Characterization of *Candida* isolates from South African pregnant and non-pregnant women

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I, Gloria Sukali declare that

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As the candidate's supervisor, I have read the thesis and have given my approval for submission for examination

Supervisor: Prof Nathlee Abbai

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To my sweet Ally and Samantha for emotional support

I am dedicating my Masters qualification to my late grandparents and my mother

ABSTRACT

Candida infections are a serious health threat to women. Characterization of *Candida* isolates has become the gold standard method used in determining antimicrobial susceptibility profiles and resistance mechanisms in vaginal *Candida* infections. However, there is a lack of data on the antimicrobial susceptibility profiles of South African *Candida* isolates to amphotericin B. This study investigated antimicrobial resistance profiles and genotypes of *Candida* isolated from South African pregnant and non-pregnant women.

This study was a sub-study of a larger study which involved the diagnosis of vaginitis and vaginosis pathogens in women. For the parent study, n=150 women were recruited from the King Edward VIII hospital in Durban, KwaZulu-Natal, South Africa. The women enrolled in the parent study were; 18 years and older, were willing to provide written informed consent and were willing to provide self-collected vaginal swabs. A total of 72 *Candida* isolates were obtained by culture. Of the 72 isolates, 31 isolates were obtained from pregnant women and 41 isolates were from non-pregnant women. The isolates were typed using the ABC genotyping method. Susceptibility testing was performed using the broth microdilution assay to measure the minimal inhibitory concentrations (MICs) for clinical isolates to amphotericin B. The *Candida albicans* ATCC 10231 strain was used as a control strain, and untreated cultures of the respective isolates were used as growth controls. Descriptive characteristics of the study participants according to *Candida* status were presented as frequencies and percentages. Comparisons by *Candida* status in the descriptive characteristics were performed using Chi square tests with a 5% significance level. P-values ≤0.05 were considered significant. All analyses were conducted using STATA.

The prevalence of *Candida* in the study population was 48.0% (72/150). All the isolates (100%) were confirmed to be *C. albicans* as per the germ tube test and quantitative polymerase chain reaction (PCR) using primers and probes specific for *C. albicans*. All 72 isolates (100%) produced positive PCR results for *C. albicans*. The majority of the isolates (45/72; 62.5%) yielded a 450bp band which was assigned Genotype A. Of the 72 isolates, 19 isolates (26.4%) yielded a band size of 840bp and was assigned Genotype B. A total of 11.1% (8/72) of the isolates yielded band sizes of 450bp and 840bp which was Genotype C. Of the 72 isolates tested, 79.2% (57/72) of the isolates were resistant to amphotericin B (MIC >1ug/ml) and 20.8% (15/72) of the isolates were susceptible to amphotericin B (MIC ≤ 1 ug/ml). When

linking MIC patterns to distribution of genotypes, it was observed that the majority (80%) of the isolates which were assigned genotype A were resistant to amphotericin B. When linking clinical symptoms with the distribution of genotypes, it was observed that the majority (58.8%) of women who reported having current symptoms of abnormal vaginal discharge carried genotype A. Genotype A was most prevalent in women who had been treated for vaginal infections in the past and in women who were HIV positive with prevalence of 64.1% and 60.8%, respectively. genotype A was most prevalent in the non-pregnant women with a prevalence of 63.4%. Genotype A was prevalent (61.3%) amongst the pregnant women and the majority (66.7%) of the HIV negative women had *Candida* infections which belonged to genotype A.

The prevalence of *Candida* was shown to be high in both pregnant and non-pregnant women in this study. This study also found a high level of resistance to the antifungal amphotericin B. Currently in our local setting, resistance patterns to the commonly used antifungals to treat *Candida* infections are not being monitored. There is a need for antifungal resistance monitoring in order to reduce the risk of future persistent and untreatable infections.

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LIST OF ABBREVIATIONS

HIV	Human Immunodeficiency Virus
Bp	Base Pair
BREC	Biomedical Research Ethics Committee
DNA	Deoxyribonucleic acid
MIC	Minimum Inhibitory Concentration
NC	Negative Control
PC	Positive Control
PCR	Polymerase Chain Reaction
PCR-RFLP	Polymerase Chain Reaction-Restriction Fragment Length Polymorphism
RAPD	Random Amplification of Polymorphic DNA
MLST	Multilocus Sequence Typing
STI	Sexually Transmitted Infections
AMR	Antimicrobial Resistance
WHO	World Health Organization

CONTENTS

INTRODUCTION

Candida species are opportunistic organisms that cause several infections in women (3). Studies have found that vaginal *Candida* infections affect up to 75% of women in their lifetime (4). Symptoms associated with *Candida* infection include: redness around the genital area, inflammation of the genital tract, itchiness and thick white discharge (4). Causes for vaginal *Candida* infections in women include; pregnancy, diabetes, and use of broad-spectrum antibiotics (5, 6). Different studies have found that pregnant women experience high rates of *Candida* infections, which affect the health of unborn babies leading to early births that result in low birthweight babies (7). Low birth weight babies are at risk of neonatal invasive *Candida* infections which increases morbidity and mortality in these babies (8). Diabetic women experience high vaginal *Candida* infections due to high glucose concentrations and this creates favourable conditions for *Candida* growth in the epithelial cells of the vagina (9). Women who are younger are also at high risk of being infected with vaginal *Candida* infections when compared to older women, since younger women engage in higher sexual risk behaviour (10).

Despite treatment of vaginal *Candida* infections, women still experience a high reoccurrence of *Candida* infections. This reoccurrence could be the result of excessive use of antifungal drugs and overuse of broad-spectrum antibiotics which leads to antimicrobial resistance (11). In a study conducted by Mohammadi-Ghalehbin and colleagues on women who were referred to a health centre in Iran, a 17.5% resistance to amphotericin B was reported (12). Despite, *C. albicans* is responsible for most vaginal *Candida* infections (70-90%), there has been an increase in other *Candida* isolates displaying antimicrobial resistance patterns (4, 13).

Characterization of *Candida* isolates from women who are culture positive is very important for identifying the species of *Candida* responsible for the infection as well as determining antimicrobial susceptibility profiles and resistance mechanisms (14). Many molecular methods, such as Southern blotting hybridization, Multilocus Sequence Typing, and DNA microsatellite analysis, have been used to genotype *Candida*. The genotype analysis places strains in clades (a clade is a group composed of one ancestor and its descendants). Genotyping studies have shown that the distribution of clades is influenced by geographic locations as well as antifungal resistance patterns. Antifungal resistance has been associated with particular clades (15). The ABC genotyping method is mostly used for the characterization of *C. albicans* whereby different band sizes are used to determine the genotype of isolates. The isolates are classified into genotypes: A (450bp), B (840bp), C (450bp and 840bp), and D (1080bp) after

amplification of the *25S ribosomal DNA* by the Polymerase Chain Reaction (PCR) (14). A study by Jafarian and colleagues reported a prevalence of 57.9% for genotype A, 31.6% for genotype B and 10.5% for genotype C in a population of 933 patients in which 23 were confirmed to be *Candida* positive (16).

In this study, the antimicrobial resistance profiles of *Candida* clinical isolates to amphotericin B were investigated. The ABC genotyping method was performed on the isolates and the correlation between genotypes and clinical factors and genotypes and resistance profiles was also determined.

LITERATURE REVIEW

Biology of *Candida*

Candida species are known to have a commensal relationship with the human body as they are part of the natural flora of the human body (17). There are different types of *Candida species* such as: *C. albicans*, *C. glabrata*, *C. krusei*, and *C. tropicalis* (17). the diploid nature of *C.albicans* may increase its virulence. *Candida* species go through different growth types namely: pseudo-hyphae and true hyphae. Pseudo-hyphae are completely different from hyphae since they are formed by buds that stick together as shown in Figure 1 and the pseudo-hyphae eventually elongate at different rates. Once elongation has terminated, a bud is formed which also elongates. The continuous repetition of the budding and elongation processes leads to the excessive formation of filaments. In contrast hyphal growth does not have any buds at the tip of the hyphae as shown in Figure 1. *C. albicans* show different phenotypes which are white and opaque. Gene expression and virulence differ in white-opaque switching phases (18). Phenotypic switching is stimulated by environmental conditions such as the host, however, the extent of this stimuli is not well known. The switch from white cells to opaque cells is necessary for mating to occur (18, 19).

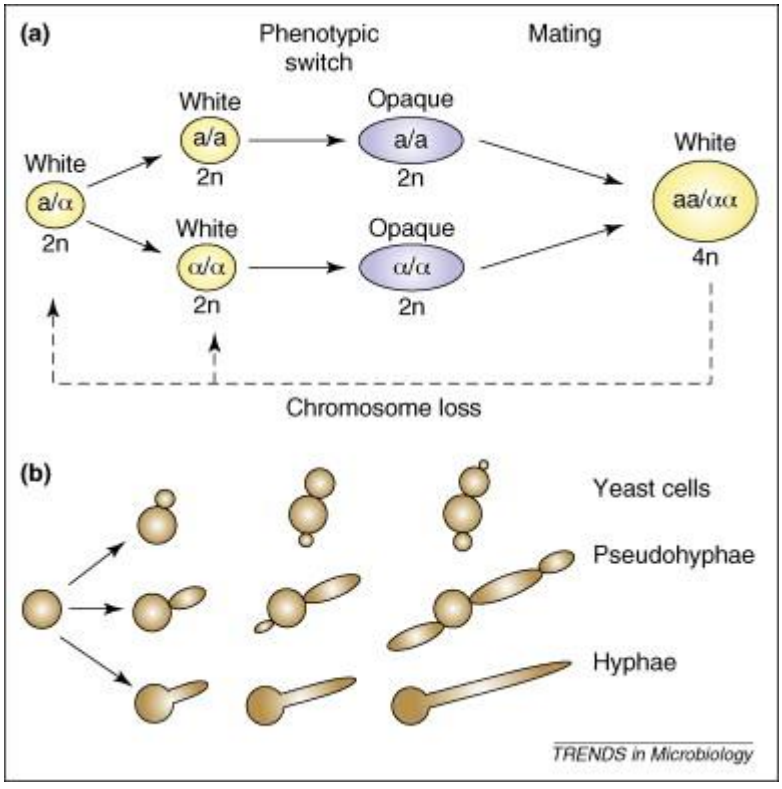


Figure 1: Life cycle of *Candida albicans* https://microbenotes.com/candida-albicans/.

In Figure 1, there is a clear description of the life cycle of *Candida*. *Candida* is an asexual microorganism with three growth types which are: yeast, pseudo-hyphae and true hyphae. They have a para-sexual life cycle which enables them to switch to different phenotypes. They have a diploid stage that consists of a homozygous phenotypic switch, which changes from white to opaque cells. Heterozygous phenotypic switch does not allow for change from white to opaque cells. (19). *Candida* can flourish on different niches of the human body such as the oral cavity, vagina, skin and guts (19).

Candida albicans as a natural member of the vaginal microbiota

The vaginal microbiota is dominated by different species namely: *Lactobacillus*, *Gardnerella*, *Candida* and many others. Amongst the species mentioned above, the *Lactobacillus* species is responsible for maintaining a healthy vaginal environment (20). *C. albicans* makes up about 30% of the vaginal microbiota (21, 22). The vaginal microbiota is usually balanced until an opportunistic pathogen becomes infectious, which leads to infections such as vaginal *Candida* infections (20). The mechanism in which vaginal *Candida* infection develops is not fully clear as it is normally a result of an imbalance in the vaginal microbiota. Risk factors for vaginal *Candida* infections include: pregnancy, broad-spectrum use of antifungals, diabetes and HIV (23). *Lactobacillus* species in the vagina produces hydrogen peroxide and lactic acid which are essential in controlling the growth of *Candida* species in the vagina. Reproduction hormones such as glycogen, when produced in high levels produces carbon which is responsible for overgrowth of *Candida* species, this overgrowth of *Candida* species has a significant factor on the transition from commensal harmless yeasts to hyphae form which causes vaginal *Candida* infections (23).

Pathogenesis of *Candida*

For *Candida* pathogenesis to occur, the host needs to have a favourable environment that allows adhesion of *Candida* species to host epithelial cells. The success of *Candida* causing an infection depends on the first contact it makes with epithelial cells. Fungal adhesion is aided by hydrolytic enzymes which are important for hyphae formation, hyphae only interact through contact sensing which facilitates a deeper adhesion to epithelial cells (24). There are several factors that contribute to the pathogenesis of *Candida* infections such as: host recognition molecules (which forms adhesins and are important for attachment), the secretion of enzymes (hydrolytic enzymes) and phenotypic change (25). The matrix consists of extracellular compounds secreted by the cells (26, 27). Each *Candida* species has a different

extracellular matrix composition and therefore causes different reactions to antifungal drugs. Figure 2 represents the biofilm matrix formed by *C. albicans*. (28). The matrix of *C. albicans* consists of three major polysaccharides (β-1,3 glucan, β-1,6 glucan and α-1,6 mannan with α-1,2 linked branches.

The extracellular matrix influences antifungal susceptibility by:

1. Acting as a physical barrier which limits antifungal penetration to the biofilm, which makes it difficult to treat fungal cells within the biofilm compared to planktonic cells.
2. Biofilm fungi experience slow growth which is then responsible for reduced metabolic activity and lower susceptibility.
3. Quorum sensing is one of the ways in which biofilm fungi communicates. Biofilms have a higher cell density which may potentially trigger susceptibility to antifungals (29).

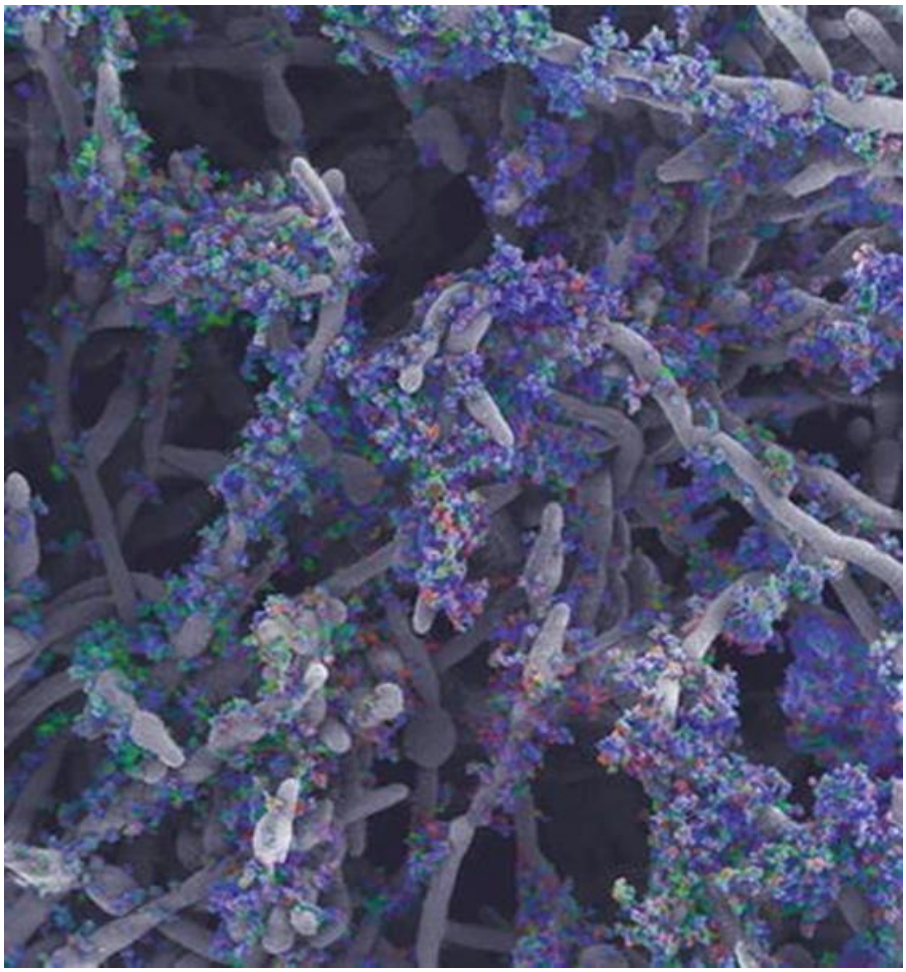


Figure 2: Represents *Candida albicans* biofilm obtained through a scanning electron micrograph. The three major polysaccharides (β-1,3 glucan, β-1,6 glucan and α-1,6 mannan with α-1,2 linked branches) of the extracellular matrix are coloured in blue, red and green (1).

Hydrolytic enzymes produced by *Candida* species are: proteases, phospholipase and esterase, these enzymes play a critical role in microbial pathogenicity. Phospholipids and protease enzymes are important for the interaction between *Candida* species and the host. The enzyme protease breaks down surface proteins and obstructs normal immunity which leads to tissue invasion (19, 30). Phospholipid components of the cell membrane are degraded by the enzyme phospholipase this leads to cell damage and lysis which prompts its dissemination. Esterase enzymes breaks down Ester bonds which further prompts its tissue invasion (30).

Candida infection may be attributed to different morphologies. These morphologies are:

1. Yeast form: are considered as the most prevalent form of *Candida*, they are non-invasive and exist as a single cell. Immunocompromised individuals are easily targeted by this form of *Candida*.
2. Pseudo hyphae: are elongated chains of connected yeasts cells, they are able to adhere effectively to host tissue and deeply invade host cells and tissue.
3. Hyphae: are the most dangerous form of *Candida*. They are long, have branches and are filamentous, this allows for deeper penetration to host tissues. This form of *Candida* are responsible for severe *Candida* infections (29).

Transmission of *Candida albicans*

C. albicans can be transmitted through high risk sexual behaviours (anogenital and orogenital) or through vertical transmission (mother-to-child transmission) (31). According to Reed and colleagues, oral sex is identified as a high-risk sexual behaviour in both men and women. In addition, women engaging in high-risk sexual behaviours are prone to infection with vaginal *C. albicans.* This is confirmed by previous studies which suggested that *C. albicans* isolated from the oral cavity of male sexual partners was the same as the *C. albicans* found in the vaginal cavity of the women (32, 33). New-born babies are exposed to oral *C. albicans* infection immediately after birth and this is a very serious health concern specially in immune compromised babies (34). According to Al-Rusan and colleagues children who had been

delivered vaginally from mothers who were infected with *C. albicans* had high oral colonization when compared to babies born through caesarean section (35). A study showed that 39% of low birth weight babies born to mothers who had *C. albicans* infection experienced vertical transmission. These findings suggest that low birth weight babies are at a higher risk of developing neonatal complications (36). It is therefore important for pregnant women to be screened for vaginal infections such as *C. albicans* to prevent neonatal consequences.

Diagnosis of *Candida*

Over the years the prevalence of *Candida* infections has drastically increased. It has therefore become important to identify which diagnostic methods provide fast and accurate diagnosis. Laboratory techniques such as microscopy and culture methods are used for the diagnosis of *Candida* (37). A study conducted on 71 women who complained of vaginal discharge and itching found that 23 of the patients (32%) presented with positive *Candida* cultures. This study also concluded that patients who had positive *Candida* cultures had positive Gram stain results. Both microscopy and laboratory diagnostic methods had a sensitivity and specificity of 75% and 100%, respectively (37). A study conducted on women who complained about having abnormal discharge and itching around the genital area found that only 60-80% of women had positive yeast cultures and also concluded that when culture is used as a diagnostic method there could be overdiagnosis (37). This accounts for misleading or false results which leads to patients not getting the right treatment (38).

Germ tube tests are conducted to assess the presence of *C. albicans* (39). A study conducted on 150 women attending Gombe specialist hospital in Nigeria from which vaginal swabs were collected found that 132 women (88%) had positive Gram stains. Of the 132 Gram stain positives, 120 women (80%) had positive germ tube results and were therefore concluded to be *C. albicans*. The remaining 12 samples were germ tube negative and therefore were non-*albicans Candida* (39).

Polymerase Chain Reaction (PCR) assays such as Restriction Fragment Length Polymorphism (RFLP) and Random Amplified Polymorphic DNA (RAPD) analysis have been used for the detection or diagnosis of *Candida* (40, 41). According to Tapia and colleagues, who conducted a study on pregnant women which assessed recurrent vaginal *Candida* infection, RAPD is a good diagnostic technique for assessing *Candida* variation. RFLP has been documented to be a fast diagnostic method and provides accurate results (40). A study conducted on women to

identify the prevalence of vaginal *Candida* infection attending a local gynaecology and midwifery setting in Iran concluded that RFLP is a reliable diagnostic method and provides accurate results (42).

The BD affirm ™ VPIII assay for *Candida*, is a DNA probe hybridization test used to identify species of *Candida* (43). A study conducted to assess the performance of molecular assays (the BD affirm ™ VPIII assay for *Candida* and The BD MAX ™ assay for *Candida)* on women who had vaginal inflammation found that 33% (66/200) of the women tested positive for *Candida*. While using the BD affirm ™ VPIII assay for *Candida* detection that study found that the BD affirm assay had a sensitivity and specificity of 69.4% and 87.3% respectively, compared to 98.4% and 99.2% for the BD MAX ™ assay. Furthermore the BD MAX ™ assay outperformed the BD affirm ™ VPIII assay due to its high sensitivity of 98.4% compared to 69.4% (43, 44).

The BD MAX ™ assay for *Candida,* uses real-time PCR to amplify specific DNA targets, which helps in detecting and differentiating DNA from *Candida* species which include: *C. albicans, C. krusei, C. glabrata, C. tropicalis and C. parapsilosis* (43, 44). The results obtained from this assay are qualitative (44). A study conducted on 195 women with symptoms of vaginal discharge found that 35.9% (70/195) tested positive for *Candida*. The BD MAX ™ assay had a sensitivity and a specificity of 86.4% and 86.0%, respectively in the studied population (45).

Prevalence and risk factors of vaginal *Candida* in women

Candidiasis is a yeast infection that is responsible for severe vaginal infections in women (46). In a study conducted by Idowu and colleagues, the prevalence of *Candida* in Nigerian women was found to be 40%. The relatively high prevalence of vaginal candidiasis among the women was likely due to a combination of factors such as poor personal hygiene, the use of contraceptives, and drug abuse (47). In another study conducted in Vietnam, the prevalence of vaginal yeast colonization in non-pregnant women was 51.3%. In that study, nine different yeast species were identified. Among the species identified, *C. albicans* (51.37%) was the most frequent (48). According to Potokoué and colleagues a study conducted on 152 women in Congo, Brazzaville found that child bearing age was associated with the high prevalence of vaginal *Candida* infection and this may be due to the high hormone production that women experience during this time (49). A study conducted in Libya on 115 women showed that 43.8% of pregnant women had *Candida* infection and 37.8% non-pregnant women tested positive for

Candida. The high prevalence of *Candida* in pregnant women is due to high levels of estrogen production which enables the growth of *Candida* with *C. albicans* being the most prevalent (92%) (50). A study conducted on 168 women attending a tertiary hospital in central India (128 non-pregnant and 40 pregnant) found that *Candida* positivity in pregnant women was higher (45.0%) when compared to non-pregnant women (28.9%), thereby making pregnant women a higher risk group when compared to non-pregnant women (51). A study by Altayyar and colleagues found that 43.8% of pregnant women had vaginal *Candida* infections compared to the 37.8% of non-pregnant women (50). A high percentage of 45.0% of pregnant women compared to the non-pregnant who had a prevalence of 28.9% (51). A study conducted in Nigeria showed that 46% of pregnant women with *Candida* infections had early births which resulted in low-birth-weight babies. That study also suggested that despite women in early stages of pregnancy receiving antifungal treatment, they still experience persistent *Candida* infections (7). In addition, low birth weight babies are at a higher risk of experiencing *Candida* colonization and neonatal invasive candidiasis which then increases morbidity and mortality in babies (8).

In a study conducted in India, on 152 pregnant women, the prevaence of *Candida species* was reported to be 72.3% with *C. albicans* being the most prevalent (52). In another study conducted by Kanagal and colleagues on 118 pregnant women, found that 42.37% of the women tested positive for *Candida*, with *C. albicans* being the most prevalent (69.23%) followed by *C. glabrata* (23.07%) and *C. tropicalis* (7.96%) (53). A study conducted in Yemen showed that the prevalence of *Candida* infections in pregnant women was 51.6% and suggested that women who are in their adolescent ages do not normally get infected by vaginal *Candida* compared to women who have passed adolescent ages (20-29 years). This study also highlighted that the most prevalent *Candida species* was *C. albicans*. The higher prevalence of vaginal *Candida* infection observed in that study may be attributed to risk factors such as pregnancy, age and sociodemographic status (54).

A study conducted in South Africa suggested that pregnancy increases the prevalence of vaginal *Candida* infection and pregnant women are considered a compromised group (55). Another study in South Africa suggested that women who are older than 35 years of age are at a lower risk of being infected with vaginal *Candida* when compared to women who are younger (56). *Candida* infection in pregnant women has many consequences such as: low birth weight infants, premature labour and complications that may lead to abortions, it is therefore

important that these women are screened for infections so that they can be managed appropriately.

Vaginal *Candida* infection in women is also influenced by factors such as broad-spectrum use of antifungals and being diabetic.

Over the years many antifungal drugs have been developed to treat *Candida* infections, some of these drugs include: polyene antifungals, the imidazoles, the echinocandins and the azoles (57, 58). Repeatedly using antifungal drugs causes the immune system to become resistant to the antifungal agents that the patients had previously been administered. Patients with a compromised immune system stand a higher chance of being infected with *Candida*. It is therefore important to reduce broad-spectrum use of antifungals to ensure that patients do not develop resistance to antifungal drugs. This can be achieved by proper diagnosis of the infection and thereafter treatment (11).

Diabetes plays a very critical role in vaginal *Candida* infection in women. A study conducted in Iran showed that women with diabetes experienced a repetition of *Candida* infections when compared to the normal population. The incidence of *Candida* infections in diabetic women increases due to the lack of proper regulation of blood sugar levels (59). Another study conducted in Pakistan on diabetic and non-diabetic pregnant women showed that there is a high frequency of vaginal *Candida* infection in diabetic women, they found that 45% of pregnant diabetic women had vaginal *Candida* infection when compared to the non-diabetic women who only accounted for 27% of the infections (60). In addition, according to Sadaqat and colleagues diabetic pregnant women showed a high frequency of vaginal *Candida* infection when compared to non-diabetic pregnant women (9). It is important for pregnant women to control their blood glucose levels in order to reduce the incidence of vaginal *Candida* infection and reduce neonatal complications.

Treatment of vaginal *Candida* infections in South Africa

South Africa follows the syndromic management approach recommended by the World Health Organization (WHO) for the treatment of vaginal Candidiasis. The treatment algorithm for vaginal discharge syndrome is presented in the Figure 3. The treatment algorithm is applicable for non-pregnant and pregnant women who complain of symptoms of abnormal vaginal discharge/dysuria/vulvar itching or burning are treated with a clotrimazole vaginal cream or pessary (44, 61).

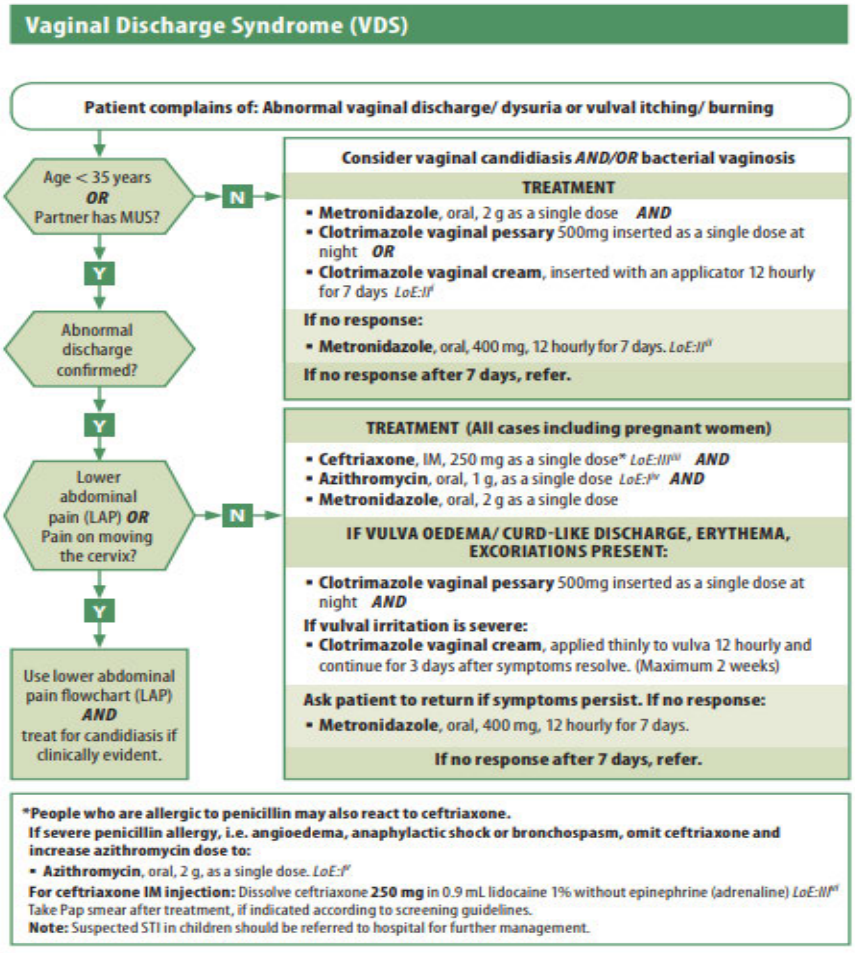


Figure 3: Treatment guidelines for women diagnosed with reproductive tract and sexually transmitted infections. South African Department of Health: Sexually Transmitted Infections management guidelines, 2015 (61).

Antifungal classes available for treatment of *Candida*

Candida isolates have shown resistance to antifungals that are currently available for treatment, and this has had major clinical consequences on patients, especially on immune compromised patients (62).

Table 1: Antifungals available for the treatment of *Candida* (2)

Antifungals	**Function (treatment and target site)**
5-flucytosine	Metabolism of pyrimidine is affected after it converts to 5-fluorouracil, affecting nucleic acid and protein synthesis
Echinocandins	Halts formation of enzyme 1,3-β-D glucan, which obstruct cell wall formation
Polyene	Attaches to ergosterol and affects cell membrane, which leads to the loss of sugars, electrolytes, and metabolites
Azoles	Changes formation of ergosterol by stopping the function of the enzyme P450 14α-sterol demethylase

Amphotericin B for the treatment of *Candida* infection

Amphotericin B is an antifungal drug that was developed in the 1950s for the treatment of serious fungal infections (63). Before the development of amphotericin B, there were no antifungal drugs that proved to be effective against fungal infections. For this reason, amphotericin B was considered to be the gold standard for fungal treatment in the early 1960s before the development of azoles and triazoles (64). Amphotericin B is part of the polyene group (64) and achieves its efficacy by binding to ergosterol, which are sterols found in the cell membrane of the fungi. Once binding has been achieved, cell membrane permeability is affected which leads to a variety of metabolic disruptions and the outflow of micro molecules resulting in cell death (65).

Amphotericin B promotes inflammation or inflammatory cytokine production using toll-like receptors (TLR)-2, in which T helper-1 (Th1) brings about immune responses. The immune response is important for the stimulation of macrophages and plays an important role in producing superoxide and nitric acid, these immune responses facilitate host responses to infections (66, 67).

Mechanism of action of amphotericin B

Amphotericin B has more than one mode of action on fungal cells, the first mechanism allows for the drug to bind to the sterols and the second mechanism allows a pro-oxidant process that causes oxidative damage in the fungal cell (68, 69).

When amphotericin B is administered in small concentrations it causes loss of small components such as potassium and sodium through pores and when administered in high concentrations it causes major metabolic obstruction which leads to cell death (70). After loss of potassium, hydrogen ions from the environment are transferred which leads to an inflow of protons which increases acid concentration in the cytoplasm, this then leads to precipitation of cytoplasmic components (69). Higher concentrations of amphotericin B have lethal effects on *C. albicans*, due to oxidation effects that cause cell damage (70).

Resistance to polyenes such as amphotericin B

The polyene, such as amphotericin B has been the first drug of choice for fungal treatment (63). Polyene resistance has gradually increased over the years. This can be due to the complex interaction observed between amphotericin B and the fungal plasma membranes (68). Another mechanism of resistance is observed during the growth of the fungal cell. In the log phase of fungal growth, break down and resynthesis takes place at very high rates, however, during the stationary phase the rate at which break down and synthesis of fungal cells occurs is reduced which leads to amphotericin B resistance (71, 72). The emergence of resistance to amphotericin B (polyene) is due to the high affinity of oxosterols and low affinity of 3-hydroxy. Recent studies suggests that ergosterols are gradually being replaced by biosynthesis precursors such as: lanosterol and lichesterol which therefore reduces ergosterol content to polyenes and leads to amphotericin B resistance (72). Genes acquiring mutations may lead to the development of resistance to polyenes. Biosynthesis of ergosterol is achieved with the use of two enzymes namely: C8 sterol isomerase and C5 sterol desaturase. Fecosterol is converted into episterol

with the aid of C8 sterol which is regulated by the *ERG2* gene. Episterol is converted into ergosterol when C5 sterol desaturase is catalyzed, the *ERG3* gene regulates this process. Any changes observed in the *ERG2* and *ERG3* genes results in amphotericin B resistance (2, 72, 73).

Genotyping of *Candida albicans*

There are different methods used for *Candida* genotyping, these methods include: Random Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP) analyses and Multilocus Sequence Typing (MLST). A study conducted by Tapia and colleagues which assessed the types of *Candida* causing recurrent vaginal *Candida* infections in pregnant women suggested that the RAPD technique for genotyping is a good method to be used when assessing *Candida* variation (74). Another study suggested that the MLST genotyping method is a good method to be used to determine whether recurrent vaginal *Candida* infection is as a result of reinfection or a relapse (75).

ABC genotyping is an important method used for *Candida* species variation and it is also important for assessing virulence and antimicrobial resistance of *Candida* isolates (16). ABC genotyping focuses on the *25S rDNA* gene depending on the presence or absence of an intron. The size of the amplified PCR products determines the group in which *Candida* isolates will be classified into: genotype A (450 bp), genotype B (840 bp) or genotype C (450 and 840 bp). A study by Jafarian and colleagues reported a prevalence of 57.9% for genotype A, 31.6% for genotype B and 10.5% for genotype C in a population of 933 patients in which 23 were confirmed to be *Candida* positive (16).

Candida genotyping is of great importance, which helps in identifying which strain the patient is infected with. Different variants show different phenotypes and this may be responsible for contributing to drug resistance (41). A study conducted on both pregnant and non-pregnant women found that they all had different genotypes of *Candida,* which has led to a diverse variation of *Candida* making it difficult for treatment to be effective (41). According to Fornari and colleagues, women infected with vaginal Candidiasis had complicated infections that were caused by different strains of *Candida*. Furthermore, their study highlighted the importance of genotyping which is an important genomic process to be performed on different *Candida* isolates. This will ensure that the patients are being treated for not only the right disease but also identifying which strain the patient is infected with so that they may be given other antifungal drugs that will be more effective in the treatment of the identified variant (14).

Rationale of the study

Candida infections have previously not been treated as STIs. This has led to great concerns as over the years the number of women who experience *Candida* infections has increased (13). The use of the syndromic management in South Africa for the treatment of vaginal infections has led to the overuse of antifungals which is a contributory factor to the emergence of antifungal resistance. Currently, there is a lack of data on the antifungal susceptibility profiles of South African isolates of *Candida* to amphotericin B. In addition, there is also limited data on the genotyping profiles of *Candida* isolates circulating in our local population of women. This study will attempt to fill in these gaps in the literature. The current study involved the genotyping of *C. albicans* isolates obtained from pregnant and non-pregnant women. The ABC genotyping method was used to assign the genotypes. This study also determined the antifungal resistance profiles of the isolates to amphotericin B using the microbroth dilution method.

Hypothesis

We hypothesize that there will be genetic variation in the isolates of *Candida* investigated in this study. We also hypothesize that patterns of resistance to amphotericin B will be identified.

Aims

To investigate the antifungal resistance profiles and genotypes of *Candida* isolated from South African pregnant and non-pregnant women.

Objectives

- To determine the antifungal susceptibility patterns of isolates of *Candida* to amphotericin B using the microbroth dilution method
- To genotype the *Candida* isolates using the ABC genotyping method
- To link the genotypes and clinical factors such as: symptoms of infection, past treatment for infections and history of STIs with patterns of amphotericin B resistance

METHODOLOGY

Ethics Consideration

Approval for this study was obtained from the Biomedical Research Ethics Committee (BREC) of the University of KwaZulu-Natal (UKZN) with reference number (BREC/00005018/2022).

Study population

This study was a sub-study of a larger study which was approved by BREC (BREC/00003674/21) and involved the diagnosis of vaginitis and vaginosis pathogens in women. For the parent study, n=150 women were recruited from the King Edward VIII hospital in Durban, KwaZulu-Natal, South Africa. The women enrolled in the parent study were; 18 years and older, were willing to provide written informed consent and were willing to provide self-collected vaginal swabs. Instructions on the sample collection were provided by the study team. Data on the woman's sexual behaviour, clinical history and socio-demographic information was obtained from all enrolled women using a structured questionnaire administered by the study team. The study population was recruited from January to August 2022.

Following sample collection, the swab which were used for the detection of *Candida* was placed in Cary Blair Transport media and transported to the Clinical Medicine Laboratory, University of KwaZulu-Natal for the culture analysis. Upon arrival at the laboratory, the swab was streaked onto Sabouraud Dextrose Agar (SDA) plates containing chloramphenicol and incubated for 48 hours at 35°C (76). Following incubation, 72 isolates were obtained. Of the 72 isolates, 31 isolates were obtained from pregnant women and 41 isolates were from non-pregnant women.

Confirmatory assays for the isolates

To confirm the identity of the isolates, TaqMan assays using primers and probes specific for *C. albicans, C. lusitaniae, C. dubliniensis, C. parapsilosis, C. glabrata, C. tropicalis* and *C. krusei* were performed. The germ tube test was also used to differentiate *albicans* from *non-albicans Candida* species (77)

DNA isolation

Prior to performing the TaqMan assays, DNA was extracted from the isolates using the PureLink Microbiome Kit (ThermoFisher Scientific, United States) according to the manufacturer's instructions. Briefly, single colonies of the isolates were resuspended in Brain Heart Infusion broth and centrifuged for 30 minutes at 14 000 xg. The supernatant was discarded and 800µl of S1 lysis buffer was added to the pellet and pipetted up and down to mix the sample. The sample was then transferred to the bead tube and 100µl of S2 lysis enhancer was added to the bead tube, capped and vortexed briefly. This was incubated at 95^{o}C for 10 minutes, followed by vortexing at a maximum speed for 7 minutes and further centrifuged at 14 000 xg for 1 minute. Thereafter, 500µl of the supernatant was transferred to a clean microcentrifuge tube, avoiding the bead pellet and any cell debris. To bind DNA to the column, 900µl of binding buffer was added and vortexed briefly. Following this, 700µl of the sample mixture was loaded onto a spin column-tube and centrifuged at 14 000 xg for 1 minute. The flow through was discarded and the spin column was centrifuged at 14 000 xg for 30 seconds. The spin column was placed in a clean tube and 50µl of S6 elution buffer was added. The tube was incubated at room temperature for 1 minute. After 1 minute, the spin column was centrifuged at 14 000 xg for 1 minute, the column was discarded. The purified DNA was stored at -20°C. The concentration and purity of the DNA was assessed using a Nanodrop Spectrophotometer (ThermoFisher Scientific, United States).

The germ tube test

The germ tube test was performed to differentiate *C. albicans* from other *Candida* species such as *C. krusei, C. glabrata, C. tropicalis and C. parapsilosis*. For this assay, 0.5ml of foetal calf serum was added to a small tube. A single colony of the culture growing on the SDA plate was added to the serum and mixed. The tubes were incubated at 37°C for 2 to 4 hours. After incubation, a drop of the sample was placed on a glass slide and viewed under the microscope. A positive germ tube result was observed when there were short hyphal (filamentous) extensions arising laterally from the yeast cells, with no constriction at the point of origin as noted for *C. albicans*. A negative result was observed when there were no hyphal (filamentous) extensions arising from the yeast cells, or short hyphal extensions constricted at the point of origin as observed for *C. tropicalis*, *C. glabrata* and other yeast (77).

Genotyping of the *Candida* isolates

The isolates were typed using the ABC genotyping method. Using the already extracted DNA from the isolates, the *25S rDNA* gene was amplified using the following primers: 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′. (76)

The PCR master mix contained 200nM of the forward and reverse primers,12.5µl of DreamTaq (2x) Master Mix (ThermoFisher Scientific, United States), 9.5µl of water and 2µl of template DNA. Thereafter, the PCR tubes were placed into a BioRad thermal cycler. The PCR cycling conditions were as follows; 95℃ for 2 minutes; followed by 35 cycles of 95℃ for 30 seconds, 60℃ for 1 minute, and 72℃ for 1 minute and a further extension at72℃ for 7 minutes.

The PCR products were electrophoresed on a 1% agarose gel and viewed using a UV transilluminator. The *Candida* isolates were classified as: Genotype A (450bp), Genotype B (840 bp), Genotype C (450bp and 840bp), and Genotype D (1080bp) based on the band sizes yielded.

Antifungal Susceptibility testing

Susceptibility testing was performed using the broth microdilution assay to measure the minimal inhibitory concentrations (MICs) for *C. albicans* clinical isolates to amphotericin B. Briefly, two-fold serial dilutions of amphotericin B were performed in Mueller Hinton broth. The resulting concentrations ranged from 0.5 to 32µg/ml. An inoculum of *C. albicans* was prepared for each isolate which was equivalent to a 0.5 McFarland standard. Thereafter, a 1:10 dilution of the prepared inoculum was performed to yield a final concentration of 1.5x10 ^{6}cfu/ml. The *C. albicans* ATCC 10231 strain was used as a control strain and untreated cultures of the respective isolates were used as growth controls. The microtiter plates were incubated at 35°C for 48 hours. Plates were read after 24 and 48 hours. The MIC was defined as the lowest concentration of amphotericin B in which no visible growth was observed after 48 hours of incubation. Breakpoints suggested by Lass-Flörl and colleagues were used (78). MIC $\leq$ 1 µg/ml was considered susceptible and MIC $\geq$ 1 µg/ml was considered resistant. All experiments were performed in duplicate for each *C. albicans* isolate.

Data analysis

Descriptive characteristics of the study participants were presented by *Candida* status, as frequencies and percentages of the categorical variables. Comparisons by *Candida* status in the

descriptive characteristics were performed using Chi square tests with a 5% significance level. P-values ≤0.05 were considered significant. All analyses were conducted using STATA.

RESULTS

Prevalence and factors associated with *Candida* infections

The prevalence of *Candida* in the study population was 48.0% (72/150). Table 2 describes the characteristics of the study population stratified by *Candida* infection status.

Of the women who tested *Candida* positive, a higher percentage 29.1% were between the ages of 31-39 years old when compared to 22.2% who were between the ages of 18-24 years old. Similarly, within the group of *Candida* negative women, 46.1% of the women were older, 40 years and older when compared to 12.8% who were between the ages of 18-24 years old. There was a significant association between age and *Candida* status, p=0.019. The majority of the women 76.3% who tested *Candida* positive did not report symptoms of abnormal vaginal discharge at study enrolment when compared to 23.6% who reported having discharge. Similarly, the majority of the women who tested *Candida* negative did not report symptoms of abnormal vaginal discharge 80.7% when compared to 19.2% who reported having discharge. There was no significant association between symptoms of discharge and *Candida* status, p=0.513. Of the women who tested *Candida* positive, 81.9% had attended college when compared to 5.5% who had completed high school. Of the women who tested *Candida* negative, 70.5% had attended college when compared to 3.84% who had completed high school, there was no significant association between level of education and *Candida* status, p=0.471. The majority of women 86.1% who tested positive for *Candida* were not married when compared to 13.8% who were married. Similarly, the majority of women 80.7% who tested negative for *Candida* were not married when compared to 19.2% who were married. There was no significant association between marital status and *Candida* status, p=0.380. Of the women who tested *Candida* positive, 15.2% did not have a regular sex partner when compared to 84.7% who had a regular sex partner. Of the women who tested negative for *Candida,* 26.9% did not have a regular sex partner when compared to 73.0% who had a regular sex partner. There was no significant association between having a regular sex partner and *Candida* status, p=0.082. Of the women who tested positive for *Candida,* 68.05% did not live with their regular sex partner when compared 31.94% who lived with their regular sex partner. Similarly, of the women who tested negative for *Candida*, 64.10% did not live with a regular sex partner when compared to 35.89% who lived with a regular sex partner. There was no significant association between living with a regular sexual partner and *Candida* status, p=0.610. The majority of women (84.72%) who tested positive for *Candida* had their sexual debut between the ages of 15-20 when compared to 4.16% who had their sexual debut at

younger than 15 years of age. Of the women who tested negative for *Candida,* 73.07% had their sexual debut between the ages of 15-20 years when compared to 2.56% who had their sexual debut at younger than 15 years of age. There was no significant association between the age of sexual debut and *Candida* status, p=0.120. Of the women who tested positive for *Candida,* 65.27% had 2 to 4 life time sexual partners when compared to 11.11% who had 1 life time sexual partner. Similarly, of the women who tested negative for *Candida,* 57.69% had 2 to 4 life time sexual partners when compared to 12.82% who had 1 life time sexual partner. There was no significant association between the number of life time sexual partners and *Candida* status, p=0.629. Of the women who tested positive for *Candida,* 51.38% did not know if their partners had other sexual partners compared to 15.27% who knew that their partners had other sexual partners. Similarly, of the women who tested negative for *Candida,* 47.43% did not know if their partner had other sexual partners compared to 11.53% who knew that their partners had other sexual partners. There was no significant association between partners having other sexual partners and *Candida* status, p=0.555. The majority of women 69.44% who tested positive for *Candida* did not use condoms during their last sexual act when compared to 30.55% who did use condoms during their last sexual act. The majority of women 62.82% who tested negative for *Candida* did not use condoms during their last sexual act compared to 37.17% who did use condoms during their last sexual act. There was no significant association between condom use during last sexual act and *Candida* status, p=0.392. Of the women who tested positive for *Candida,* 88.88% did not smoke when compared to 11.11% who smoked. Similarly, of the women who tested negative for *Candida,* 88.46% did not smoke when compared to 11.53% who smoked. There was no significant association between smoking and *Candida* status, p=0.934. Of the women who tested positive for *Candida,* 70.83% did not consume alcohol when compared to 29.16% who consumed alcohol. Similarly, of the women who tested negative for *Candida,* 74.35% did not consume alcohol when compared to 25.64% who consumed alcohol. There was no significant association between alcohol consumption and *Candida* status, p=0.628. The majority of women 81.94% who tested positive for *Candida* did not use contraceptives when compared to 2.77% who used pills. Similarly, the majority of women 70.51% who tested negative for *Candida* did not use contraceptives when compared to 3.84% who used IUCDs/Implants. There was no significant association between contraception method and *Candida* status, p=0.412. Of the women who tested positive for *Candida,* 44.44% did not have past abnormal vaginal discharge when compared to 55.55% who reported having past abnormal vaginal discharge. Similarly, of the women who tested negative for *Candida,* 37.17% did not have past abnormal vaginal discharge when compared

to 62.82% who reported having past abnormal vaginal discharge. There was no significant association between past abnormal vaginal discharge and *Candida* status, p=0.365. Of the women who tested positive for *Candida,* 45.83% were not treated for STIs in the past when compared to 54.16% who were treated for past STIs. Similarly, of the women who tested negative for *Candida*, 47.43% were not treated for past STIs when compared to 52.56% who were treated for past STIs. There was no significant association between treatment for past STIs and *Candida* status, p=0.844. The majority of women 56.94% who tested positive for *Candida* were not pregnant compared to 43.05% who were pregnant. Similarly, the majority of women 83.33% who tested negative for *Candida* were not pregnant when compared to 16.66% who were pregnant. There was a significant association between pregnancy status and *Candida* status, p=0.000. Of the women who tested positive for *Candida,* 29.16% were HIV negative when compared to 70.83% who were HIV positive. Of the women who tested negative for *Candida,* 35.89% were HIV negative when compared to 64.10% who were HIV positive. There was no significant association between HIV status and *Candida* status, p=0.380 (Table 2).

Table 2: Characteristics of the study population stratified by *Candida* infection status

Variables	***Candida* Negative**	***Candida* Positive**	**Total**	
	N (%)	N (%)	N (%)	p-value
Overall	78 (52)	72 (48)	150 (100)	
Age group				0.019
18-24	10 (12.8)	16 (22.2)	26 (17.3)	
25-30	17 (21.7)	19 (26.3)	36 (24.0)	
31-39	15 (19.2)	21 (29.1)	36 (24.0)	
40+	36 (46.1)	16 (22.2)	52 (34.6)	
Current abnormal vaginal discharge				0.513
No	63 (80.7)	55 (76.3)	118 (78.6)	
Yes	15 (19.2)	17 (23.6)	32 (21.3)	
Highest level of education				0.471
College	55 (70.5)	59 (81.9)	114 (76)	
High school	3 (3.84)	4 (5.5)	7 (4.6)	

Primary school	2 (2.5)	0 (0)	2 (1.3)	
Married				0.380
No	63 (80.7)	62 (86.1)	125 (83.3)	
Yes	15 (19.2)	10 (13.8)	25 (16.6)	
Has a regular sex partner				0.082
No	21 (26.9)	11 (15.2)	32 (21.3)	
Yes	57 (73.0)	61 (84.7)	118 (78.6)	
Living with regular sexual partner				0.610
No	50 (64.10)	49 (68.05)	99 (66)	
Yes	28 (35.89)	23 (31.94)	51 (34)	
Age of sexual debut				0.120
15-20 years old	57 (73.07)	61 (84.72)	118 (78.66)	
21-25 years old	19 (24.35)	8 (11.11)	27 (18)	
<15 years old	2 (2.56)	3 (4.16)	5 (3.33)	
Life time number of sexual partners				0.629
1	10 (12.82)	8 (11.11)	18 (12)	
2-4	45 (57.69)	47 (65.27)	92 (61.33)	
>4	23 (29.48)	17 (23.61)	40 (26.66)	
Partner has other partners				0.555
No	23 (29.48)	20 (27.77)	43 (28.66)	
Yes	9 (11.53)	11 (15.27)	20 (13.33)	
Don't know	37 (47.43)	37 (51.38)	74 (49.33)	
Condom use during last sexual act				0.392
No	49 (62.82)	50 (69.44)	99 (66)	
Yes	29 (37.17)	22 (30.55)	51 (34)	
Smokes				0.934
No	69 (88.46)	64 (88.88)	133 (88.66)	
Yes	9 (11.53)	8 (11.11)	17 (11.33)	
Drinks alcohol				0.628
No	58 (74.35)	51 (70.83)	109 (72.66)	
Yes	20 (25.64)	21 (29.16)	41 (27.33)	
Contraception method				0.412

None	55 (70.51)	59 (81.94)	114 (76)	
Implant	3 (3.84)	4 (5.55)	7 (4.66)	
Sterilization	8 (10.25)	3 (4.16)	11 (7.33)	
Injectables	6 (7.69)	4 (5.55)	10 (6.66)	
Pills	4 (5.12)	2 (2.77)	6 (4)	
Other (traditional methods)	2 (2.56)	0 (0)	2 (1.33)	
Past abnormal vaginal discharge				0.365
No	29 (37.17)	32 (44.44)	61 (40.66)	
Yes	49 (62.82)	40 (55.55)	89 (59.33)	
Treated for past STIs				0.844
No	37 (47.43)	33 (45.83)	70 (46.66)	
Yes	41 (52.56)	39 (54.16)	80 (53.33)	
Pregnancy status				0.000
Not pregnant	65 (83.33)	41 (56.94)	106 (70.66)	
Pregnant	13 (16.66)	31 (43.05)	44 (29.33)	
HIV status				0.380
Negative	28 (35.89)	21 (29.16)	49 (32.66)	
Positive	50 (64.10)	51 (70.83)	101 (67.33)	

Culturing and confirmatory assays for the obtained isolates

All the isolates (100%) were confirmed to be *C. albicans* as per the germ tube test (Figure 4). The isolates were further confirmed to be *C. albicans* by the quantitative PCR assay using primers and probes specific for *C. albicans*. Table 3 shows the amplification results obtained for the TaqMan assay using the probes and primers specific for *C. albicans*. All samples produced positive amplification. The positive and negative controls produced the desired results.

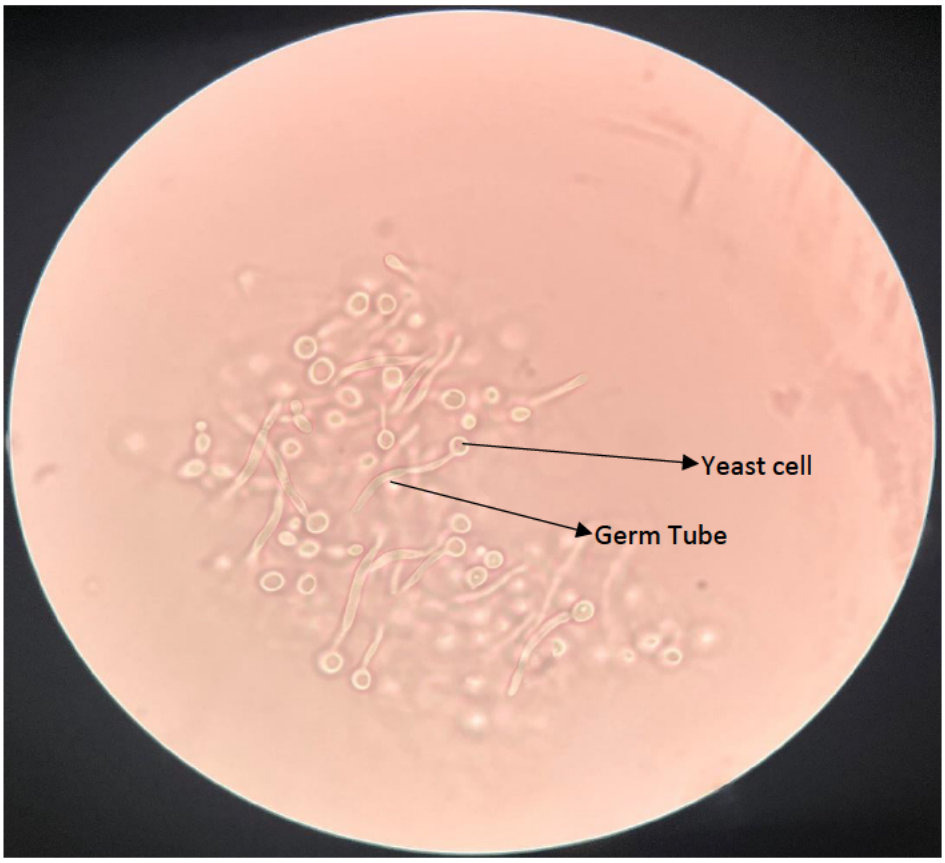


Figure 4: Microscope slide depicting the results of the germ tube test which was conducted. The slide was viewed with oil immersion under 100X magnification.

Table 3: Results of the TaqMan assay using primers and probes specific for *Candida*

Isolate Number	Results of the TaqMan assay (Cycling threshold values)	
CA 1	Positive (CT = 15.9)	
CA 10	Positive (CT = 30.8)	
CA 11	Positive (CT= 17.5)	
CA 12	Positive (CT = 16.7)	
CA 14	Positive (CT = 19.8)	
CA 17	Positive (CT = 20.2)	
CA 18	Positive (CT = 26.3)	
CA 20	Positive (CT = 32.9)	
CA 21	Positive (CT = 14.0)	
CA 23	Positive (CT = 12.3)	
CA 25	Positive (CT = 15.3)	
CA 27	Positive (CT = 14.5)	
CA 28	Positive (CT = 29.1)	

CA 29	Positive (CT = 30.9)	
CA 30	Positive (CT = 15.3)	
CA 32	Positive (CT = 28.6)	
CA 34	Positive (CT = 17.8)	
CA 35	Positive (CT = 14.9)	
CA 37	Positive (CT = 19.6)	
CA 40	Positive (CT = 33.1)	
CA 41	Positive (CT = 17)	
CA 42	Positive (CT = 31.3)	
CA 43	Positive (CT= 17.7)	
CA 44	Positive (CT = 24.9)	
CA 47	Positive (CT = 16.2)	
CA 53	Positive (CT = 28.6)	
CA 54	Positive (CT = 17.4)	
CA 56	Positive (CT = 17.8)	
CA 58	Positive (CT = 17.9)	
CA 59	Positive (CT = 18.9)	
CA 60	Positive (CT = 15.8)	
CA 62	Positive (CT = 24.7)	
CA 63	Positive (CT = 16.8)	
CA 65	Positive (CT = 26.6)	
CA 67	Positive (CT = 19.3)	
CA 68	Positive (CT = 29.6)	
CA 69	Positive (CT = 25.1)	
CA 71	Positive (CT = 12.4)	
CA 72	Positive (CT = 21.5)	
CA 75	Positive (CT = 18.5)	
CA 77	Positive (CT = 17)	
CA 79	Positive (CT = 27.9)	
CA 80	Positive (CT = 19.2)	
CA 81	Positive (CT = 22)	
CA 82	Positive (CT = 29.0)	
CA 83	Positive (CT = 16.5)	
CA 84	Positive (CT = 20.2)	
CA 85	Positive (CT = 19.1)	
CA 86	Positive (CT = 15.4)	
CA 87	Positive (CT = 18.8)	
CA 88	Positive (CT = 16.9)	
CA 89	Positive (CT = 16.4)	
CA 91	Positive (CT = 14.6)	
CA 94	Positive (CT = 14.9)	
CA 95	Positive (CT = 18.8)	
CA 96	Positive (CT = 15.1)	
CA 97	Positive (CT = 16.3)	

CA 98	Positive (CT = 17.9)	
CA 99	Positive (CT = 28.3)	
CA 102	Positive (CT = 16.0)	
CA 103	Positive (CT = 15.2)	
CA 107	Positive (CT = 27.2)	
CA 110	Positive (CT = 31.1)	
CA 119	Positive (CT = 18.8)	
CA 128	Positive (CT = 15.9)	
CA 132	Positive (CT = 15.5)	
CA 135	Positive (CT = 32.2)	
CA 141	Positive (CT = 14.9)	
CA 142	Positive (CT = 31.1)	
CA 145	Positive (CT = 31.7)	
CA 146	Positive (CT = 13.6)	
CA 147	Positive (CT = 13.7)	

Antifungal susceptibility assays

Of 72 isolates which were tested, 79.2% (57/72) of the isolates were resistant to amphotericin B since they displayed MICs of >1µg/ml and 20.8% (15/72) of the isolates were susceptible to amphotericin B (MIC ≤ 1 µg/ml). The exact MIC values for each isolate are shown in Table 4.

Table 4: *C. albicans* isolates MIC values to amphotericin B

Isolate name	**MIC (µg/ml)**	**Susceptibility profile**
CA (ATCC)	1	Susceptible
CA 1	2	Resistant
CA 10	32	Resistant
CA 11	> 32	Resistant
CA 12	32	Resistant
CA 14	4	Resistant
CA 17	1	Susceptible
CA 18	4	Resistant
CA 20	2	Resistant
CA 21	1	Susceptible
CA 23	4	Resistant

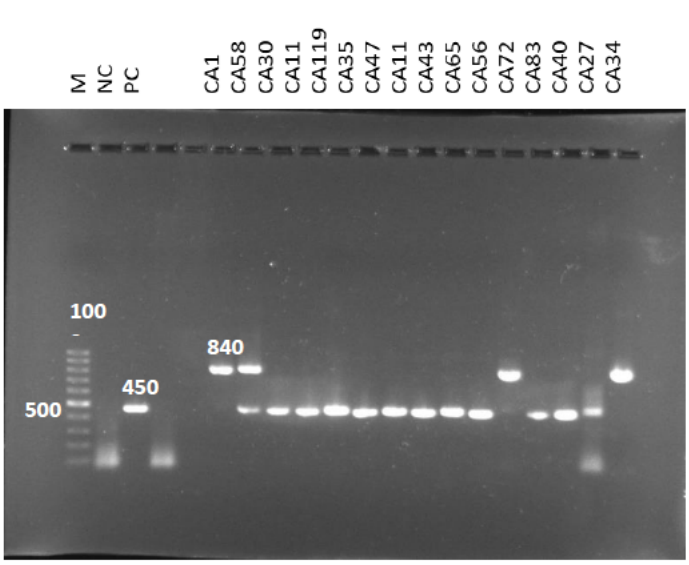
CA 25	4	Resistant
CA 27	> 32	Resistant
CA 28	4	Resistant
CA 29	> 32	Resistant
CA 30	1	Susceptible
CA 32	> 32	Resistant
CA 34	1	Susceptible
CA 35	> 32	Resistant
CA 37	4	Resistant
CA 40	> 32	Resistant
CA 41	4	Resistant
CA 42	> 32	Resistant
CA 43	> 32	Resistant
CA 44	8	Resistant
CA 47	> 32	Resistant
CA 53	1	Susceptible
CA 54	4	Resistant
CA 56	16	Resistant
CA 58	4	Resistant
CA 59	1	Susceptible
CA 60	0,5	Susceptible
CA 62	32	Resistant
CA 63	32	Resistant
CA 65	16	Resistant
CA 67	4	Resistant
CA 68	4	Resistant
CA 69	1	Susceptible
CA 71	8	Resistant
CA 72	16	Resistant
CA 75	4	Resistant
CA 77	4	Resistant
CA 79	> 32	Resistant

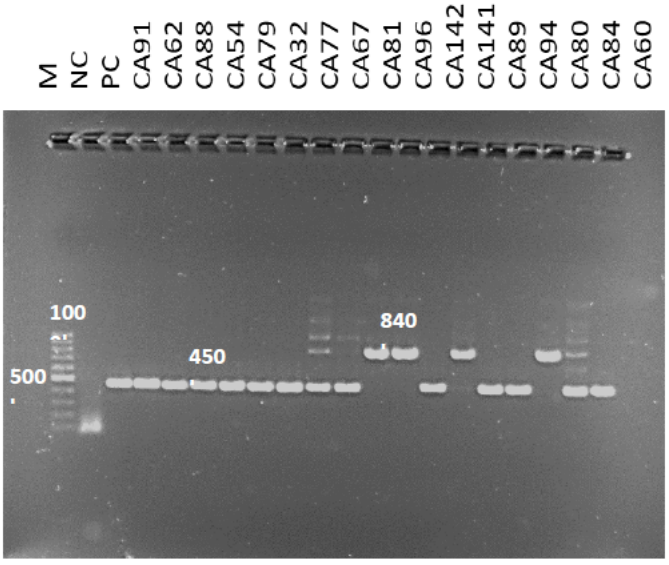
CA 80	2	Resistant
CA 81	> 32	Resistant
CA 82	1	Susceptible
CA 83	16	Resistant
CA 84	2	Resistant
CA 85	16	Resistant
CA 86	> 32	Resistant
CA 87	4	Resistant
CA 88	8	Resistant
CA 89	2	Resistant
CA 91	> 32	Resistant
CA 94	> 32	Resistant
CA 95	2	Resistant
CA 96	1	Susceptible
CA 97	4	Resistant
CA 98	4	Resistant
CA 99	1	Susceptible
CA102	1	Susceptible
CA103	4	Resistant
CA107	> 32	Resistant
CA110	> 32	Resistant
CA119	> 32	Resistant
CA128	1	Susceptible
CA132	1	Susceptible
CA135	> 32	Resistant
CA141	4	Resistant
CA142	8	Resistant
CA145	4	Resistant
CA146	1	Susceptible
CA147	2	Resistant

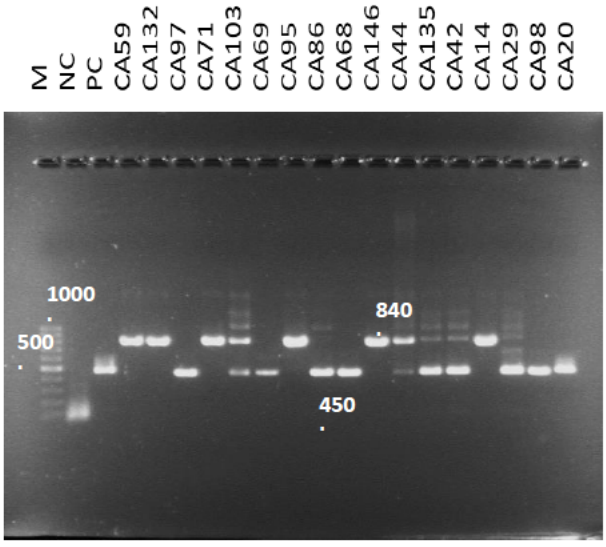
Genotyping analysis

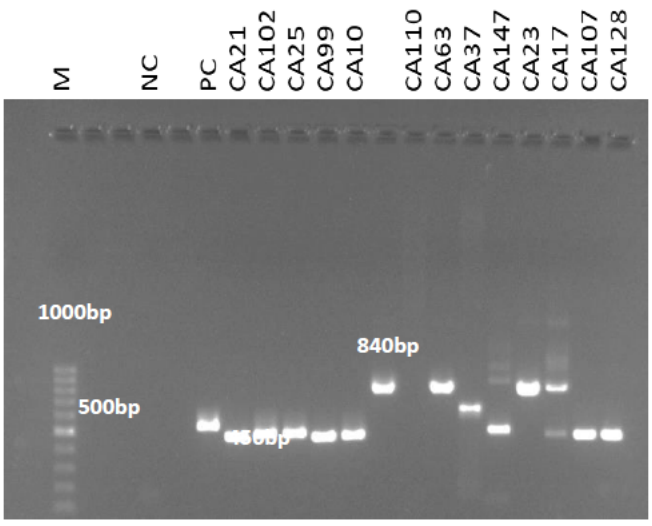
All 72 isolates (100%) produced positive PCR results (Figures 5,6,7,8,9, and 10). The majority of the isolates (45/72; 62.5%) yielded a 450bp band which was assigned Genotype A. Of the 72 isolates, 19 isolates (26.4%) yielded a band size of 840bp and was assigned Genotype B. A total of 11.11% (8/72) of the isolates yielded two band sizes of 450bp and 840bp which was assigned Genotype C. None of the isolates were assigned Genotype D.

A summary of the assigned genotypes is shown in Table 5.









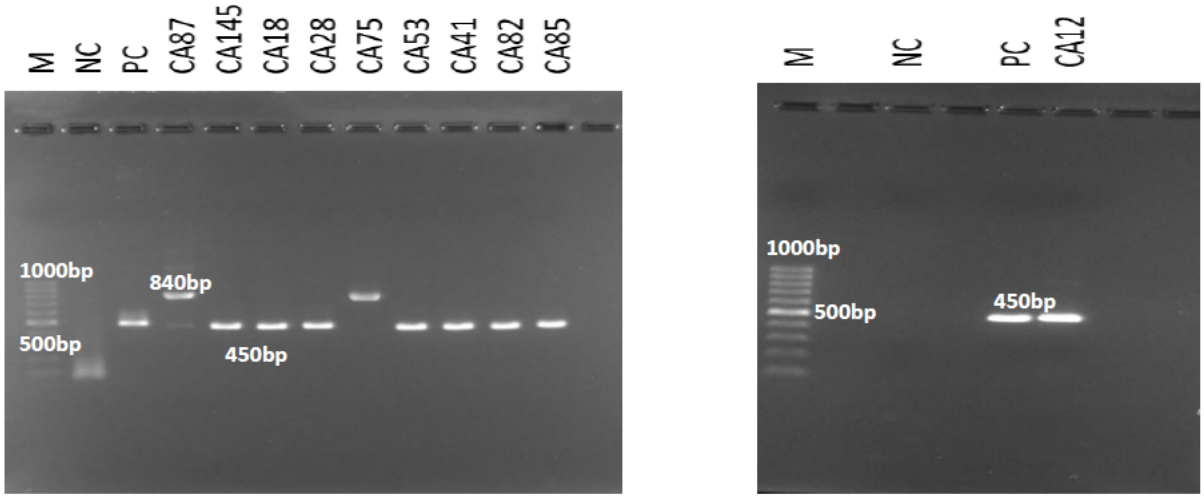


Figure 5: Agarose gel showing positive amplicons generated for *Candida* isolates. Band sizes of 450bp, 840bp and combined 450bp and 840bp were observed. M: 100 bp DNA molecular ladder (ThermoFisher Scientific), NC: negative control (no template DNA added), PC: positive control (C. *albicans* ATCC 10231 strain) and clinical isolates of *C. albicans*.

Table 5: Assignment of Genotypes for the individual isolates based on the banding patterns obtained

Isolate Name	PCR product size	Genotype
CA 1	840 bp	B
CA 10	450 bp	A
CA 11	450 bp	A
CA 12	450 bp	A
CA 14	840 bp	B
CA 17	450 and 840 bps	C
CA 18	450 bp	A
CA 20	450 bps	A
CA 21	450 bp	A

CA 23	840 bp	B
CA 25	450 bp	A
CA 27	450 bp	A
CA 28	450 bp	A
CA 29	450 bp	A
CA 30	450 bp	A
CA 32	450 bp	A
CA 34	840 bp	B
CA 35	450 bp	A
CA 37	840 bp	B
CA 40	450 bp	A
CA 41	450 bp	A
CA 42	450 and 840 bps	C
CA 43	450 bp	A
CA 44	450 and 840 bps	C
CA 47	450 bp	A
CA 53	450 bp	A
CA 54	450 bp	A
CA 56	450 bp	A
CA 58	450 and 840 bps	C
CA 59	840 bp	B
CA 60	450 bp	A
CA 62	450 bp	A
CA 63	840 bp	B
CA 65	450 bp	A
CA 67	450 bp	A
CA 68	450 bp	A
CA 69	450 bp	A
CA 71	840 bp	B
CA 72	840 bp	B
CA 75	840 bp	B
CA 77	450 and 840 bps	C

CA 79	450 bp	A
CA 80	840 bp	B
CA 81	840 bp	B
CA 82	450 bp	A
CA 83	450 bp	A
CA 84	450 and 840 bps	C
CA 85	450 bp	A
CA 86	450 bp	A
CA 87	840 bp	B
CA 88	450 bp	A
CA 89	450 bp	A
CA 91	450 bp	A
CA 94	450 bp	A
CA 95	840 bp	B
CA 96	840 bp	B
CA 97	450 bp	A
CA 98	450 bp	A
CA 99	450 bp	A
CA 102	450 bp	A
CA 103	450 and 840 bps	C
CA 107	450 bp	A
CA 110	840 bp	B
CA 119	450 bp	A
CA 128	450 bp	A
CA 132	840 bp	B
CA 135	450 and 840 bps	C
CA 141	840 bp	B
CA 142	450 bp	A
CA 145	450 bp	A
CA 146	840 bp	B
CA 147	450 bp	A

Correlation between clinical factors and genotypes

Table 6 shows the distribution of the genotypes according to clinical factors. Of the women who reported having current abnormal vaginal discharge at enrolment, the majority of the women had *Candida* infections which belonged to Genotype A (58.8%). Similarly, of the women who reported that they had been treated for vaginal infections in the past and also had experienced symptoms of abnormal vaginal discharge in the past, the majority of the women had *Candida* infections which belonged to Genotypes A (64.1%% and 62.5%), respectively. The majority of the pregnant women had *Candida* infections which belonged to Genotype A (61.3%) and the majority of the non-pregnant women had *Candida* infections which belonged to Genotype A (63.4%%). The majority of the HIV positive women had *Candida* infections and the majority of the HIV negative women had *Candida* infections which belonged to Genotype A (60.8% and 66.7%) respectively.

Table 6: Distribution of the different genotypes in association with clinical factors

CLINICAL FACTORS	GENOTYPES (n=72)		
	A (n=45)	B (n=19)	C (n=8)
Current abnormal vaginal discharge			
Yes	10 /17 (58.8%	6/17 (35.3%	1/17 (5.9%)
No	35/55 (63.6%)	13/55 (23.6%)	7/55 (12.7%)
Past treatment of STI			
Yes	25/39 (64.1%)	10/39 (25.6%)	4/39 (10.3%)
No	20/33 (60.6%)	9/33(27.3%)	4/33 (12.1%)
Past abnormal vaginal discharge			
Yes	25/40 (62.5%)	11/40 (27.5%)	4/40 (10%)
No	20/32 (62.5%)	8/32 (25%)	4/32 (12.5%)
Pregnancy status			
Pregnant	19/31 (61.3%)	8/31 (25.8%)	4/31 (12.9%)
Non-pregnant	26/41 (63.4%)	11/41 (26.8%)	4/41 (9.8%)
HIV status			
Positive	31/51 (60.8%)	13/51(25.5%)	7/51 (13.7%)
Negative	14/21 (66.7%)	6/21 (28.6%)	1/21 (4.8%

Correlation between susceptibility profiles and genotypes

Table 7 shows the correlation between amphotericin B susceptibility patterns and genotypes. For the isolates which were assigned as Genotype A, 80% of the isolates were resistant to amphotericin B and 20% were susceptible. Similarly, for isolates which were assigned Genotype C, a larger proportion of the isolates were resistant to amphotericin B (87.5%) and 12.5% were susceptible. For isolates assigned Genotype B, the majority of the isolates were resistant to amphotericin B (73.7%) when compared to 26.3% which were susceptible.

Table 7: Correlation between susceptibility profiles and genotypes

Genotypes	Susceptibility pattern	
	Susceptible (n= 15)	Resistant (n= 57)
A	9/45 (20%)	36/45 (80%)
B	5/19 (26.3)	14/19 (73.7%)
C	1/8 (12.5%)	7/8 (87.5%)

DISCUSSION

Characterization of *Candida* isolates plays a critical role in determining antimicrobial susceptibility profiles and resistance mechanisms in vaginal *Candida* infections. Drug resistance leads to various treatment failures, compromising the patient's overall health (79). Over the years there has been an increase in drug resistance observed in patients with vaginal *Candida* infections with many studies reporting that *Candida* species were the leading cause of vaginal infections in women (25, 80-82). In the current study, the prevalence of vaginal *Candida* infection was 48.0%. This prevalence was higher when compared to the prevalence observed in Yemen, Nigeria and India where the prevalence of vaginal *Candida* infection was found to be 22.1%, 30.0% and 43.0% respectively (5, 6, 83).

The prevalence of *C. albicans* in the current study was a 100%. Studies from other countries have reported that for patients with vaginal *Candida* infection, *C. albicans* is the most prevalent causative pathogen (50, 53, 84). This is consistent with the findings of the current study. However, some studies have highlighted the rise in non-*albicans Candida* causing vaginal infections (6, 52, 53, 83).

The current study was part of a larger study which included questions on socio-demographics, sexual behaviour and clinical history. We included this information in the current study so that a full description of the study population from which the *Candida* isolates were obtained was provided. In the current study, of the women who tested *Candida* positive, a higher percentage of the women were older (31-39 years old) and this was significant, p=0.019. A study conducted in Yemen found that women aged 30-34 years old were at higher risk of being infected with vaginal *Candida* (85) and these findings are consistent with the results obtained in our study. In this study, the prevalence of vaginal *Candida* infection in pregnant women was 43.05% and the prevalence of vaginal *Candida* infection in non-pregnant women was 56.94 % and this was significant, p=0.000. A study conducted in India reported that 72.37% of pregnant women had vaginal *Candida* infections (52). A study conducted in Kenya reported the prevalence of vaginal *Candida* infection to be 42.7% in pregnant women (6). Another study conducted in Nigeria found that the prevalence of vaginal *Candida* in pregnant women was 30% (5). A previous study found that the prevalence of vaginal *Candida* infection in non-pregnant women was 22.1% which was lower than the prevalence observed in the current study (84). A study conducted on women attending a tertiary hospital in central India found that the *Candida* positivity in pregnant women was 45.0%, which was higher when compared to non-

pregnant women (28.9%) (51). A study conducted in Vietnam found that 51.3% of non-pregnant women had vaginal *Candida* infection (48). The prevalence in Vietnam was lower when compared to the prevalence estimates reported in the current study. The prevalence of *Candida* infection is higher in pregnant women due to the immune suppression which women undergo during pregnancy as well as the high levels of estrogen production which makes this a very conducive environment for the growth of *Candida* (6, 50). The high prevalence of vaginal *Candida* infection in pregnant women results in early births which results in low birth weight babies whom are at a higher risk of being colonized by *Candida* which leads to neonatal complications, thereby increasing morbidity and mortality rates (7, 8). In the current study, of the women who tested positive for *Candida*, the majority of the women were HIV positive. A study conducted in Nigeria found that the prevalence of *Candida* infection was higher in women who were HIV positive (88.8%) when compared to women who were HIV negative (58.6%) (86). Another study conducted in Brazil found that the prevalence of vaginal *Candida* infection in HIV positive women was 29.7% and 14.5% in HIV negative women (87). HIV and *Candida* infections have a strong association in pregnant women (88). A study conducted in Tanzania highlighted HIV as a risk factor for vaginal *Candida* infections (89), these results corroborate with the results that were observed in our study.

Fluconazole is currently the antifungal of choice for the treatment of vaginal *Candida* infections (80). However, in the current study, we looked at resistance to amphotericin B. The rationale behind this is as follows; the current study was a sub-study of another study which already investigated susceptibility to the antifungal, fluconazole. In that study, we did observe resistance to fluconazole (manuscript in progress). For this study, we then decided to now look at another antifungal to see if there is resistance to different classes of antifungals in our isolates. The antifungal susceptibility results showed that of the 72 isolates, 79.2% were resistant to amphotericin B and 20.8% were susceptible to amphotericin B. This study observed a high prevalence of resistance to amphotericin B. A study conducted in Iran on women infected with vaginal *Candida* infections found that 150 of the cultured isolates were *C. albicans* and 89 isolates were non-*albicans Candida*, they also found that all the *C. albicans* isolates were susceptible to amphotericin B (82). In Brazil, a study which was conducted on asymptomatic and symptomatic women who were assessed for vaginal *Candida* infection found that *C. albicans* was the most frequently isolated species with prevalence rates of 40% and 90%, respectively followed by *C. glabrata* with prevalence rates of 13% and 6%,

respectively. In that study, all the isolates were susceptible to amphotericin B (90). A study conducted in Egypt reported a 100% prevalence for *C. albicans* and found that all the isolates (100%) were susceptible to amphotericin B (25). The current study showed a high resistance to amphotericin B with prevalence of 79.2%. The difference in susceptibility patterns to amphotericin B in *C. albicans* isolates may be due to different geographic locations and difference in populations sampled.

ABC genotyping is one method that has been used in the identification of *Candida* variation and has been of important use in linking genotypes to antifungal resistance (74). The majority of the isolates in the current study (62.5%) were assigned Genotype A and (26.4%) were assigned Genotype B, and 11.1% of the isolates were assigned Genotype C. Our results corroborate with another study which found Genotype A to be the most prevalent genotype with a prevalence of 75.7% (14). Another study conducted on women who had vaginal *Candida* infection in Brazil also found Genotype A to be the most prevalent when compared to other ABC genotypes followed by Genotype C and D with prevalence rates of 93.6% and 6.4%, respectively. That study also highlighted that Genotype B was not detected (91). However, in an Iranian study, 83.5% of the vaginal *Candida* isolates had Genotype C, followed by Genotypes B and A with prevalence rates of 12.6% and 3.8%, respectively (92). The differences in the ABC genotypes observed may be due to different geographical regions.

The current study also described the distribution of the genotypes in association with clinical factors as well as the distribution of the genotypes in relation to amphotericin B susceptibility patterns since this information was lacking in our setting. We wanted to investigate if specific genotypes are driving antifungal resistance which may impact future treatment of *Candida* as well as contribute to clinical manifestations associated with this infection.

In this study, for the women who reported having current symptoms abnormal vaginal discharge, Genotype A was most prevalent. Having been treated for vaginal infections in the past was linked to Genotype A, being HIV positive was linked to Genotype A and being non-pregnant was linked to genotype A. Genotype A was prevalent amongst the pregnant women and the majority of the HIV negative women had *Candida* infections which belonged to Genotype A. To the best of our knowledge there are no studies that have linked genotypes to clinical factors, this study now provides this missing data. In this study, the majority of the isolates which were assigned Genotype A were resistant to amphotericin B. Currently, there is a lack of published studies on the link between genotypes and amphotericin B susceptibility

patterns. This study now fills in this gap in the literature. The limitations of this study were as follows; the sample size was small and this could have been a reason for the study being unable to detect non-*albicans Candida*. In addition, this study was conducted at one geographical location and is not representative of the general population

Conclusion

The prevalence of *Candida* was shown to be high in both pregnant and non-pregnant women in this study. During pregnancy it is important for the women to be managed appropriately for this infection in order to prevent neonatal morbidity and mortality. This study also found a high level of resistance to the antifungal amphotericin B. Future research can now focus on the mechanisms associated with resistance in our local isolates. Currently in our local setting, resistance patterns to the commonly used antifungals to treat *Candida* infections are not being monitored. There is a need for antifungal monitoring in order to reduce the risk of future untreatable infections.

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