane target (Medoff *et al.*, 1983).

AMB specificity for fungal ergosterol versus mammalian cholesterol was demonstrated by Readio and Bittman, (1982) with AMB binding 10-fold more tightly to unilamellar vesicles composed of ergosterol than those containing cholesterol. After binding to ergosterol via its two hydrophobic chains (Bastert *et al.*, 2001), AMB induces formation of minute pores, causing leakage of K+ and influx of Na+ which results in an ionic imbalance (Medoff *et al* 1983, Schell, 1993 and Hsu and Burnett, 1993). This permeability has been hypothesized to cause leakage of additional intracellular components, resulting in the subsequent death of the fungal cell (McGinnis and Rinaldi, 2003).

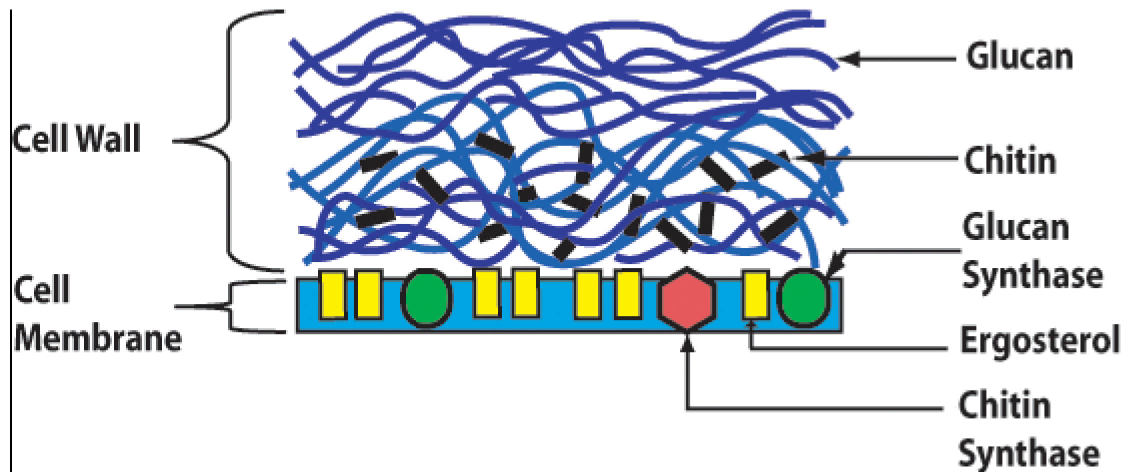


Figure 1. Representation of select fungal cell wall and membrane components. (Sited from Wey *et al*, 1988).

Amphotericin B has also been proposed to have both immunostimulatory and immunosuppressive effects on the host. AMB has an immunostimulatory effect on macrophages by increasing oxidative bursts (Brajtburg *et al.*, 1990) and killing of fungal conidia (Jahn *et al.,* 1998).

Amphotericin B causes immunosuppression by disrupting the cell membranes of polymorphonuclear lymphocytes (PMN), thereby preventing the PMN's oxidative burst (Jullien *et al.,* 1991). Another immunosuppressive property of AMB is that it can induce lipid peroxidation of the mammalian cell membranes, causing fragility in the membrane that may lead to increased permeability (Brajtburg and Bolard 1996 and Barwicz *et al.,* 2000).

2.6.2 Mechanism of action of Azoles

The ergosterol synthesis pathway and proposed azole target are shown in Figure 2. The primary mode of azole action has been demonstrated to be inhibition of ergosterol biosynthesis through the selective inhibition of the enzyme, P450 14α-demethylase ($P450_{14\alpha dm}$) following the stochiometric interaction of the N-3 (imidazole) or the N-4 (triazoles) substituents of the azole ring with the heme of $P450_{14\alpha dm}$ (Joseph and Hollomon 1997, Yoshinda, 1998). This interaction prevents $P450_{14\alpha dm}$'s ability to remove the C-14methyl group from lanosterol, resulting in the accumulation of 14α methylsterols in the cell membrane instead of ergosterol (Groll *et al.*, 1998). While physiochemical similarities exist between ergosterol and many of its precursors, few of these intermediates are able to replace ergosterol as a fluidity regulator within the cell membrane (Rodreguez *et al.,* 1985, Nes *et al.,* 1993, Joseph and Hollomon, 1997).

Emerging resistance to commonly used antifungal drugs in Kenya has been noted (Wanzala and Bii, 2000). This is attributed to irrational use of antibiotics and increasing population of immunocompromised individuals. Azole resistant has drastically emerged over the past decades making it necessary to carry out constant antifungal drug resistance surveillance.

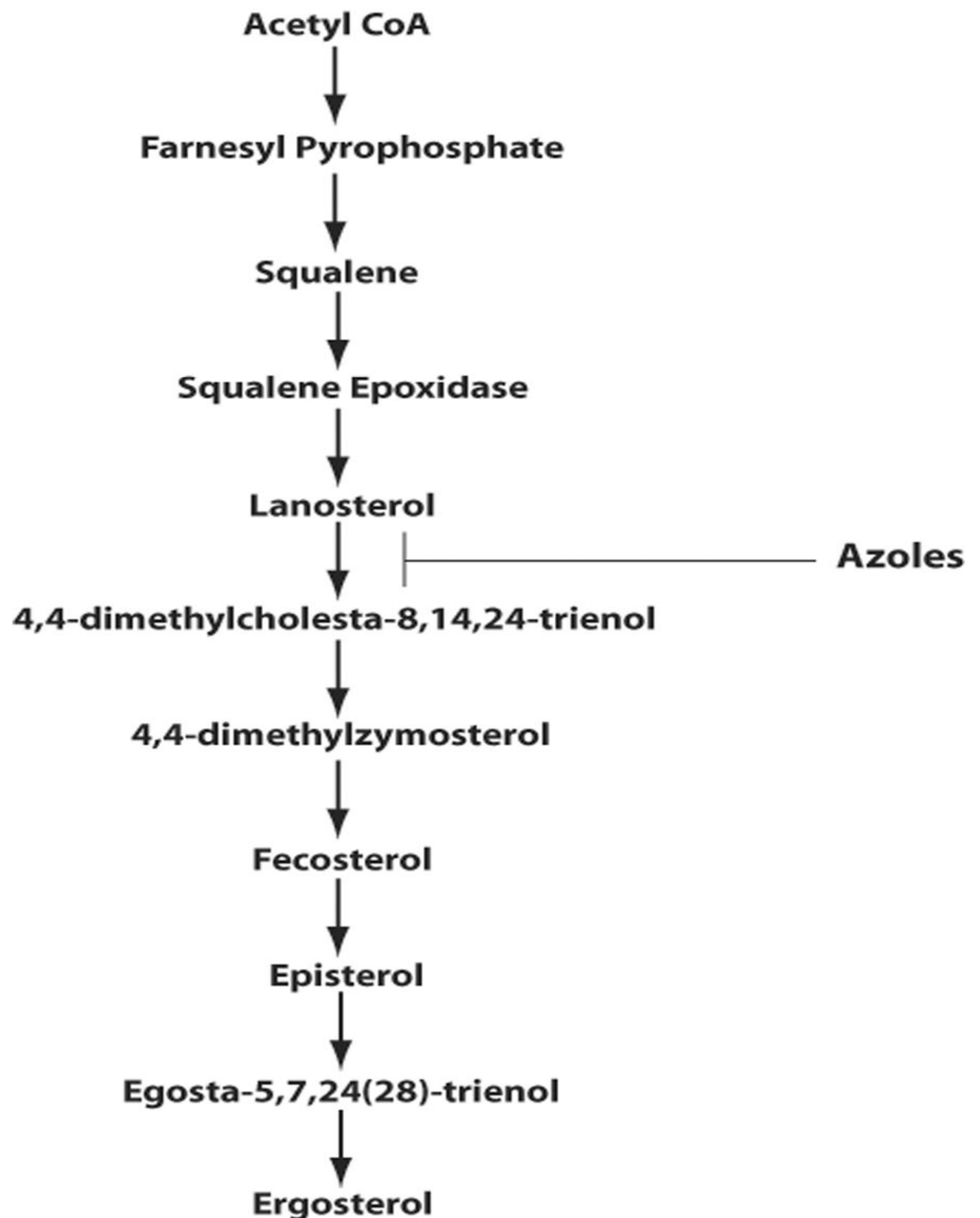


Figure 2. Ergosterol synthesis pathway and target of azoles.
(Sited from Wey *et al*, 1988).

2.7 Antifungal Drug Susceptibility Testing

In vitro antifungal susceptibility testing is used to determine the susceptibility of a clinical fungal isolate to an antifungal drug *in vitro*. The ultimate goal of this testing is to use the resulting *in vitro* minimum inhibitory concentration (MIC) of antifungal drug as a predictor of antifungal efficacy in the patient (i.e. *in vivo*). In addition to its clinical use, susceptibility testing can also be used in both drug development, as a screening tool, and epidemiological studies, to determine and / or monitor patterns of susceptibility or resistance in defined populations (Sandven *et al.*, 1998).

Until new antifungal agents were introduced in the 1980's, antifungal susceptibility testing was not considered necessary as there was only one therapeutic option (potassium iodide) available for most mycoses (Medoff *et al.*, 1983). Susceptibility testing has since become necessary due to increasing numbers of available antifungal drugs, antifungal resistance, numbers of opportunistic fungal pathogens, and incidence of fungal infections (Seibold and Tintelnot, 2003).

There has been some debate as to whether antimicrobial susceptibility testing is the best testing format for determining the effect of antifungal drugs on fungi *in vitro*.

While susceptibility testing has been used traditionally for both bacteria and fungi to determine the minimum inhibitory concentration (MIC) of a drug, it may not detect low level resistance or resistance mechanisms expressed under certain conditions (i.e. *in vivo*). The alternative to susceptibility testing is antimicrobial resistance testing, a testing format designed to detect phenotypic resistance (Diaz-Guerra *et al.*, 2003). The ultimate goal of both testing formats is to extrapolate the results to predict clinical efficacy in the patient (Nes *et al*, 1993). Despite the apparent advantages of resistance testing, the majority of antifungal susceptibility tests have been developed in the susceptibility testing format. Antifungal resistance tests are starting to be developed, however, as molecular mechanisms of resistance, i.e. phenotypic resistance, can now be determined by polymerase chain reaction (PCR) and real-time PCR based techniques (Mann *et al.*, 2003).

Regardless of the development of these resistance tests, investigators developing new or modifying old antifungal susceptibility tests need to optimize testing conditions to facilitate detection of both antifungal susceptibility and resistance (Nascimento *et al.*, 2003).

CHAPTER THREE

3. MATERIALS AND METHODS

3.1 Experimental Design

The study was laboratory based experiment where *Candida* species isolated between 1998 and 2004 were characterized. The isolates were from previous KEMRI/JICA Opportunistic infection (OI) project and acute respiratory Infections (ARI). It was conducted at Mycology Laboratory, Center for Microbiology Research, Kenya Medical Research Institute (KEMRI).

3.1.1 Sample size

Azole resistance in yeasts is estimated at 11.0 % (Bii *et al.,* 2006). 1t was therefore necessary that a sample size expected to detect 89 % of non-azole resistant *Candida* be used. The formula according to Fischers *et al.*, (1998) was used to calculate and arrive at a sample size of 150 isolates (Appendix 3).

3.1.2 Inclusion and exclusion Criteria

Yeast isolates from clinical sources from 1998 - 2004 that grew on SDA and on CHROMagar *Candida* were used in the study.

Isolates that failed to grow on SDA and non *Candida* yeasts were excluded from the study. This is because all yeasts grow on SDA.

3.1.3 Sources of Isolates

This study investigated a total of 150 *Candida* isolates from clinical sources in Nairobi. They were from culture collections from previous studies in Mycology Laboratory, Kenya Medical Research Institute. They were from both HIV positive and HIV negative patients. Isolates were recovered from swabs, urine, sputum, aspirates, Cerebral Spinal Fluid (CSF), blood and bile. Majority of the isolates were from swabs especially vaginal and throat swabs, most of the specimens were from females. These were from various Health institutions in Kenya namely Cottolego, St Thomas Bernados, Mbagathi district hospital, Aga Khan Hospital and Kenyatta National Hospital.

Table 1. Sources of the isolates investigated in the study

Specimen	Number of isolates
Swabs (vaginal, throat, cervical and endocervical)	56
Urine	50
Sputum	25
Aspirates	12
Blood	5
CSF	1
Bile	1
Total	150

3.2 Characterization of *Candida* species

3.2.1 Phenotypic characterization

3.2.1.1 Growth on Sabourauds Dextrose Agar (SDA)

Primary isolation from stock cultures was done using SDA (Oxoid LTD Basingstoke, Hampshire, England, appendix 1a). The SDA media was prepared as follows: 47 grams of SDA was weighed and dissolved in 1litre distilled water. This was boiled and autoclaved at 121 °C for 15 minutes. Approximately 20 ml of the media was then poured into sterile petri dishes and left to set at room temperature. The isolates from the stock cultures were cultured into SDA and incubated at 37 °C for 24 - 72 hours (Mcginnis, 1980). Colony characteristics and microscopic morphology were noted and recorded.

3.2.1.2 Germ tube test

This method was used as a presumptive test for identification of *Candida albicans.* The procedure was carried out as follows; a small inoculum of the test yeast cells from a pure culture was suspended in 0.5 ml horse serum. The suspension was incubated at 37 °C for three hours after which a drop of the incubated serum was placed on a microscope slide and covered with a cover slip. The wet mounts were examined for presence of germ tubes using the 40 X objective (Dalmau morphology method, Barnett *et al* 2000). The isolates were classified as either germ tube positive or germ tube negative.

3.2.1.3 Temperature tolerance

The isolates that formed germ tubes were cultured into SDA and incubated at 45 °C for 72 hours after which any growth was observed. This was to differentiate between *C. albicans* and *C. dubliensis*(Mcginnis, 1980).

3.2.1.4 Corn Meal Agar (CMA) morphology

Chlamydospore production was used as a presumptive confirmatory test for the identification of *Candida albicans.* Briefly 17 g of Corn Meal Agar (CMA) (Sigma Biomed inc. France, appendix 1b) was weighed and dispensed in 1 liter of sterile distilled water. Approximately 10 ml of Tween 80 was added after which it was sterilized by autoclaving at 121 ^{0} C for 15 minutes. The media was then allowed to solidify for at least 30 minutes. To determine the isolates ability to produce chlamydospore, test strains were inoculated on CMA plates by slide culture technique. The test involved streaking and stabbing the media with a 48 hour old yeast colony and, covered with sterile cover slip and incubated at 25 °C for 72 hours. Chlamydospore production was examined after staining with lactophenol cotton blue (Dalmau morphology method, Barnett *et al* 2000). The isolates were categorized as chlamydospore positive or negative.

3.2.2 Biochemical characterization

3.2.2.1 CHROMagar *Candida*

CHROMagar (CHROMagar Microbiology, Paris, France) was used for presumptive identification of *C. albicans, C. tropicalis* and *C. krusei.* This medium was also used for detection of mixed colonies. The method is based on the differential release of chromogenic breakdown products from various substrates following differential exoenzyme activity.

CHROMagar (CA) was purchased as powdered media and the plates were prepared according to the manufacturers' instructions (Tintelnot *et al.*, 2000).

Briefly, 47.7 grams of dehydrated CA was reconstituted in 1 litre of distilled water. It was brought to boil by repeated heatings and then cooled before approximately 20 ml of the media was dispensed into sterile petridishes. The media was allowed to solidify for at least 30 minutes. Using an inoculating needle, a single colony from a pure culture was seeded into CHROMagar media and incubated at 37 °C for 48 hours. Color changes were noted.

3.2.2.2 Analytic Profile Index (API)

API 20 C AUX system (Bio Merieux, 1 Etoile, France) was used for confirmatory purposes. The method is based on sugar and carbon assimilation and consists of 19 fermentation and assimilation tests and a control presented as a strip of cupules and accompanied by an ampoule of suspending medium. The API strip was

inoculated with the test yeast and incubated at 37 ^{0}C for 24 - 48 hours. After incubation, numerical profiles were constructed from the reaction patterns and were used to obtain identification results with the analytical profile index (Larone, 1995).

3.2.3 Molecular Characterization

3.2.3.1 DNA Extraction

Seventy five *Candida albicans* isolates were randomly selected for molecular characterization. Modified randomly amplified DNA technique known as PCR fingerprinting (Williams *et al*, 1995) was used in molecular characterization.

A small colony of each *Candida albicans* isolate was picked and suspended in 100 µl of lysis buffer in appendorf tube. This was vortexed for 10 seconds and boiled at 100 °C for 15 minutes. An equal volume (100 µl) of 2.5 M Sodium acetate was then added and kept at -20 °C for 1 hour. This was centrifuged at 13000 rpm for 5 minutes at 4 °C using a refrigerated centrifuge (Tomy Seiko CO., LTD Japan).

The supernatant was transferred into a new tube and 100 µl of phenol / chloroform / isoamyl alcohol was added, vortexed and centrifuged at 12000 rpm for 5 minutes at 4 °C.

The supernatant was transferred into a new tube and an equal volume (100 µl) Chloroform / Isoamyl alcohol (24:1) added. The mixture was vortexed and

centrifuged at 12000 rpm for 5 minutes at 4°C. The supernatant was then transferred to new tube. An equal volume (100 µl) of Isopropanol was added, vortexed and centrifuged at 12000 rpm for 15 minutes at 4 °C. The supernatant was discarded and 150 µl of 70 % cold ethanol added, mixed gently and centrifuged at 12000 rpm for 2 minutes at
4°C. The supernatant was discarded and the pellet was vacuum dried for 9 minutes after which the pellet was dissolved in 100 µl TE.

3.2.3.2 Polymerase Chain reaction (PCR)

Genotypic analysis was done using primer pairs that span the site of the transposable intron in the 25S rDNA. Primers and sequence used were; CA-INT-L (5'-ATA AGG GAA GTC GGC AAA ATA GAT CCG TAA-3') and CA-INT-R (5'-CCT TGG CTG TGG TTT CGC TAG ATA GTA GAT-3').

3.2.3.3 Master Mix

Amplification was done in a 50 µl volume containing 1µm of each primer, 1.5 Mm $MgCl_2$, 0.5 µ of Taq polymerase, 200 µm dATP, 200 µm dCTP, 200 µm dGTP and 200 µm dTTP and 1µl of template topped up to 50 µl with sterile distilled water. The mixture was subjected to thermocycling using an automated thermal cycler (PERKIN ELMER).

3.2.3.4 PCR Conditions

The PCR reactions were performed with an automated thermal cycler using the following conditions; denaturation for 3 min at 94 °C before 30 cycles of 94 °C for 1 min, 65 °C for 1 min and 72 °C for 2.5 min final extension at 72 ^{o}C for 10 min.

3.2.3.5 Electrophoresis

The PCR products were then electrophoresed through 1.5 % agarose gel in 1x TAE buffer (40 mM Tris-acetate, 0.2 Mm EDTA) for 40 min at 100 V. Bands were visualized by UltraViolet transillumination (JENCONS-PLS, Japan) after Ethidium bromide staining.

3.3 Drug Susceptibility Tests

Antifungal drug susceptibility was performed using broth microdilution method and procedures were conducted as outlined in the Clinical Laboratory Standard Institute (CLSI) document M27-A2 (National committee for clinical laboratory standards, 2002).

This method recommends the use of RPMI - 1640 medium (with glutamine and phenol red, without bicarbonate, Sigma R-7755 5t. Louis, U.S.A, appendix c). The RPMI - 1640 medium was supplemented with 0.2 % glucose and buffered to a pH of 7.0 with 0.165 mol / L Mops (3-[N-morpholino] propanesulfonic acid) (Sigma M-6270).

3.3.1 Antifungal agents

Standard powders of the following antifungal agents were obtained from their respective manufacturers: Fluconazole, Clotrimazole and Nystatin (Sigma, St. Louis, and U.S.A) and Amphotericin B (MP Biomedicals, Inc, France).

Fluconazole (10) mg was weighed and dissolved with 1 ml of sterile Dimethylformamide. This was diluted in 14.63 ml of RPMI medium to make an initial concentration of 64 μg / ml. Serial dilutions were done to give dilution range of 0.125 - 64 μg / ml i.e., (640, 320, 160, 80, 40, 20, 10, 5, 2.5 and 1.25).

Clotrimazole powder (2.9 mg) was weighed and dissolved in 1 ml ethanol. This was diluted with 17.13 ml of RPMI - 1640 to make an initial concentration of 160 μg / ml which was serially diluted to give a dilution range of 0.03 μg / ml - 16 μg / ml ((i.e. 160, 80, 40, 20, 10, 5, 2.5, 1.25, 0.6, and 0.3).

5000 μg / ml of Nystatin was weighed and dissolved in 1ml Dimethyl Sulfoxide (DMSO). This was then diluted in 27 ml RPMI medium to make an initial concentration of 185 μg / ml. Nystatin was tested over a range of 18.5 - 0.07 μg / ml (i.e.185, 92.5, 46, 23, 11.5, 5.8, 2.9, 1.4, 0.7).

Amphotericin B powder (5 mg) was weighed dissolved in 1 ml DMSO and kept in darkness to avoid destruction by light.

One millilitre (5000 μg / ml) Amphotericin-B was diluted in 30.25 ml RPMI and this made the initial concentration of 160 μg / ml. Amphotericin B was tested over a range of 0.03 μg / ml - 16 μg / ml (i.e. 160, 80, 40, 20, 10, 5, 2.5, 1.25, 0.6, and 0.3).

Each drug range was prepared as recommended by NCCLS document, 2002.

One hundred microlitre of the antifungal drug dilutions were dispensed into u-shaped microtiter plates (Greiner Bio-one, Holland). The test isolates were suspended in RPMI medium to 1 McFarland standard suspension. 100 μl of the mixture was inoculated into each microtiter well containing different dilutions of the test drug diluting the drug ten times to the required concentration. The inoculated microtitre plates were incubated at 37 ^{o}C for 24 hours. MIC was scored as the lowest concentration with 100 % inhibition for Amphotericin B, Nystatin and Clotrimazole and 80 % reduction in turbidity for Fluconazole.

3.3.2 Quality Control strains

Prior to antifungal susceptibility testing each isolate was sub-cultured at least twice on SDA for 24 hours before use. This was to obtain a pure culture of each isolate. The QC strain, *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258, were included in each batch of susceptibility tests to ensure quality control and the results were accepted only when the MIC of the QC strain were within the CLSI limits.

CHAPTER FOUR

4. RESULTS

4.1 Phenotypic Characterization

4.1.1 Growth on Sabaraud's Dextrose Agar

All the 150 *Candida* isolates showed growth on Saboraud Dextrose agar (SDA). Colonies were white to cream colored, smooth, glabrous and yeast-like in appearance. Microscopic morphology showed spherical to subspherical budding yeast-like cells or blastoconidia, 2.0-7.0 x 3.0-8.5 um in size (Figure 3). The observed morphological characteristics were compared with those in the Dalmau morphology identification chart (Barnett *et al*., 2000).

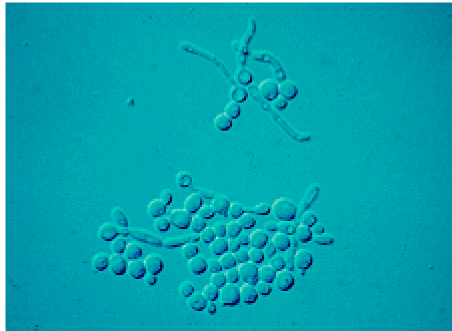


Figure 3. Budding yeast cells of *Candida* species on SDA. (Magnification X40)

4.1.2 Germ Tube Test

Of the *C. albicans* isolates tested, 86.1 % were germ tube positive (Figure 4). The rest 13.9 % were germ tube negative. The observed morphological characteristics

were compared with those in the Dalmau morphology identification chart (Barnett *et al*., 2000).

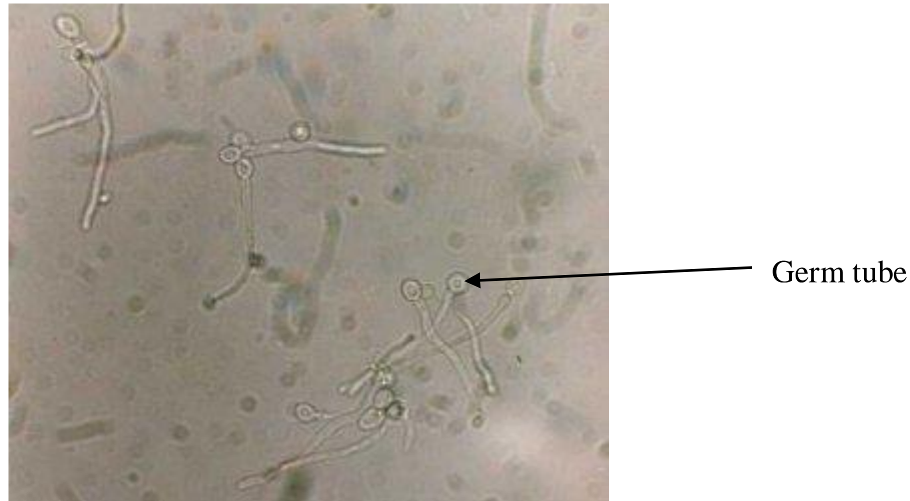


Figure 4. Germ tube positive *C. albicans* after incubation for 3 hours at 37°C on Horse serum. (Magnification X40)

4.1.3 Growth on Corn Meal Agar (CMA)

All the *Candida* isolates showed growth on CMA. *C. albicans* species produced abundant chlamydospores and pseudohyphae with clusters of spores (Figure 5) on CMA after 72 hours incubation at 25 °C.

C. tropicalis formed blastoconidia singly and long pseudohyphae on CMA. *C. parapsilosis* formed blastoconidia along curved pseudohyphae and giant mycelial cells. *C. guilliemondii* formed fairly short, fine pseudohyphae and clusters of blastoconidia at septa. *C. krusei* formed pseudohyphae with cross-matchsticks or

tree-like blastoconidia. *C. glabrata* formed small, oval, single terminal budding, non encapsulated yeast cells, formation of pseudohyphae was not seen. The observed morphological characteristics were compared with those in the Dalmau morphology identification chart (Barnett *et al*., 2000).

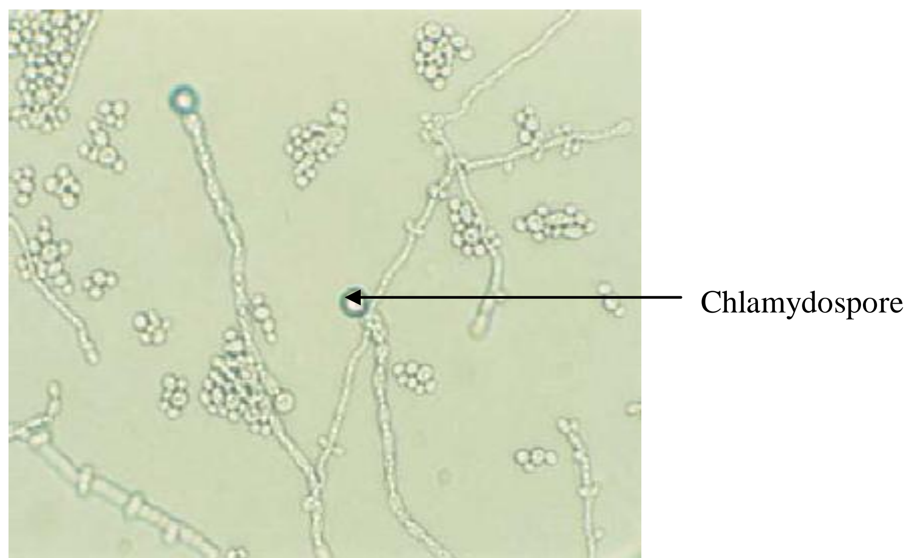


Figure 5. *Candida albicans* with abundant chlamydospores and pseudohyphae on Corn Meal Agar. (Magnification X40).

4.1.4 Temperature Tolerance

All the 130 *C. albicans* grew at both 37 °C and 45 °C temperatures on SDA.

4.2 Biochemical characterization

4.2.1 Analytic Profile Index (API)

All the isolates were confirmed using API C AUX 20 system. One hundred and thirty isolates were identified as *C. albicans*. The Other 20 *Candida* isolates were identified as: 6 (4%) *C. parapsilosis,* 4 (2.7%) *C. tropicalis,* 4 (2.7%) *C. krusei,* 2 (1.3%) *C. guilliemondii,* 2 (1.3%) *C. glabrata*, and 2 (1.3%) *C. famata using* the same method.

4.2.2 Growth on CHROMagar *Candida*

Nearly all isolates of *Candida* species tested gave colonies with colors ranging from white through pink, pinkish purple, blue and purple after 48 hours of incubation on CHROMagar *Candida* at 37 °C (Table 2, figure 6).

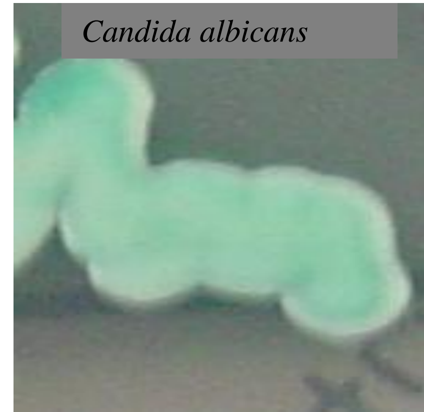
Of the 150 isolates 130 (86.7%) yielded several shades of green colonies after 48 hours of incubation in CHROMagar *Candida.* They were identified as *C.albicans* (Fig. 6 b). Four isolates developed a distinctive dark blue or blue-gray color after 48 h of incubation on CA and were identified as *C. tropicalis* (Fig.6 c). Six isolates developed a pinkish color and were identified as *C. parapsilosis*

(Fig. 6 a). Nearly all isolates of *Candida* species tested gave colonies with colors described as ranging from white, through pink, pinkish purple, and purple after 48 h of incubation on CA at 37 °C (Table 2). The observed morphological characteristics were compared with those in the Dalmau morphology identification chart (Barnett *et al*., 2000, Tintelnot *et al.*, 2000).

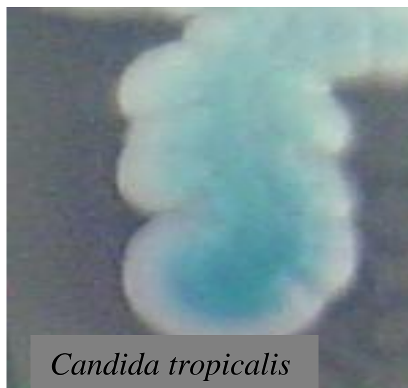
Table 2. Colony colors of *Candida* isolates on CHROMagar *Candida* after 48 h at 37 °C

Species	**Total No. Of Isolates**	**Colors Observed**
C. albicans	130	Green.
C. parapsilosis	6	Pink
C. tropicalis	4	Dark blue
C. krusei	4	Whitish Pink
C. guilliemondii	2	Pinkish Purple
C. glabrata	2	light Pinkish
C. famata	2	White, Light Pink

(a)



(b)



(c)

Figure 6. Different *Candida* species on CHROMagar *Candida* after incubation for 48 hours;
(a) *Candida parapsilosis* showing pink coloration (b) *Candida albicans* showing green coloration and (c) *Candida tropicalis* showing blue coloration.

4.3 Molecular Characterization

Genotypic analysis of seventy five randomly selected *C. albicans* isolates indicated that 60 % belonged to genotype A (Figure 7 b). In this genotype only one band of 450 base pair was amplified. The band size was similar to that of reference strain *C. albicans* ATCC 90028 genotype A which was used as standard. The second common genotype was C (16%) which exhibited two bands of approximately 450 and 650 base pairs (Figure 7 a). Eight percent were categorized as genotype B with one band of approximately 650 base pairs. Four percent of the isolates gave a faint band of approximately 550 base pairs (figure 7 c). These isolates did not correspond with any of the earlier described genotypes (A, B, C, D and E, Tamura *et al,* 2001); therefore it was placed in a new genotype F. However, 12 % of the isolates used did not show any specific band.

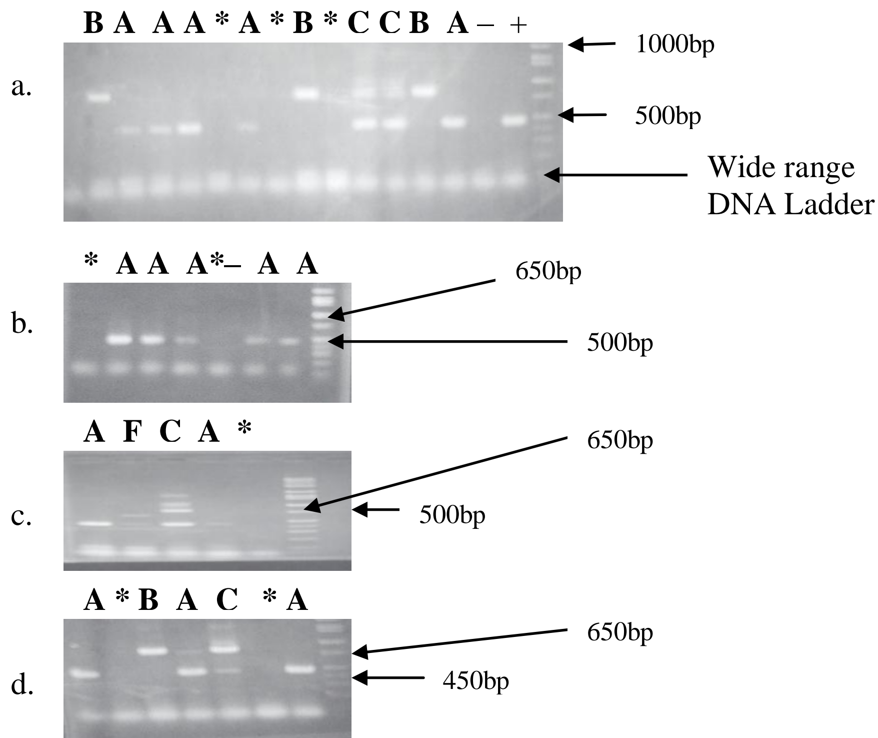


Figure 7. Representative gel showing different genotypes of *Candida albicans* after electrophoresis, ethidium bromide staining and photographed with polaroid camera.

A Genotype A with 450 bp, **B** Genotype B with 650 bp, **C** Genotype C with 2 bands, 450 and 650 bp, **F** Genotype F with 550 bp**.** (DNA ladder 100kb marker)

* No specific band amplified, + *Candida albicans* ATCC 90028 used as positive control and -negative control.

4.4 Antifungal Drug Susceptibility tests

4.4.1 Fluconazole Susceptibility

4.4.1.1 Susceptibility of *Candida albicans* isolates to Fluconazole

Most of the *C. albicans* isolates were fairly susceptible to Fluconazole. (Table 3, Fig. 8). Most (73.1 %) of the *C. albicans* isolates were susceptible (MIC ≤ 8 µg / ml) to Fluconazole, 14.6 % of the isolates were susceptible dose dependent (MIC 16 - 32 µg / ml) while 12.3 % were resistant (MIC ≥ 64 µg / ml) to Fluconazole. The MIC_{90} to Fluconazole was 64 µg / ml while MIC_{50} was 4.0 µg / ml.

Table 3. Response of *Candida albicans* isolates to various Fluconazole concentrations after incubation for 24 hrs at 37°C.

Category	MIC range (µg / ml)	Frequency (%)
Susceptible (S)	≤ 8	73.1
Susceptible dose dependant (S-DD)	16-32	14.6
Intermediate (I)	-	-
Resistant (R)	≥ 64	12.3
	Total	100

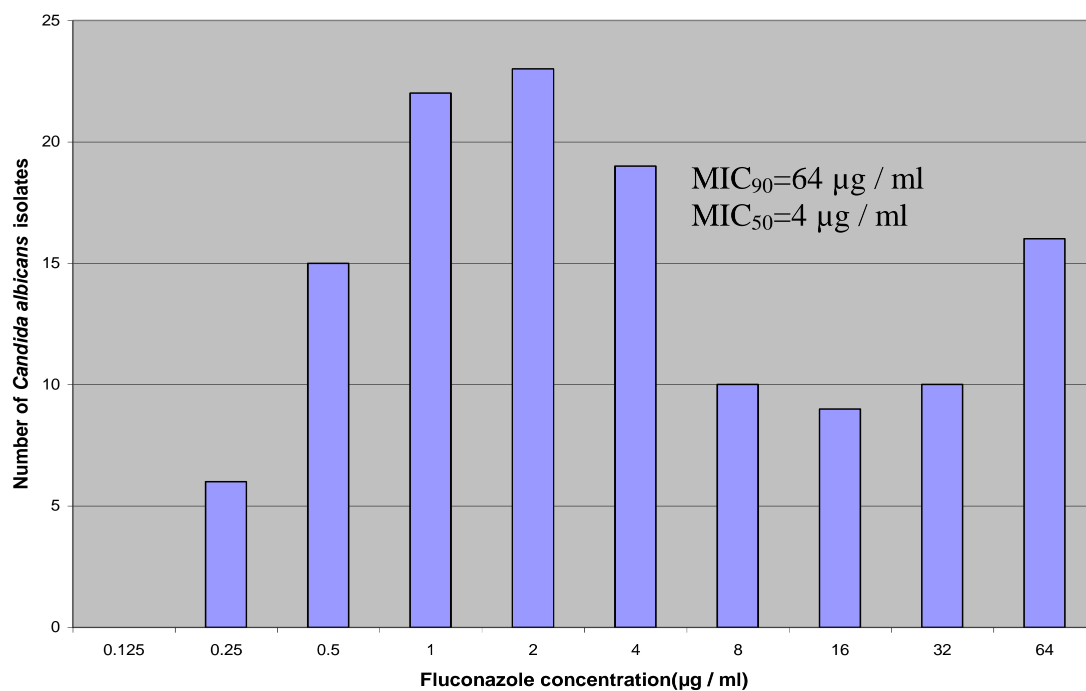


Figure 8. The number of *Candida albicans* isolates inhibited at various concentrations of Fluconazole after incubation for 24 hours at 37°C.

4.4.1.2 Susceptibility of non *albicans* C*andida* isolates to Fluconazole

The non *albicans* C*andida* were fairly susceptible to Fluconazole. Table 4 shows the distribution of the 20 non *albicans Candida* at various concentrations of Fluconazole.

Table 4. Response of non *albicans Candida isolates* to various Fluconazole concentrations after incubation for 24 hrs at 37°C.

Category	*C. parapsilosis*	*C. tropicalis*	*C. krusei*	*C. guilliemondii*	*C. famata*	*C. glabrata*
Susceptible (S) (≤ 8)	5	4	0	0	2	0
Susceptible dose dependant (S-DD) (16-32)	0	0	1	2	0	0
Intermediate (I)	0	0	0	0	0	0
Resistant (R) (≥ 64)	1	0	3	0	0	2
Total	6	4	4	2	2	2

Out of the six *C. parapsilosis* isolates, 5 were susceptible to Fluconazole and one was resistant. Isolates of *C. tropicalis* and *C. famata* were susceptible to Fluconazole. Three isolates of *C. krusei* were resistant to Fluconazole while one isolate was susceptible dose dependent.

C. guilliemondii isolates were susceptible dose dependent to Fluconazole while *C. glabrata* isolates were resistant to Fluconazole.

4.4.2 Clotrimazole Susceptibility

4.4.2.1 Susceptibility of *Candida albicans* isolates to Clotrimazole

C. albicans isolates were fairly susceptible to Clotrimazole. Sixty one percent of *C. albicans* isolates had an MIC ≤ 1 μg / ml while the rest 39 % had an MIC of ≥ 1 μg / ml to Clotrimazole. Twenty seven percent of the isolates had an MIC of between 2 - 8 μg / ml to Clotrimazole while 12.2 % of the isolates had an MIC ≥ 16 μg / ml (Table 5).

The MIC_{90} to Clotrimazole was 16.0 μg / ml while MIC_{50} was 1.0 μg / ml. Figure 9 shows the distribution of the 130 *C. albicans* isolates to various concentrations of Clotrimazole.

Table 5. Response of *Candida albicans* isolates to various Clotrimazole concentrations after incubation for 24 hours at 37°C.

	Clotrimazole Concentration (μg / ml)			
	0.03-0.25	0.5-1	2-8	≥16
Number of isolates (%)	30	30.8	27	12.2

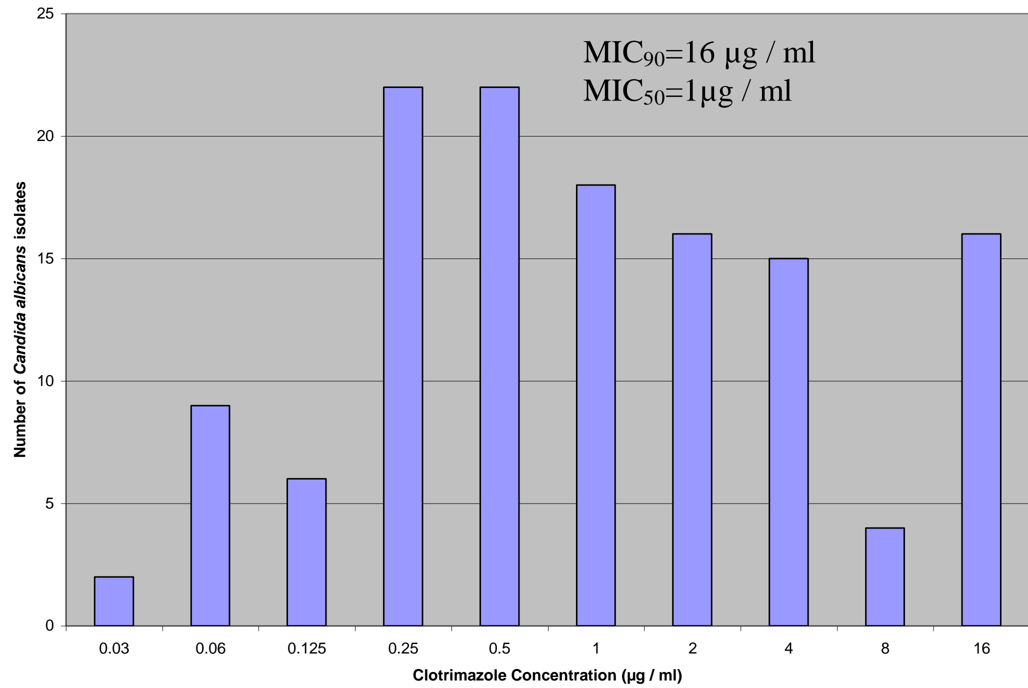


Figure 9. The number of *Candida albicans* isolates inhibited at various concentrations of Clotrimazole after incubation for 24 hours at 37 °C.

4.4.2.2 Susceptibility of non *albicans* C*andida* isolates to Clotrimazole

The non *albicans* C*andida* were fairly susceptible to Clotrimazole. Table 6 shows the distribution of the 20 non *albicans Candida* isolates at various concentrations of Clotrimazole. Four of the 6 *C. parapsilosis* isolates had MIC of between 0.25 – 2 µg / ml to Clotrimazole while the other 2 isolates had MIC of 16 µg / ml. *C. krusei* and *C. famata* isolates were susceptible to Clotrimazole with an MIC of between 0.06 - 2 µg / ml. *C. tropicalis* was fairly susceptible to Clotrimazole. Out of the 4 isolates of *C. tropicalis,* 2 were susceptible while 1 was resistant and the other one had an MIC of 4 µg / ml. One isolate of *C. glabrata* was susceptible while the other one was resistant.

Table 6 . Response of non *albicans Candida* isolates to various Clotrimazole concentrations after incubation for 24 hours at 37°C.

Candida species	Clotrimazole concentrations (µg / ml) and the number of isolates (%)			
	0.03-0.25	0.5-1	2-8	≥16
Candida parapsilosis	1	2	1	2
Candida tropicalis	2	0	1	1
Candida krusei	2	1	1	0
Candida guilliemondii	2	0	0	0
Candida famata	1	0	0	1
Candida glabrata	0	1	0	1
Total	8 (40 %)	4 (20 %)	3 (15)	5 (25 %)

4.4.3 Nystatin Susceptibility

4.4.3.1 Susceptibility of *Candida albicans* isolates to Nystatin

C. albicans isolates were fairly susceptible to Nystatin. Eighty five percent of *C. albicans* isolates had an MIC of ≤ 1.15 µg / ml to Nystatin, while 14.6 % of the isolates had MIC ≥ 1.15 µg / ml. 20.8 % of the isolates had an MIC of between 1.15 and 9.25 µg / ml. 3.8 % of the isolates had an MIC ≥ 18.5 µg / ml. The MIC_{90} of Nystatin was 18.5 µg / ml and MIC_{50} was 0.29 µg / ml (Table 7, Fig. 10).

Table 7. Response of *Candida albicans* isolates to various concentrations of Nystatin after incubation for 24 hours at 37°C.

	Nystatin Concentration (µg / ml)			
	0.07-0.29	0.58-1.15	2.3-9.25	≥18.5
Number of isolates (%)	76(58.5)	35(26.9)	14(10.8)	5 (3.8)

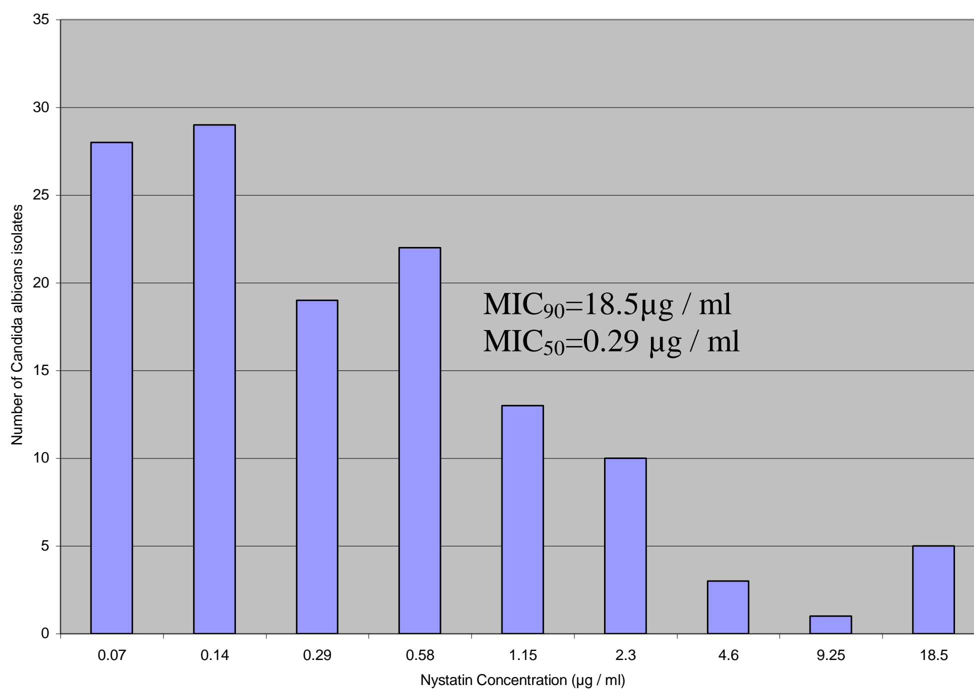


Figure 10. The number of *Candida albicans* isolates inhibited at various concentrations of Nystatin after incubation for 24 hours at 37°C.

4.4.3.2 Susceptibility of non *albicans* C*andida* isolates to Nystatin

Ninety percent of the non *albicans* C*andida* were fairly susceptible (MIC ≤ 1.15 µg / ml) to Nystatin. Table 8 shows the distribution of the 20 non *albicans Candida* at various concentrations of Nystatin. Only 10 % of non *albicans Candida* had MIC between 2.3 - 9.25 µg / ml while 35 % had MIC between 0.58 - 1.15 µg / ml. All the other non *albicans Candida* were susceptible with MIC between 0.07 - 0.29 µg/ml. None of the non *albicans Candida* isolates had MIC of ≥ 18.5 µg / ml.

Table 8. Response of non *albicans Candida* isolates to various Nystatin concentrations after incubation for 24 hours at 37°C.

Candida species	Nystatin concentrations (µg / ml) and the number of isolates (%)			
	0.07-0.29	0.58-1.15	2.3-9.25	≥18.5
Candida parapsilosis	5	0	1	0
Candida tropicalis	4	0	0	0
Candida krusei	0	3	1	0
Candida guilliemondii	0	2	0	0
Candida famata	1	1	0	0
Candida glabrata	1	1	0	0
Total	11(55 %)	7(35 %)	2(10 %)	0

4.4.4 Amphotericin B Susceptibility

4.4.4.1 Susceptibility of *Candida albicans* isolates to Amphotericin B

Most isolates of C. *albicans* were fairly susceptible to Amphotericin B. Majority (93.1 %) of *C. albicans* isolates had an MIC ≤ 1 µg / ml while the rest 9.7 % had an MIC of ≥ 1 µg / ml. Only 4.6 % of the isolates had an MIC between 2 - 8 µg / ml and 2.3 % of the isolates had an MIC ≥ 16 µg / ml to Amphotericin B. The MIC_{90} and MIC_{50} to Amphotericin B were 1 and 0.25 µg / ml respectively (Table 9, Fig.11).

Table 9. Response of *Candida albicans* isolates at various Amphotericin B concentrations after incubation for 24 hours at 37 °C.

	Amphotericin B concentration in µg/ml and number of isolates (%)			
	0.03-0.25	0.5-1	2-8	≥16
Number of isolates (%)	89(68.5)	32(24.6)	6(4.6)	3 (2.3)

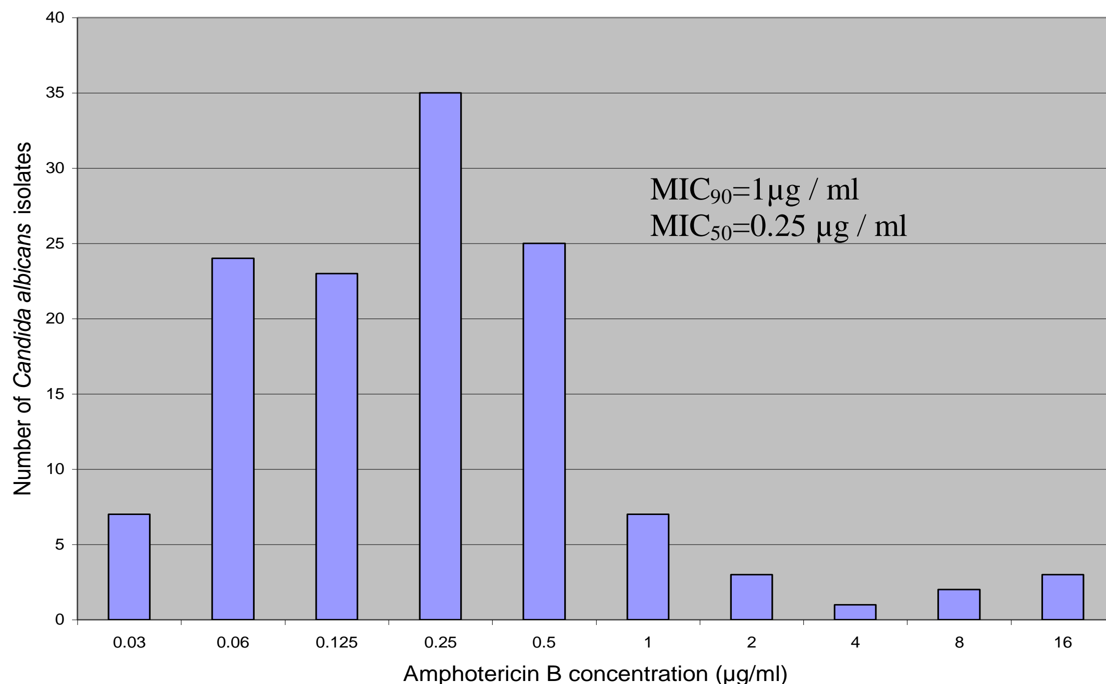


Figure 11. The number of *Candida albicans* isolates inhibited at various concentrations of Amphotericin B after incubation for 24 hours at 37°C.

4.4.4. 2. Susceptibility of non *albicans* C*andida* isolates to Amphotericin B

Most (75%) of the non *albicans* C*andida* isolates were fairly susceptible to Amphotericin B. Nearly all *C. parapsilosis* isolates were susceptible to Amphotericin B with an MIC range of between 0.03 - 8 µg / ml while 3 out of 4 *C. tropicalis* isolates were susceptible to Amphotericin B (MIC 0.03 - 0.25 µg / ml). One isolate of *C. tropicalis had* an MIC ≥ 16 µg / ml. All isolates of *C. Krusei, C. guilliemondii, C. glabrata* and *C. famata* had MIC range of between 0.03 -2 - µg / ml (Table 10).

Table 10. Distribution of non *albicans Candida* isolates at various concentrations of Amphotericin B.

Candida species	Amphotericin B concentrations (µg / ml) and the number of isolates (%)			
	0.03-0.25	0.5-1	2-8	≥16
Candida parapsilosis	4	1	1	0
Candida tropicalis	3	0	0	1
Candida krusei	3	0	1	0
Candida guilliemondii	2	0	0	0
Candida famata	2	0	0	0
Candida glabrata	1	1	0	0
Total	15 (75%)	2 (10%)	2 (10%)	1(5%)

CHAPTER FIVE

5. DISCUSSION

C. albicans was the most frequently isolated yeast pathogen accounting for 86.8 % of the isolates. This is in line with previous investigations where *C. albicans* accounted for up to 80 % of the candidiasis infection (Wade, 1993). The figure is slightly higher than that reported probably because of the type of patients, their immune status or the sample size. Although *C. albicans* was the most frequently isolated yeast pathogen, other *Candida* species were identified. These species included *C. glabrata*, *C. krusei, C. parapsilosis, C. famata, C. guilliermondii* and *C. tropicalis.* Previous studies have reported other non *albicans Candida* as emerging significant pathogens (Coleman *et al.,* 1988, and Moran *et al.,* 2003).

In the recent years the number of serious opportunistic yeast infections, particularly in immunocompromised patients has dramatically increased (Richardson *et al.,* 2003). Among them, *Candida* species accounts for a large number of serious opportunistic yeasts. Majority of the isolates were from swabs especially vaginal and throat swabs, most of the specimens were from females, an indication that vaginal candidiasis is common. Very few isolates were obtained from blood and CSF probably because these are normally sterile body fluids and candidaemia and *Candida* meningitis is not commonly reported and carry grave prognosis (Reef and Mayer, 1995).

In this study, all the *Candida* isolates grew on Sabaraud's Dextrose Agar (SDA, Odd, 1991) medium (the medium most widely used for the isolation of *Candida* and other yeast species from clinical specimens). This is a general purpose medium that supports the growth of most pathogenic fungi. However SDA is not a differential medium and colonies of different pathogenic yeast species grown on this agar cannot be easily distinguished from each other. Careful observers are often able to recognize mixtures of different yeast species when they occur on a single plate but the absence of any differential indicator in SDA means that there is no guarantee that mixed yeast cultures will be detected. In this study mixtures of different yeasts was observed and were differentiated by the subsequent phenotypic methods.

CHROMagar *Candida* used in this study was able to identify 3 *Candida* species on the basis of color (Figure 6).To be of value for routine isolation and presumptive differentiation of yeasts, an indicator medium should exhibit several properties. It should support the growth of yeasts but not of bacteria. If the medium also facilitates the growth of filamentous fungi, it is not a disadvantage, since in many clinical samples it is not possible to predict whether yeast or filamentous fungi will be isolated. The differential property of the medium should also allow unambiguous presumptive discrimination between the yeast species most commonly encountered in clinical samples. Finally it should facilitate the

recognition of specimens containing mixtures of yeast species, and exposure of the fungi to the differential indicator substances should not affect their viabilities for subsequent subculture.

Out of 130 *C. albicans* species, 3.9 % were germ tube negative. This supports other investigations by scientists that up to 5 % of *Candida albicans* are germ tube negative (Reef, 1995). The non *albicans Candida* did not form any chlamydospores and API C AUX 20 confirmed that they were non *albicans Candida*. Since *C. albicans* is the yeast species most often isolated from clinical material, most clinical laboratories approach yeast identification by applying simple rapid tests such as germ tube formation to distinguish *Candida albicans* from other species which require more extensive testing for proper identification.

All the *C. albicans* were positive for chlamydospores typical of *C. albicans.* They all showed distinct growth at 37 ^{o}C and 45 ^{o}C temperatures. *Candida dubliensis* was therefore not detected in this investigation. The ability to easily differentiate between *C. albicans* and other *Candida* species in routine laboratory practice remains a technical problem. Evidence for the inducible stable fluconazole resistance *in vitro* in *C. dubliensis* strain have been demonstrated in immunocompromised patients receiving long – term fluconazole prophylaxis (Moran *et al.,* 1997 and Pfaller *et al*., 1999). This situation has necessitated that

clinical laboratories be able to isolate and identify yeasts of medical importance as rapidly and accurately as possible.

C. dubliensis is very similar to *C. albicans* in many characteristics like germ tube formation and chlamydospore production (Ruhnke *et al.* 1997 and Sullivan et *al.*, 1998). It is therefore likely that *C. dubliensis* strains have been and will continue to be identified in the clinical laboratory as *C. albicans* (Sullivan et *al.*, 1998). Several methods for identification of *C. dubliensis* and discrimination from *C. albicans* have been reported. They include formation of dark green colonies on CHROMagar *Candida* (Tintelnot *et al.*, 2000), no or strictly reduced growth at 45 ^{o}C (Gales *et al.,* 1999) and lack of ability to assimilate xylose (Salkin et *al.*,1998, Schoofs *et al*.,1999 and Pincus *et al*.,1999) .

In the present study, *C. albicans* isolates were genotyped using the method described by McCullough *et al* (1997). This is the first study to report on the genotype distribution of *C. albicans* from clinical setting in Kenya. Using primers that span the site of the transposable intron in the 25S rDNA, *C. albicans* can be classified into five genotypes (A, B, C, D and E). Four *C. albicans* genotypes were identified. However, slight differences in the amplicon sizes with that previously reported by McCullough *et al* (1997) were noted. As expected the *C. albicans* genotype A amplified one band of 450 base pairs. However genotype B which was supposed to give one band of 840 base pair band amplified one band of 650 base

pairs instead. In genotype C, two bands of 450 and 650 instead of 450 and 840 base pairs were amplified. There were 4 % of the *C. albicans* isolates in which a faint band of 550 base pair was amplified which could not be categorized as either genotype D or E with expected amplicon size of 1,080 and 1400 base pairs respectively. These strains were designated *C. albicans* genotype F. Genotype E with amplicon size of 1,080 base pair which represent *C. dubliniensis* was not detected which further supports our phenotypic characterization which did not identify any *C. dubliniensis* isolates. Although *C. dubliniensis* has a world wide distribution, it was not detected neither has it been reported in Kenya previously. It was noted that some (12 %) of the *C. albicans* isolates yielded no specific band and therefore could not be genotyped using the primer set used. These could be an indication that there could be other genotypes of *C. albicans* circulating in Kenya that are different from those in Japan which was also noted with the differences in amplicon sizes. Possible explanation could be that the primers used were designed using *C. albicans* strains from elsewhere and did not include strains from Africa or Kenya. Strains from different geographical regions could exhibit slight differences in genotypic features and may need specific probes.

Genotype A was the most predominant (60 %). This is in consistent with other studies in Japan showing that majority of *C. albicans* belongs to genotypes A followed by genotypes B, C and D respectively (Tamura et al., 2001). However, in

the present study the prevalence distribution was; genotype A (60%), C (16%), B (8%) and F (4%). *C. albicans* genotype D and E were not detected.

Most *Candida albicans* were susceptible (73.1 %) to Fluconazole with an MIC ≤ 8 µg / ml. However 12.2 % were resistant (Table 3). This is in agreement with previous studies by scientists whereby Fluconazole resistant *C. albicans* accounted for 9 - 12% of the isolates from patients with candidiasis (Newman, 1994). Since *C. albicans* is a part of the intestinal and vaginal normal flora, and thrush is merely its overgrowth, drugs that keep candidiasis at its check may never completely eradicate it. The continued presence of the organism during treatment makes the selection of drug resistant fungal organisms more likely. As prophylactic use of azoles increase, the incidence of azole resistant *in Candida* is also likely to increase (Pfaller, 1995). The increasing incidence of AIDS and the recent development of a new treatment strategy for patients with hematological malignancies and organ transplants have led to an increase in the number of immunocompromised patients at risk of fungal infections (Sandhu *et al.*, 1995). The majority of these diseases are caused by *Candida albicans* (Dixon *et al.*, 1996).

Azole antifungal agents have therapeutic activity against different *Candida* species. Among azole drugs, Fluconazole is more tolerated with wider spectrum of efficiency. During the last decade, the higher incidence of fungal infections in hospitalized patients has resulted in the use of systemic antifungal agents

especially Fluconazole, which remains a first line antifungal agent (Redding *et al.*, 1994). However in the recent years increasing resistance with Fluconazole has appeared and antifungal drug resistance is quickly becoming a major problem in immunocompromised patients (Wheat, 1998). Majority of the non *albicans Candida* used in this investigation were susceptible to Fluconazole. However a marked resistance was observed among *C. glabrata* and *C. krusei* (Table 4). This is in agreement with past studies by Pfaller *et al.* (1999) that there has been an emerging resistance to Fluconazole among the two species.

Seventy nine percent (79 %) of *C. albicans* had an MIC of ≤ 1µg / ml to Clotrimazole. However 12.2 % of the isolates had an MIC of ≥ 16 µg / ml, which is an indication of resistance to the drug. Clotrimazole has been shown to inhibit all the major fungi causing systemic infection, at a concentration of 1µg / ml, with efficacy against *Candida, Histoplasma and Aspergillus* species (Grahame-Smith *et al.,* 1992). Although favorable results from systemic treatment of candidiasis and aspergillosis have been described, most *C. albicans* still show high MICs to Clotrimazole. Induction of low blood pressure and toxicity are some of the draw backs for its systemic use. Despite the high MIC to Clotrimazole, the drug is extensively used in Kenya for management of vaginal candidiasis and for dermatological conditions. On the other hand 25 % non *albicans Candida* had an MIC ≥ 16 µg / ml, which also indicates an emerging resistance to Clotrimazole.

Clotrimazole is a tritylimidazole derivative [bis-phenyl (2 chlorophenyl) 1-imidazolylmethane]. Initially developed by Bayer in 1968, it was the first commercially available azole antifungal drug (Holt *et al.*, 1972). Released in 1975 as a topical antifungal agent, Clotrimazole has been a well-tolerated and frequently administered drug for mucocutaneous candidiasis. Clotrimazole is approved for both treatment and prevention of oral candidiasis.

Initial *in vitro* studies of Clotrimazole used various methodologies (Bergan *et al.*, 1983, Hamilton 1972 and Hoeprich *et al.*, 1976). Previous testing of the antifungal activity of Clotrimazole faced several difficulties. As Clotrimazole is highly insoluble in water, the drug must first be dissolved with an organic solvent such as dimethylformamide ethanol, chloroform, polyethylene glycol, or DMSO (Holt *et al.*, 1972, Hussain *et al.*, 1986). Further dilution of the drug can be completed using an aqueous growth medium. Hoeprich *et al.*, (1976) demonstrated that using undefined growth media may affect the MICs of azoles such as Clotrimazole and Miconazole. As recommended by NCCLS standard M27-A, the use of a defined growth medium, such as RPMI, could obviate this problem. Inoculum size also was previously reported to influence MICs of Clotrimazole (Plempel *et al.*, 1969).

At present, there are no established interpretive breakpoint criteria by which to designate a *Candida* isolate as either susceptible or resistant to Clotrimazole. Interpretive breakpoint criteria have been defined for Fluconazole and Itraconazole against *Candida* species. (Rex *et al.*, 1997). Logically, an isolate is labeled as

resistant to a drug if its MIC exceeds the amount of the drug attainable in the infected tissue of a patient. However, the correlation between the antifungal activity of *in vitro* levels and levels of the drug in tissue is often variable. Clotrimazole troches, for example, may achieve salivary concentrations of 5.2 to 15 µg / ml for as long as 3 h after dissolution (Shadomy, 1971). Factors such as protein binding, physiologic temperature, and local pH and osmolality may alter the antifungal activity of the same drug concentration *in vitro* and *in vivo*. Correlation between *in vitro* concentrations and clinical response is a key variable in determining breakpoint criteria. In Kenya a one percent topical cream application for dermatomycoses and a 100 mg pessary for vaginal candidiasis are still widely used.

Majority of the *C. albicans* isolates were susceptible to Amphotericin B with 2.3 % of the isolates having an MIC $\geq$ 16 µg / ml. The non *albicans Candida* were also sensitive to Amphotericin B drug with only one non *albicans Candida* resistant to Amphotericin B. Amphotericin B is still widely used as the drug of choice for most fatal disseminated fungal infections. However the high cost of the drug makes it unaffordable to the majority of patients especially in the developing world. It should also be noted that Amphotericin B can only be administered in low doses due to its toxicity.

Most of the *C. albicans* and non *albicans Candida* were susceptible to Nystatin with majority of isolates having an MIC range of 0.07 - 1.15 µg / ml. Nystatin has been considered effective against *Candida* oesophangitis (Nyst *et al.,* 1992). A cure rate of less than 10 % has been reported in Democratic Republic of Congo (Nyst *et al.,* 1992) and 21.6 % in Uganda (Ravera *et al.*, 1999).

The susceptibility pattern revealed that most of the *Candida* isolates were susceptible to Amphotericin B, Fluconazole and Nystatin. *Candida* species were most sensitive to Amphotericin B. Prevalence of resistance to Amphotericin B was lowest followed by Nystatin and azoles. *Candida* has shown reduced susceptibility to Clotrimazole. Fungal infections are often challenging to manage; caution has to be exercised in the use of antifungal drugs to arrest any further increase in the resistance. They have to be taken only under a clinician's prescription.

6. CONCLUSION AND RECOMMENDATIONS

From the study *C. albicans* was the most prevalent isolate hence the most common cause of candidiasis. Non *albicans Candida* were also identified which indicates their emergence as opportunistic pathogens especially in HIV / AIDS patients. All the *C. albicans* formed chlamydospores and grew at both 37 °C and 45 °C ruling out the possibility of *C. dubliensis*.

This was the first study to report genotype distribution of *Candida albicans* from a clinical setting in Kenya. In addition to the previously classified genotypes of *C. albicans* in Japan by Tamura *et al*., (2001), a new genotype F was detected. This could be an indication that there could be other genotypes circulating in Kenya different from those of Japan implying that strains from different geographical regions exhibit slight differences in genotypic features.

This study showed increased Minimum inhibitory concentrations (MICs) to commonly used antifungal drugs. The results call for further investigations on fungal resistance especially in the context of opportunistic infections in HIV/AIDS. Further studies need to be carried out in order to understand the mechanisms of resistance to commonly used antifungal drugs. There is also need to conduct genotypic analysis of clinical isolates as there may be existing genotypes that are unidentified. This is important in understanding pathogenicity and for clinicians to make appropriate and accurate treatment choices especially in the management of Candidiasis.

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8.0 APPENDICES

1. MEDIA COMPOSITION

a) Saboraud Dextrose Agar (SDA) (Emmons Modification)

Dextrose…………………………..20g

Peptone……………………………10g

Agar………………………………17g

Distilled Water…………………...1,000 ml

Final pH 6.9

Saboraud Dextrose Agar (SDA) with Antibiotics

To SDA after boiling and after autoclaving, add

Cyclohexamide ………….500mg

Chloramphenicol………….50mg

Dissolve Cyclohexamide in 10 ml of acetone and add it to the medium after autoclaving at 121 ^{o}C for 15 minutes. Mix well.

Dissolve the Chlorampenicol in 10 ml of 95 % ethanol add it to the medium after autoclaving at 121 ^{o}C for 15 minutes. Mix well.

Dispense in sterile petridishes.

b) Corn Meal Agar

Corn Meal…………………………………40 g

Agar………………………….. … ………..20 g

Tween 80 (Polysorbate 80)………………10 ml

Distilled Water…………………………..1,000 ml

Mix Corn Meal Agar (40g) well with 500 ml of water; heat to 65 ^{o}C for 1 hour.

Filter through gauze and then paper until clear, restore to original volume. Adjust to PH 6.6 - 6.8. Add agar dissolved in 500 ml of water. Add Tween 80. Autoclave at 121 ^{o}C for 15 minutes.

c) <u>RPMI (Roswel Park Memorial Institute)-1640 Media Composition (with glutamine and phenol red but without bicarbonate)</u>

Constituent	**g/l Water**	**Constituent**	**g/l Water**
L-arginine (free base)	0.200	Biotin	0.0002
L-aspargine(anhydrous)	0.050	D-pantothenic	0.00025
L-aspartic acid	0.020	Chlorine chloride	0.003
L-cystine . HCL	0.0652	Folic acid	0.001
L-glutamic acid	0.020	Myoinositol	0.001
L-glutamine	0.300	Niacinamide	0.0002
Glycine	0.010	PABA	0.001
L-histidine (free base)	0.015	Pyridoxine HCL	0.001
L-hydroxyproline	0.020	Riboflavin	0.0002

L-isoleucine	0.050	Thiamine HCL	0.001
L-leucine	0.050	Vitamin B_{12}	0.0000 05
L-lysine.HCl	0.040	Calcium nitrate x H_2O	0.100
L-methionine	0.015	Potassium chloride	0.400
L-phenyllalanine	0.015	Magnesium sulfate (anhydrous)	0.0488 4
L-proline	0.020	Sodium chloride	6.000
L-serine	0.030	Sodium phosphate, dibasic (anhydrous)	0.800
L-threonine	0.020	D-glucose	2.000
L-tryptophan	0.005	Glutathione, reduced	0.001
L-tyrosine .2Na	0.02883	Phenol red, Na	0.0053
L-valine	0.020		

2. SUMMARY OF SOURCE AND PHENOTYPIC CHARACTERISTICS OF ALL THE 150 *Candida* ISOLATES

	SEX	SPECIMEN	SDA	CHROM	GROWTH AT 45°C	GTT	CMA	API CONFIRM-ATION
1	F	Swabs(R+L cheeks)	Growth	*C.albicans*	+	+	Chlamydospores	
2	M	Pleural effusion	Growth	*C.albicans*	+	+	Chlamydospores	
3	M	Urine	Growth	*C.albicans*	+	+	Chlamydospores	
4	F	HVS	Growth	*C.albicans*	+	+	Chlamydospores	
5	F	HVS	Growth	*C.albicans*	+	+	Chlamydospores	
6	F	Urine	Growth	*C.albicans*	+	+	Chlamydospores	
7	F	Urine	Growth	*C.albicans*	+	+	Chlamydospores	
8	F	HVS	Growth	Pink			Absent	C. parapsilosis
9	F	Bile	Growth	Pink			Absent	C. parapsilosis
10	M	Blood	Growth	*C.albicans*	+	+	Chlamydospores	
11	F	HVS	Growth	*C.albicans*	+	+	Chlamydospores	
12	F	Vaginal swab	Growth	*C.albicans*	+	+	Chlamydospores	
13	F	Urine	Growth	Pink			Absent	*C.glabrata*
14	F	Urine	Growth	*C.albicans*	+	+	Chlamydospores	
15	M	Sputum	Growth	*C.albicans*	+	+	Chlamydospores	
16	F	HVS	Growth	*C.albicans*	+	+	Chlamydospores	
17	M	Tracheal aspirate	Growth	*C.albicans*	+	+	Chlamydospores	
18	M	Sputum	Growth	*C.albicans*	+	+	Chlamydospores	
19	F	Urine	Growth	*C.albicans*	+	+	Chlamydos	

							pores	
20	M	Urine	Growth	Pink			Absent	*C.glabrata*
21	F	Urine	Growth	*C.albicans*	+	+	Chlamydos pores	
22	F	Peritoneal aspirate	Growth	*C.albicans*	+	+	Chlamydos pores	
23	F	Urine	Growth	*C.albicans*	+	+	Chlamydos pores	
24	F	Vaginal swab	Growth	*C.albicans*	+	+	Chlamydos pores	
25	F	Urine	Growth	*C.albicans*	+	+	Chlamydos pores	
26	F	Endocervical swab	Growth	*C.albicans*	+	-		
27	F	Urine	Growth	*C.albicans*	+	+		
28	F	Urine	Growth	*C.albicans*	+	+		
29	F	Urine	Growth	Pink			Absent	*C. parapsilosis*
30	F	Urine	Growth	*C.albicans*	+	+	Chlamydos pores	
31	M	Urine	Growth	*C.albicans*	+	+	Chlamydos pores	
32	F	Sputum	Growth	*C.albicans*	+	+	Chlamydos pores	
33	M	Sputum	Growth	*C.albicans*	+	+	Chlamydos pores	
34	M	Sputum	Growth	Pink			Absent	*C.krusei*
35	M	Catheter tip	Growth	*C.albicans*	+	+	Chlamydos pores	
36	F	Sputum	Growth	*C.albicans*	+	+	Chlamydos pores	
37	F	HVS	Growth	*C.albicans*	+	+	Chlamydos pores	
38	F	Urine	Growth	*C.albicans*	+	+	Chlamydos pores	
39	F	Urine	Growth	*C.albicans*	+	+	Chlamydos pores	
40	F	Vulvovaginal swab	Growth	*C.albicans*	+	+	Chlamydos pores	
41	F	Urine	Growth	*C.albicans*	+	+	Chlamydos pores	
42	M	Sputum	Growth	*C.albicans*	+	+	Chlamydos pores	

43	F	HVS	Growth		+	+	Chlamydos pores	
44	F	HVS	Growth	*C.albicans*	+	+	Chlamydos pores	
45	F	HVS	Growth	*C.albicans*	+	-	Chlamydos pores	
46	M	Urine	Growth	*C.albicans*	+	+	Chlamydos pores	
47	F	Urine	Growth	Blue			Absent	*C. tropicalis*
48	F	HVS	Growth	Pink			Absent	*C. parapsilosis*
49	F	HVS	Growth	*C.albicans*	+	+	Chlamydos pores	
50	M	CVP line tip	Growth	Pink			Absent	*C. famata*
51	F	Urine	Growth	Pink			Absent	*C. famata*
52	F	Urine	Growth	*C.albicans*	+	+	Chlamydos pores	
53	M	Urine	Growth	*C.albicans*	+	+	Chlamydos pores	
54	F	Urine	Growth	*C.albicans*	+	+	Chlamydos pores	
55	F	Pancreatic cyst aspirate	Growth	*C.albicans*	+	+	Chlamydos pores	
56	F	HVS	Growth	*C.albicans*	+	+	Chlamydos pores	
57	F	HVS	Growth	Blue			Absent	*C. tropicalis*
58	M	Throat swab	Growth	*C.albicans*	+	+	Chlamydos pores (constricted)	
59	F	HVS	Growth	*C.albicans*	+	+	Chlamydos pores	
60	F	HVS	Growth	*C.albicans*	+	+	Chlamydos pores	
61	M	Sputum	Growth	*C.albicans*	+	+	Chlamydos pores	
62	F	Blood	Growth	*C.albicans*	+	+	Chlamydos pores	
63	F	Urine	Growth	*C.albicans*	+	+	Chlamydos pores	
64	M	Urine	Growth	*C.albicans*	+	+	Chlamydos pores	
65	F	Urine	Growth	*C.albicans*	+	+	Chlamydos	

							pores	
66	F	Urine	Growth	*C.albicans*	+	+	Chlamydos pores	
67	F	Urine	Growth	*C.albicans*	+	+	Chlamydos pores	
68	F	Urine	Growth	*C.albicans*	+	-	Chlamydos pores	
69	M	Sputum	Growth	*C.albicans*	+	+	Chlamydos pores	
70	M	Tracheal tube end	Growth	*C.albicans*	+	+	Chlamydos pores	
71	F	HVS	Growth	*C.albicans*	+	+	Chlamydos pores	
72	F	HVS	Growth	*C.albicans*	+	+	Chlamydos pores	
73	F	PV swab	Growth	*C.albicans*	+	+	Chlamydos pores	
74	M	Sputum	Growth	*C.albicans*	+	+	Chlamydos pores	
75	F	HVS	Growth	*C.albicans*	+	+	Chlamydos pores	
76	F	HVS	Growth	*C.albicans*	+	+	Chlamydos pores	
77	M	Tracheal aspirate	Growth	*C.albicans*	+	+	Chlamydos pores	
78	M	Sputum	Growth	*C.albicans*	+	+	Chlamydos pores	
79	F	Cervical Swab	Growth	*C.albicans*	+	+	Chlamydos pores	
80	F	Urine	Growth	*C.albicans*	+	+	Chlamydos pores	
81	M	Sputum	Growth	Pink			Absent	*C. guilliemondii*
82	F	Tracheal aspirate	Growth	*C.albicans*	+	+	Chlamydos pores	
83	F	HVS	Growth	*C.albicans*	+	+	Chlamydos pores	
84	F	Sputum	Growth	*C.albicans*	+	+	Chlamydos pores	
85	M	Sputum	Growth	*C.albicans*	+	+	Chlamydos pores	
86	F	HVS	Growth	Blue			Absent	*C. tropicalis*
87	M	Sputum	Growth	*C.albicans*	+	+	Chlamydos	

								pores	
88	F	Urine	Growth	*C.albicans*	+	+	Chlamydos pores	
89	M	Sputum	Growth	*C.albicans*	+	+	Chlamydos pores	
90	F	HVS	Growth	*C.albicans*	+	+	Chlamydos pores	
91	F	HVS	Growth	*C.albicans*	+	+	Chlamydos pores	
92	F	HVS	Growth	*C.albicans*	+	+	Chlamydos pores	
93	F	HVS	Growth	*C.albicans*	+	+	Chlamydos pores	
94	M	Urine	Growth	Pink	+		Absent	*C. guilliemondii*
95	F	HVS	Growth	*C.albicans*	+	+	Chlamydos pores	
96	F	Urine	Growth	*C.albicans*	+	+	Chlamydos pores	
97	F		Growth	*C.albicans*	+	+	Chlamydos pores	
98	F	Urine	Growth	*C.albicans*	+	+	Chlamydos pores	
99	M	Urine	Growth	Blue			Absent	*C. tropicalis*
100	F	Tracheal aspirate	Growth	*C.albicans*	+	-	Chlamydos pores	
101	M	HVS	Growth	*C.albicans*	+	+	Chlamydos pores	
102	F	Sputum	Growth	*C.albicans*	+	+	Chlamydos pores	
103	F	Sputum	Growth	*C.albicans*	+	+	Chlamydos pores	
104	F	HVS	Growth	Pink			Absent	*C. parapsilosis*
105	F	Urine	Growth	*C.albicans*	+	+	Chlamydos pores	
106	F	HVS	Growth	*C.albicans*	+	+	Chlamydos pores	
107	F	HVS	Growth	*C.albicans*	+	+	Chlamydos pores	
108	F	HVS	Growth	*C.albicans*	+	+	Chlamydos pores	

109	F	Endocervical swab	Growth	Pink			Absent	*C. krusei*
110	F	Urine	Growth	*C.albicans*	+	+	Chlamydos pores	
111	F	Urine	Growth	*C.albicans*	+	+	Chlamydos pores	
112	F	Vaginal swab	Growth	*C.albicans*	+	+	Chlamydos pores	
113	F	HVS	Growth	*C.albicans*	+	+	Chlamydos pores	
114	M	Tracheal aspirate	Growth				Absent	*C. parapsilosis*
115	F	Sputum	Growth	*C.albicans*	+	+	Chlamydos pores	
116	F	HVS	Growth	*C.albicans*	+	+	Chlamydos pores	
117	F	HVS	Growth	*C.albicans*	+	+	Chlamydos pores	
118	F	Urine	Growth	*C.albicans*	+	+	Chlamydos pores	
119	F	Urine	Growth	*C.albicans*	+	+	Chlamydos pores	
120	M	Urine	Growth	*C.albicans*	+	+	Chlamydos pores	
121	M	Urine	Growth	Blue	+	+	Absent	*C. krusei*
122	M	Urine	Growth	*C.albicans*	+	+		
123	M	Sputum	Growth	Pink	+	+	Absent	*C. krusei*
124	F	Sputum	Growth	*C.albicans*	+	+	Chlamydos pores	
125	F	Liquor swab	Growth	*C.albicans*	+	+	Chlamydos pores	
126	F	HVS	Growth	*C.albicans*	+	+	Chlamydos pore	
127	F	HVS	Growth	*C.albicans*	+	+	Chlamydos pores	
128	F	HVS	Growth	*C.albicans*	+	+	Chlamydos pores	
129	M	Blood	Growth	*C.albicans*	+	+	Chlamydos pores	
130	F	CSF	Growth	*C.albicans*	+	+	Chlamydos pores	
131	F	HVS	Growth	*C.albicans*	+	+	Chlamydos pores	

132	F	HVS	Growth	*C.albicans*	+	+	Chlamydos pores	
133	F	HVS	Growth	*C.albicans*	+	-	Chlamydos pores	
134	F	HVS	Growth	*C.albicans*	+	+	Chlamydos pores	
135	F	Tracheal Aspirate	Growth	*C.albicans*	+	+	Chlamydos pores	
136	F	Sputum	Growth	*C.albicans*	+	+	Chlamydos pores	
137	F	Sputum	Growth	*C.albicans*	+	+	Chlamydos pores	
138	M	Sputum	Growth	*C.albicans*	+	+	Chlamydos pores	
139	F	Pv Swab	Growth	*C.albicans*	+	+	Chlamydos pores	
140	F	Vaginal discharge	Growth	*C.albicans*	+	+	Chlamydos pores	
141	M	Blood	Growth	*C.albicans*	+	+	Chlamydos pores	
142	M	Sputum	Growth	*C.albicans*	+	+	Chlamydos pores	
143	F	Urine	Growth	*C.albicans*	+	+	Chlamydos pores	
144	M	Urine	Growth	*C.albicans*	+	+	Chlamydos pores	
145	F	Urine Catheter specimen	Growth	*C.albicans*	+	+	Chlamydos pores	
146	F	HVS	Growth	*C.albicans*	+	+	Chlamydos pores	
147	M	Urine	Growth	*C.albicans*	+	+	Chlamydos pores	
148	M	Blood	Growth	*C.albicans*	+	+	Chlamydos pores	
149	F	HVS	Growth	*C.albicans*	+	+	Chlamydos pores	
150	F	HVS	Growth	*C.albicans*	+	+	Chlamydos pores	

3. FORMULA FOR OBTAINING SAMPLE SIZE

$N = Z^2 p q / d$

Where;

N= Minimum sample size required

Z = Standard errors from the mean corresponding to 95% confidence level for 2-tailed test=1.96

P=Estimated prevalence (proportion) of azole resistant *Candida* species = 11%

q =Estimated prevalence (proportion) of non-azole resistant *Candida* species (1-p) = 89%

d=0.05 absolute precision

Therefore

$N = 1.96^2 \times (0.11 \times 0.89)$

0.0025

One hundred and fifty (150) *Candida* species were therefore characterized.