The Effect of a Combination Therapy of Fluconazole and Amphotericin B on the Growth and *CDR1* Gene Expression of *Candida glabrata*

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Keywords

Candida glabrata

*Cg*CDR1 gene

Fluconazole

Amphotericin B

Combination therapy

Real-Time polymerase chain reaction

Abstract

Introduction:

Candida glabrata (*C. glabrata*/ *Cg*) is a pathogenic organism that is increasingly developing frank innate and acquired resistance to the most commonly used antifungal agents, namely, azole group of antifungals. Furthermore, *C. glabrata*-associated oropharyngeal infections affecting immunocompromised patients, are more difficult to treat and the development of resistance worsen the prognosis.

Molecular studies related the emergence of resistance in *C. glabrata* to the upregulation of ATP-binding cassette (ABC) transporter genes, which work by reducing drug concentration within the cell via drug efflux mechanism, and among these genes, *Cg*CDR1 is considered to play a major role in resistance development. Thus, in order to overcome this problem, several combinations of antifungal agents are being studied.

Aim: To evaluate the effect of a combination therapy of fluconazole and amphotericin B on the growth and CDR1 gene expression of *C. glabrata*.

Research design and methodology: This *in-vitro* study evaluated the effect of a combination therapy of fluconazole and amphotericin B on the growth of *C. glabrata* and related it to the expression of *Cg*CDR1 resistance gene. *C. glabrata was* revived in brain heart infusion (BHI) broth and later inoculated onto agar plates. Following overnight incubation, 5 colonies were transferred using a sterile loop into 2 ml of phosphate buffered saline (PBS) solution to establish McFarland (Mcf) standard. Later, the solution was diluted by transferring 200 µL to 400 ml of yeast peptone dextrose (YPD) agar (flask 1). From (flask 1), 90 ml, 99 ml and 89 ml of inoculum were allocated into 3 separate flasks, into which 10 ml fluconazole, 1 ml amphotericin B and 11 ml combination (10 ml fluconazole + 1 ml amphotericin B) were added, respectively. The inoculums were left to settle for 20 minutes, then incubated at 37^{o}C with serial dilutions carried after 30 minutes, 2, 4, 6 and 24 hours. From the 96-microtiter plate, 10 µL for each treatment arm and time interval were transferred from selected wells and onto 30 Casein-peptone Soymeal-peptone (CASO) agar plates, and incubated for 24 hours. After incubation, the number of colonies were counted using an automated colony counter, to establish colony forming unit (CFU)/ml.

*Cg*CDR1 gene expression was analyzed using real time polymerase chain reaction. After 6 hours of incubation, a sample was taken from each treatment arm, transferred into CASO agar

plates and incubated for 24 hours at 37ºC. After establishing Mcf, gene extraction and gene expression were carried out according to manufacturer's instructions.

Results and discussion: No significant difference between the effect of the combination and amphotericin B was evident regarding *C. glabrata* growth. However, the combination therapy was more effective against *C. glabrata* than fluconazole, with a marked decrease in candidal growth at 30 minutes and 6 hours. Furthermore, the expression of *Cg*CDR1 gene at 6 hours contact time was more pronounced in the samples of *C. glabrata* treated with the combination therapy, compared to that of the monotherapy.

Conclusion: The combination therapy had better effect on the growth of *C. glabrata* than fluconazole monotherapy. On the other hand, the expression of *Cg*CDR1 was detected in the samples of *C. glabrata* treated with the combination therapy, suggesting the ability of the yeast to adapt and develop resistance in such environment.

Declaration

I, the undersigned hereby declare that the mini-thesis titled "The effect of a combination therapy of fluconazole and amphotericin B on the growth and CDR1 gene expression of *Candida glabrata*" is originally my work, and has not been previously submitted in its entirety or in part at any university for a degree or examination, and all the sources used or quoted herein, have been indicated and acknowledged by complete references.

Signature 

Full name: Nada Abdelrahman Nurein Mohamed Date: 1/11/2019

Dedication

I dedicate this mini-thesis to my loving, caring parents and my gorgeous sisters, who without their unwavering help, support and encouragement, this work would not have seen the light of day.

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First and foremost, all praise be to Allah for blessing me with an incredible amount of strength and patience to complete this mini-thesis.

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Abbreviations

ABC	ATP binding cassette
BHI	Brain heart infusion broth
CASO	Casein-peptone Soymeal-peptone agar
CLSI	Clinical and Laboratory Standards Institute
C. glabrata	*Candida glabrata*
*Cg*CDR1	*Candida glabrata* Cerebellar Degeneration Related Protein 1
CFU	Colony forming units
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
Mcf	McFarland standards
OPC	Oropharyngeal candidiasis
PBS	Phosphate buffered saline
YPD	Yeast peptone dextrose

Chapter 1: Introduction

1.1 Study outline

Herein, an outline of the framework of this mini-thesis which is composed of six chapters, is projected. **Chapter 1** presents the background for the study and the purpose for conducting it. **Chapter 2** provides a review of the literature that elaborates in detail the oral infections caused by *C. glabrata* and the different antifungal agents commonly used to eradicate the infection. A review of the previous reports that documented the different mechanisms via which *C. glabrata* develops resistance to antifungal treatments, is included. Lastly, the chapter outlines the different ways generated to investigate and counteract the resistance.

Chapter 3 lists the study aim and objectives. **Chapter 4** details the research design and methodology used, describing the research materials and data collection method. **Chapter 5 and Chapter 6** presents the statistical methods used to analyze the data and ethical approval for conducting the research. **Chapter 7, Chapter 8 and Chapter 9** feature the study results, discussion and conclusion respectively.

1.2 Background

C. glabrata is an important pathogenic organism in the oral mucosa (Redding *et al*., 2000, 2004). It is common in patients with advanced cancer (Davis *et al*., 2006), patients receiving radiation therapy for head and neck cancer (Redding *et al*., 2000, 2004), the elderly and the denture wearers (Coco *et al*., 2008). Studies further reported that *C. glabrata*-associated oropharyngeal candidiasis (OPC) in human immunodeficiency virus (HIV) and cancer patients, is more aggressive and more difficult to treat, compared to infections caused purely by *candida albicans* (*C. albicans*) (Canuto *et al.*, 2000; Redding *et al.*, 2000, 2002, 2004).

C. glabrata is rapidly developing inborn resistance to the most commonly used azole antifungal agents, such as fluconazole and itraconazole. It is also becoming inherently less susceptible to polyene class of antifungals, such as amphotericin B and Nystatin, which are used in case of azole failure (Fidel *et al*., 1999). Thus, *C. glabrata* associated infections are becoming more difficult to treat (Sanguinetti *et al*., 2005).

Many molecular mechanisms are reported to be implicated in the development of antifungal resistance in *C. glabrata.* However, the most important mechanism is the upregulation of drug

efflux genes belonging to the ABC transporter family (Niimi *et al*., 2002; Wada *et al*., 2002; Sanguinetti *et al*., 2005; Rodrigues *et al*., 2014). Studies showed that among the ABC transporters, *Cg*CDR1 is the main gene corresponding to azole resistance in *C. glabrata* (Sanguinetti *et al*., 2005; Yoo *et al.*, 2010; Abbes *et al*., 2013; Szweda *et al*., 2015; Shahrokhi *et al*., 2017).

1.3 Rationale of the study

The prolonged and frequent use of azole antifungal agents led to the development of azole resistant *C. glabrata,* presenting a problem to medicinal management (Alexander *et al*., 2013). Recent interest in evaluating the success of antifungal combinations in overcoming this resistance has increased (Johnson *et al*., 2004).

Studies demonstrated that the combination of fluconazole and amphotericin B has antifungal activity against various species. However, only few studies have assessed its activity against *C. glabrata in vitro* (Mukherjee *et al.*, 2005). In this study, the antifungal effect of a combination of fluconazole and amphotericin B on the growth and CDR1 gene expression of *C. glabrata* was evaluated.

Chapter 2: Literature review

2.1 Oral candidiasis

Oral candidiasis is considered one of the most frequent conditions affecting the oral cavity, and it is a notable source of morbidity. The disease causes chronic pain upon mastication, thus limits the nutritional intake of the immunocompromised and elderly people (Redding *et al.*, 2000). The disease arises as a result of overgrowth of *candida* species, or due to alteration of the normal physiology of the oral cavity by local and systemic host factors (Epstein, 1990).

2.1.1 Treatment of oral candidiasis

The treatment of oral fungal infections is restricted to three classes of antifungal drugs, namely, azoles, polyenes and echinocandins. The pyrimidines are also used but usually in combination with azoles or polyenes antifungal agents (Muzyka, 2005).

Fluconazole is the most frequently used azole agent for the treatment of oral candidiasis (Chapman, Sullivan *et al.*, 2008). It works through the inhibition of the enzyme involved in ergosterol biosynthesis, therefore, prevents the production and renewal of sterols (Rodrigues *et al*., 2014). The drug prevents OPC in cancer patients (Pappas, Kauffman *et al*., 2009), and effectively treat OPC in HIV patients (Vazquez 2007) and patients receiving radiation therapy (Redding *et al*., 2004). Studies showed that orally administered fluconazole can be readily absorbed through the gut and secreted in high levels in saliva (Niimi *et al*., 2010; Force 1995). However, the prolonged exposure to fluconazole and the use of immunosuppressive medications had led to the emergence of fluconazole resistance in candidal organisms (Enwuru *et al*., 2008; Andrew 2003).

Amphotericin B is a fungicidal agent that belongs to the family of polyenes. It works through binding to ergosterol molecules, and increasing cell permeability. These two mechanisms result in pore formation on the cell wall and subsequent cell lysis ensue (Rodrigues *et al*., 2014). The drug treats severe and difficult to eradicate fungal infections (Rodrigues *et al*., 2014; Williams and Lewis 2011). However, when administered in high doses intravenously, infusion-associated toxicity and nephrotoxicity side effects are exhibited (Hamill 2013). Consequently,

lipid formulations of amphotericin B had been prepared and showed improvement in diminishing toxicity (Adler-Moore and Proffitt 2008).

Amphotericin B is commonly used to treat chronic erythematous candidiasis, and prescribed in the form of oral suspension or lozenges (Williams and Lewis 2011). The oral suspensions of amphotericin B showed successful results in the management of azole-refractory and persistent oral candidiasis in HIV patients (Grim, Smith *et al*., 2002). The same was reported by Dewsnup and Stevens (1994), however, their study also reported that the culture results of those patients showed azole resistant *C. glabrata*.

2.2 *Candida glabrata*

C. glabrata is now increasingly implicated in oral infections, either as the only identifiable organism from oral fungal lesions (Canuto *et al.*, 2000; Redding *et al.*, 2000, 2002), or as a co-infecting organism with *C. albicans* (Redding *et al.*, 2002, 2004). Furthermore, it is considered the second most common cause of candidiasis in majority of the reported cases (Azie *et al*., 2012).

2.2.1 Oral carriage of *Candida glabrata*

C. glabrata was reported as the most commonly isolated *Non-Candida Albicans Candida (NCAC)* species from the oral cavity in elderly people, accounting for 29% in people above 80 years of age and rising to 58% in those who wore dentures (Lockhart *et al*., 1999).
In diabetics, the oral carriage of *C. glabrata* accounted for 9.4% of all *candida* isolates, and was identified as the second most isolated species after *C. albicans*. Furthermore, it has been reported that the incidence of *C. glabrata* had risen with the increased use of antibiotics (Kadir *et al*., 2002).

2.2.2 *Candida glabrata* associated Oral infections

Denture stomatitis, is a debilitating disease seen in denture wearers, predominantly caused by *C. albicans*. However, *C. glabrata* was isolated from 31% of patients with denture stomatitis, indicating its presence as a pathogenic yeast. Also, it was revealed that 70% of the patients had

mixed infections with *C. glabrata* and *C. albicans*; and this was thought to worsen the prognosis of inflammation in denture wearers. (Coco *et al*., 2008).

C. glabrata-associated OPC was found in 22.7% of cancer patients, and 72% of *C. glabrata* colonies were resistant to fluconazole and itraconazole (Bagg *et al*., 2003), and as the main cause of OPC in 14% of HIV positive patients. Furthermore, *C. glabrata* was the most frequent *NCAC* species isolated as a sole causative organism in pseudomembranous candidiasis in HIV patients (Canuto *et al*., 2000).

2.2.3 Virulence properties of *C. glabrata*

Formation of hyphae is not the virulence mechanism of choice for *C. glabrata*, it secretes hydrolytic enzymes and adheres to medical devices by biofilm formation (Silva *et al*., 2012; Pfaller and Diekema 2007). Biofilms are organized communities, planted in an extracellular matrix (Samaranayake *et al*., 2002). *C. glabrata* biofilms are much more resistant to treatments than the original planktonic cells (Rodrigues *et al*., 2014), and they are best formed in the presence of an increased serum environment. Thus, denture plaque induced inflammation would ease the growth of *C. glabrata* on the surface of the denture (Nikawa *et al*., 2000). Samaranayake *et al*., (2002) indicated that cell surface hydrophobicity (CSH) plays a role in *C. glabrata* adherence to denture acrylic. The study further revealed that the CSH value demonstrated by *C. glabrata* is four times greater than that of *C. albicans*, thus, it has twice the ability to adhere to denture surfaces.

C. glabrata also produces phospholipases to hydrolyze phospholipids, which results in damage to the host cell membrane, subsequent destruction of the mucosa and invasion of the tissues by the organism (Marcos-Arias *et al*., 2011). Furthermore, Luo *et al*., (2002, 2004), reported the ability of *C. glabrata* to breakdown erythrocytes *in vitro* using hemolysins. Therefore, the ability to breakdown hemoglobin and obtain iron for its metabolic processes, has been suggested (Luo *et al*., 2002, 2004).

C. glabrata has the ability to develop resistance to the most commonly used azole agents over time, and Fidel *et al*., (1999) related this phenomenon to the haploid state of *C. glabrata*. The haploid state allows *C. glabrata* to rapidly adapt to the environment and enhances genome mutation to develop resistance (Kołaczkowska and Kołaczkowski 2016).

2.2.4 Mechanism of drug resistance in *Candida glabrata*

In general, the reasons behind the recalcitrant fungal infections are plenty and include; the presence of multi-drugs resistant persister cells, over-expression of ABC drug efflux genes and sequestration of drugs by matrix components (Ramage *et al*., 2012).

Persister cells are a small subpopulation of drug tolerant cells that are highly resistant to high doses of antifungal treatment and result in recurrence of infections (Brauner *et al*., 2016). When the persister cells are challenged by an antifungal agent, they display a biphasic pattern of killing, by which a great number of the population is killed and a small proportion is allowed to survive (Lewis 2010). Moreover, when the persister cells are regrown they show the same biphasic pattern of killing, making them resistant to treatment. The production of persister cells in some strains of *C. glabrata* was reported by Li *et al*., (2015).

Recent data, demonstrated the emergence of azole, amphotericin B and caspofungin resistant isolates of *C. glabrata* (Krogh-Madsen *et al*., 2006). Although little is known on the mechanism of antifungal resistance by *C. glabrata*, it is known to show intrinsic and acquired resistance to azole antifungal agents (Bennett *et al*., 2004).

Many studies reported the importance of drug efflux mechanism, which results in the reduction of drug accumulation within the cell (Chapeland-Leclerc *et al*., 2010; Ferrari *et al*., 2009; Sanguinetti *et al*., 2005). Moreover, *C. glabrata* can increase the expression of the *CgERG11* gene, which encodes lanosterol 14α demethylase. This enzyme is responsible for the biosynthesis of ergosterol in *C. glabrata*, which is selectively targeted by azole antifungals (Miyazaki *et al*., 1998). However, *C. glabrata* commonly shows an overexpression of *C. glabrata CDR1* (*CgCDR1*), *C. glabrata CDR2* (*CgCDR2*) and *C. glabrata SNQ2* (*CgSNQ2*) genes. These genes are ATP binding cassette (ABC) transporter genes, involved in drug efflux mechanism and result in the development of azole resistance (Sanglard *et al*., 2001; Sanguinetti *et al*., 2005).

2.2.4.1 Resistance to azoles

Compared to other *Candida* species, *C. glabrata* was reported to be naturally less susceptible to azole group of antifungal agents by developing acquired resistance to fluconazole within a short time after exposure (Shen *et al*., 2010; Sanguinetti *et al*., 2005). Recently, the overexpression of ABC transporter genes, namely, *Cg*CDR1 and *Cg*CDR2 proved to play an

important role in the development of azole resistance in *C. glabrata* isolates (Miyazaki *et al*., 1998; Sanglard *et al*., 1999; Sanglard *et al*., 2002; Niimi *et al*., 2002; Bennett *et al*., 2004; Prasad *et al*., 2006; Vale-Silva *et al*., 2015).

The rate of expression of ABC transporter genes is mediated by a zinc finger transcription factor called PDR1. The gain of function mutation in *C. glabrata* PDR1 (*Cg*PDR1) results in overexpression of the ABC transporter genes (Tsai *et al*., 2006; Vermitsky *et al*., 2006; Ferrari *et al*., 2011; Paul *et al*., 2011). An increasing body of evidence has reported that the expression of *Cg*CDR1 was more closely related to azoles resistance in *C. glabrata* than the expression of *Cg*CDR2 (Sanglard *et al*., 1999; Brun *et al*., 2004; Rogers *et al*., 2006). Other studies showed that *Cg*CDR1 was significantly expressed in resistant *C. glabrata* isolates while *Cg*CDR2 was only moderately expressed (Sanglard *et al*., 2001; Sanguinetti *et al*., 2005; Yoo *et al*., 2010; Abbes *et al*., 2013; Szweda *et al*., 2015; Shahrokhi *et al*., 2017). It was further indicated that the ABC transporter *CgCDR1* contributed almost exclusively to azole resistance (Ferrari *et al*., 2011).

Szweda *et al*., (2015), showed significant increase in the level of expression of *CgCDR1* gene in resistant strains of *C. glabrata*. This increase was much higher than that recorded by Sanguinetti *et al*., (2005). Moreover, a study done by Fonseca *et al*., (2014), reported that overexpression of *Cg*CDR1 gene was not expressed in the planktonic cultures of the less resistant *C. glabrata* strains, however, their expression was noted in their biofilm counterparts.

2.2.4.2 Resistance to polyenes

In the presence of amphotericin B, carbohydrates like B-1, 3 glucans can be detected in *C. glabrata* cell wall, thus making the diffusion of the drug through the biofilm matrices more difficult. This may be an attempt by the *Candida* to protect itself against the antifungal drug (Taff *et al*., 2012). It was believed that the reduced susceptibility to polyenes was due to a missense mutation in *ERG6* gene, resulting in changes in sterol contents of the plasma membrane (Vandeputte *et al.*, 2007).

Several studies indicated that the overexpression of *CgCDR1* in azole resistant strains was associated with increased susceptibility to polyenes. They attributed that to the presence of free sterol incorporated in the plasma membrane (Brun *et al*., 2004; Bouchera *et al*., 2000).

2.2.5 Treatment of recalcitrant *C. glabrata* infections using drug Combination

Combination therapies are being used to counteract the resistance of *C. glabrata* to the most commonly used antifungal drugs. Different combinations of antifungal drugs were examined against resistant and susceptible strains of *C. glabrata* and the best results were obtained from the combination of amphotericin B and flucytosine (Alves *et al*., 2012). Also, Diphenyl Diselenide $(PhSe)_2$, a chemical compound with fungistatic and fungicidal activities *in vitro*, was combined with amphotericin B and fluconazole. The best results were obtained from the combination of $(PhSe)_2$ and amphotericin B (Denardi *et al*., 2013). Fluconazole was again combined with ascorbic acid for the treatment of oral mucositis caused by *C. glabrata*. The combination was used against *C. glabrata* biofilm, and appeared ineffective (Rodrigues and Henriques 2017).

There is an ongoing debate in regard to the interaction of fluconazole and amphotericin B as treatment of fungal infections (Johnson *et al*., 2004). *In vitro* and *in vivo* studies have demonstrated a lack of antagonism between fluconazole and amphotericin B when used concurrently (Ghannoum *et al*., 1995; Sanati, *et al*., 1997; Larsen *et al*., 2004). Furthermore, it was found that amphotericin B mechanism of action does not solely depend on binding to ergosterol, but also results in leakage of the fungal cytoplasmic material without binding to the ergosterol. Also, the phospholipid composition and the ergosterol content of the cell membrane result in increasing the susceptibility of the yeast to amphotericin B (Bolard *et al*., 1996). In addition, fluconazole only inhibits ergosterol synthesis partially, the remaining ergosterol are then lysed by amphotericin B (Sanati *et al*., 1996).

Interestingly, two contradicting theories regarding the interaction between fluconazole and amphotericin B were proposed by Mukherjee *et al.*, (2005). The first theory called depletion, and it proposes that azole results in antagonism by depleting the cells from ergosterol and thus, reducing the targets for the polyenes. The second theory called enhancement, where synergism is observed. In this theory, polyenes facilitate the ingress of azoles to the intracellular space by pore formation, and ease azoles action in inhibiting the ergosterol biosynthesis (Mukherjee *et al.*, 2005).

2.3 Methods used for *in vitro* analysis of *C. glabrata*

2.3.1 Detection of *C. glabrata in vitro*

The diagnosis of OPC can often be based on the clinical picture of the oral candidosis, but identifying the causative species is only possible via microbiological sampling and the use of genetic methods (Byadarahally Raju and Rajappa 2011). *C. glabrata* shows creamy, smooth, small and convex colonies on sabouraud dextrose agar (SDA), and shows lavender color on CHROMagar®. Under the microscope, *C. glabrata* shows a small sized cell and no pseudohyphae formation (Lopez *et al*., 2001; Rodrigues *et al*., 2014).

Identification of *Candida* based on genetic variation is a more reliable approach using molecular-based identification methods (Williams and Lewis 2000). These methods include DNA microarrays and polymerase chain reaction (PCR) based assays such as real time polymerase chain reaction (RT-PCR).

Molecular methods

Molecular methods for evaluating gene expression have been developed recently. Nevertheless, the pros and cons of each method should be considered before using. These methods include but are not limited to: Northern blot hybridization, DNA Microarrays and RT-PCR (Fryer *et al*., 2002) (Table 2- 1)

Table 2- 1: Advantages and disadvantages of molecular methods used for gene expression.

Method	Advantage	Disadvantage
Northern blot hybridization (Fryer *et al*., 2002)	Gold standard.	Time consuming, costly, only one gene can be analyzed at a time and it consumes huge amount of RNA.
DNA Microarrays	Reliable, rapid, easily reproduced and quantitatively monitor the expression levels of thousands of genes (Fryer *et al*., 2002).	Difficulty to design an array without having multiple associated DNA/RNA sequencing binding to the same probe on the array (Bumgarner 2013).
RT-PCR (Valasek and Repa 2005)	Extremely sensitive, quick, able to analyze small sample, lowers the possibility of cross contamination in the laboratory, has the ability to measure mRNA gene expression.	Variable results can be obtained, because multiple enzyme and oligonucleotides with different characteristics are available for reverse transcription step and priming step.

RT-PCR

Recently, the method of RT-PCR has evolved from the main PCR technique developed by Mullis in 1983 (Mullis 1990). RT-PCR can record the amplification progress of a specific target sequence using fluorescent method. During this process, the quantification of the gene is achieved by correlating how rapidly the fluorescent signal reaches the threshold level (C_T) with the quantity of nucleic acid at the original sample. The C_t score (or "C_T" score) is defined as the cycle number at which the PCR amplification cycle has exceeded a threshold level (Gygax *et al*., 2011).

In order to minimize errors and correct sample-to-sample variation in RT-PCR experiments, a housekeeping gene is used. This gene is a cellular RNA that is concurrently amplified with the target gene, and serves as an internal reference against which other RNA values can be normalized. The most common housekeeping genes used are β-actin (Suzuki *et al*., 2000).

2.3.2 Antimicrobial susceptibility testing

The activity of an antimicrobial can be assessed using a variety of laboratory methods. The commonly used methods are the disk-diffusion, broth microdilution methods and Time-Kill test (Table 2-2).

Table 2-2: The advantages and disadvantages of commonly used antimicrobial susceptibility testing.

Method	Advantage	Disadvantage
Disk-diffusion (Nijs *et al*., 2003)	Simple method. Cost effective. The results are easy to interpret. Measures the inhibitory activity of a drug.	Inability to distinguish fungicidal from fungistatic effect. The amount of antimicrobial diffused in the disk cannot be determined.
Broth microdilution method (CLSI 2012)	Reproducible method. Cost effective.	The approach to get a reproducible result must be controlled.
Time-Kill test (Pfaller *et al*., 2004)	The most suitable method for determining the fungicidal effect of an antifungal agent. Provide information on the interaction between the drug and the organism. Determine synergism or antagonism between antifungal agents in combination therapies.	Laborious method.

Time-Kill test:

Time kill test has been standardized and described in details by Clinical and Laboratory Standard Institute (CLSI) (CLSI 2008). The method is used to determine the killing of an organism over time by one or more antimicrobial agent under controlled conditions. The rate of killing is determined by testing the sampling control and antimicrobial containing flasks at different time intervals. Furthermore, this method is very useful in determining synergism and antagonism in combination therapy (CLSI 2008).

Chapter 3: Methodology

3.1 Aim

To evaluate the effect of a combination therapy of fluconazole and amphotericin B on the growth and CDR1 gene expression of *C. glabrata*.

3.2 Objectives

Two parameters will be observed in this experiment, the growth of *C. glabrata* in terms of (CFU/ml) and the expression of drug resistance gene (*Cg*CDR1) in terms of (C_t value).

1. To determine the effect of fluconazole, amphotericin B and the combination therapy at a constant dose on the growth of *C. glabrata* using Time-Kill test.

2. To measure the amount of CDR1 gene expression on exposure of *C. glabrata* to fluconazole, amphotericin B and the combination therapy combined.

3. To determine the relation between the expression of CDR1 resistance gene and the growth of *C. glabrata*, when exposed to fluconazole, amphotericin B and the combination therapy.

3.3 Null hypothesis

1. There is no difference in the effect of fluconazole, amphotericin B and the combination therapy on the growth of *C. glabrata.*

2. The expression of *C. glabrata* CDR1 gene is the same when exposed to fluconazole, amphotericin B and the combination therapy.

3. The expression of *C. glabrata* CDR1 gene is unrelated to *C. glabrata* growth when exposed to fluconazole, amphotericin B and the combination therapy.

Chapter 4: Research design and methodology

This chapter explores the research design and methodology used in the study, and expounds the research tools and methods.

4.1 Study design

This was an *in vitro* study to compare the effect of a combined therapy of fluconazole and amphotericin B on the growth of *C. glabrata* and relate it to the expression of CDR1 resistance gene. The latter was assessed using RT-PCR (Appendix A).

A pilot study was done to determine the number of samples required the actual experiment.

4.2. Study site

The study was conducted in the Dental Research Institute laboratory, Faculty of Dentistry, University of the Western Cape, Tygerberg campus.

4.3 Sample size

1. Assessment of *C. glabrata* growth:
   - For each treatment arm, at each time interval, we had 30 plates incubated and 9 control plates.
   - For each treatment arm, at each time interval, 3 plates were incubated after randomly selected colonies were re-exposed to the treatments.

(The growth of *C. glabrata* was always observed at 30 minutes, 2, 4, 6 and 24 hours) (Appendix A).

2. Assessment of *Cg*CDR1 at 6 hours:
   - For each treatment arm, 10 samples were assayed for RT-PCR.

4.4 Growth of *C. glabrata* following treatment with Fluconazole, Amphotericin B and the combination therapy

4.4.1 Drugs reconstitution

The selected concentration of fluconazole and amphotericin B was 16 µg/ml and 0.125 µg/mL, respectively. The selection of these two concentrations was based on the fact that any increase in fluconazole concentration might result in absolute resistance. Moreover, any decrease in the concentration might not give desirable results. On the other hand, a minimum concentration was selected for amphotericin B to minimize the side effects that usually accompanies the medicine, when used clinically.

The drugs were reconstituted according to the calculations provided by the CLSI. Fluconazole was dissolved in distilled water, and amphotericin B was dissolved in Dimethyl sulfoxide (DMSO) to obtain the required working concentration (CLSI 2008; Kiraz *et al*., 2010).

4.4.2 Preparation of pure culture

American Type Culture Collection (ATCC) strain of *C. glabrata* (ATCC 2001) was obtained from a standard stock culture collection, stored at the Oral and Dental Research Institute, University of the Western Cape. *C. glabrata* was revived and prepared using the direct colony suspension method.

This process involved the transfer of some colonies of the stock culture into brain heart infusion broth (BHI) using a sterile loop, and incubated at 37 ºC for 24 hours. Subsequently, it was inoculated into a solid media (Casein-peptone Soymeal-peptone Agar (CASO)) plate, and incubated overnight at 37ºC. An incubation time of between 24 hours to 48 hours at 37ºC to obtain pure culture is suitable for *C. glabrata,* permitting it to grow and form visible single colonies (Gygax *et al*., 2011; Kiraz *et al*., 2010). Five (5) colonies from the 24 hours culture were selected and suspended in 2 ml of phosphate buffered saline (PBS) using a sterile loop. The inoculum was then adjusted to a 0.5 McFarland (Mcf) standard (~1-5 × 10^6 CFU ml^{-6}) using Densichek$^{@}$ (Figure 4- 1).

Figure 4- 1: DensiCHEK Plus@ device, used to standardize the density of the inoculum to 0.5 McFarland.

4.4.3 Preparation for antimicrobial culture

In order to have a working solution, the adjusted inoculum was reduced to 5.0 x 10^2 cell per ml (According to CLSI recommendations) CLSI (2008), by transferring 200 µL of the adjusted solution to a sterile flask (Flask 1) containing 400 ml of YPD (yeast peptone dextrose). Flask 1 served as the positive (+ve) control (Figure 4-2). Following reduction of the inoculum concentration, the working solution was left to settle for 20 minutes before use.

From flask 1, different portions were allocated into their respective flasks, in order to have the same amount of the *candida* suspension without affecting the concentration of the drugs. Flask 2 (F) contained 90 ml inoculum and 10 ml of fluconazole, while Flask 3 (A) contained 99 ml of inoculum and 1 ml of amphotericin and Flask 4 (C) contained 89 ml of inoculum with 10 ml and 1 ml of fluconazole and amphotericin B, respectively (Figure 4-3). The four flasks were incubated in an Orbital Shaker Incubator at 37 ºC.

Figure 4-2: Flask 1 containing only the microorganism and YPD representing the positive control.

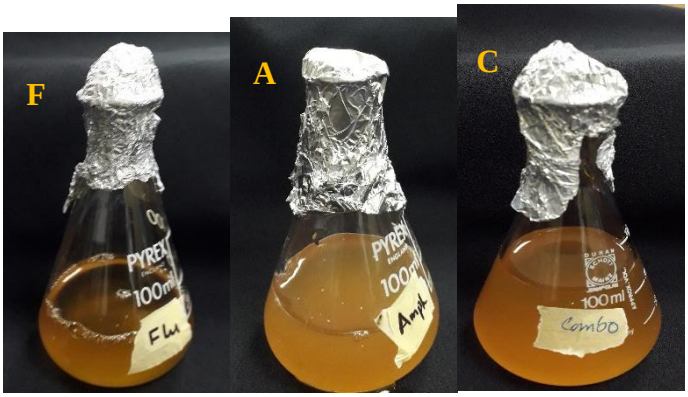


Figure 4-3: Flask 2, 3 and 4, were labelled according to the type of treatment that was proposed to be added to the suspension.

4.4.4 Serial dilution procedure

The serial dilution technique was used to evaluate the antifungal effect of fluconazole, amphotericin B and the combination therapy on *C. glabrata*, by assessing *candida* growth. 200 µL was pipetted from flask 2-4 and transferred to the first row only of each respective 96-well plates. Subsequently, 100 µL of PBS was added from row B, column 1 to row H, column 12 at each well in the 96-well microtiter plate, using the multichannel pipette. The control solution was added to the last 2 columns of each of the 96 well plates (Figure 4-4). The suspension was diluted two-folds by transferring 100 µL from the first well to the second well and so forth up to the sixth well. The last 100ul from the wells in row H was discarded.

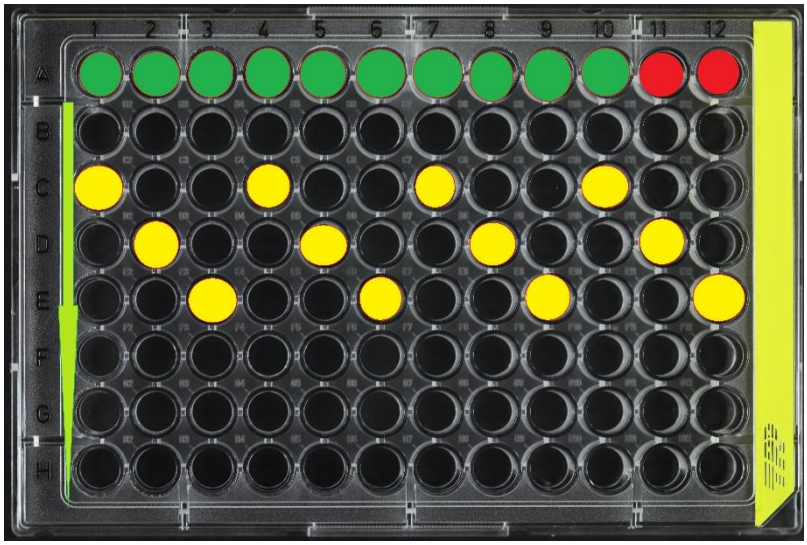


Figure 4-4: A 96-well microtiter plate exhibiting different wells containing 200 µL in the wells of the first row (A)🟢, transferred from the flasks to the corresponding 96-well plate. The last 2 wells 🔴 of the first row of each microtiter plate contained 200 µL transferred from flask 1. 100 µL from the first well (A) was transferred to the second well (B) which previously contained 100 µL of PBS. The serial dilution proceeded up to the 6th well (G).

For each 96 well plate, 10 µL were transferred from well (C), (D) and (E) 🟡 onto Casein-peptone Soymeal-peptone Agar (CASO agar) using single channel pipette, spread using a sterile hockey stick and incubated at 37°C for 24 hours under anaerobic conditions. Three CASO Agar plates were used per well dilution. This was carried out for each 96 well microtiter plate. This procedure was repeated for each treatment arm at 30 minutes, 2, 4, 6 and 24 hours respectively.

After 24 hours incubation of the CASO agar plates (n=30/ microtiter plate), the number of colony forming units (CFU) in each plate were counted using an automated colony counter (Gerber, Switzerland) (Figure 4-5).

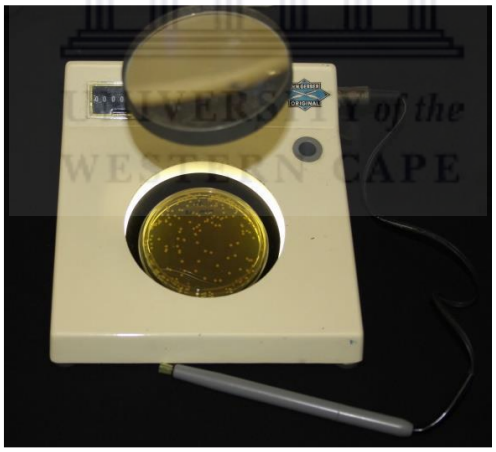


Figure 4-5: Automated colony counter used to count the number of *C. glabrata* colonies in a CASO agar plate.

4.4.5 Plate count method

The typical counting range is from 30 to 300 CFU per plate. Any number of colonies above that was considered Too Numerous To Count (TNTC) and recorded as 300 (CFU), while those

less than 30 were considered as Too Few To Count (TFTC) and recorded as zero. This is attributed to the fact that any number of CFU less than 30 is regarded as insignificant to produce infection (Sutton, 2011). Nevertheless, in this study the number of CFU/ml less than 30 were included and counted. This was done to standardize the laboratory procedure in evaluating the antimicrobial efficacy of the combination therapy against *C. glabrata.*

4.4.6 Re-exposure of *C. glabrata* to treatment

After colony counting, several persister colonies were randomly selected from each treatment at each time interval and plated in three different CASO agar plate. Subsequently, the plates were incubated at 37°C for 24 hours in an aerobic condition. After incubation, 5 colonies were selected from each plate, according to the treatment they were previously treated with and according to the time interval. The colonies were suspended in 2 ml of phosphate buffered saline (PBS) using a sterile loop. The inoculum was then adjusted to a 0.5 Mcf standard (~1-5 × 10^6 CFU ml^{-6}) using DensiCHEK$^{@}$. Following this the procedure was similar to the one previously described previously from (4.4.3 to 4.4.5). Also see (Appendix A).

4.5 *Cg*CDR1 gene expression following treatment with Fluconazole, Amphotericin B and the combination therapy

4.5.1 Preparation of antimicrobial culture

a) Preparation of pure culture:

The previously revived *C. glabrata* was transferred into BHI to ensure that it was viable, then incubated at 37 ºC for 24 hours. Subsequently, it was inoculated in CASO agar plate using a sterile loop, and incubated overnight at 37 ºC. Five (5) colonies from the 24 hours culture were selected and suspended in 2 ml of phosphate buffered saline (PBS) using a sterile loop. The inoculum was then adjusted to a 0.5 McFarland (Mcf) standard (~1-5 × 10^6 CFU ml^{-6}) using Densichek@ (Figure 4- 1).

b) Sample preparation for RNA extraction

(The first step was similar to that in (4.4.3)).
After 6 hours of incubation of the flasks, 10 ul was transferred from each treatment arm and spread onto their corresponding CASO agar plates. Subsequently, the plates were incubated at 37°C for 24 hours in an aerobic condition. After incubation, 5 colonies were selected from each plate and suspended in their corresponding tubes containing 2 ml of phosphate buffered saline (PBS), using a sterile loop, and subsequently adjusted to 0.5 Mcf standard (~1-5 × 10^6 CFU ml^{-6}) using DensiCHEK@. Afterwards, 1 ml of the adjusted solution, from each treatment arm, was transferred into their matching 2 ml Eppendorf tubes and centrifuged for 5 minutes at 5,000 rounds per minute (rpm); the media and the supernatant were discarded. The procedure was repeated until a visible pellet was seen in each of the Eppendorf tubes.

4.5.2 RNA extraction from *C. glabrata*

The extraction of total RNA was done according to prescribed protocol utilizing equipment and reagents specified by the company Stratec Molecular© (Berlin, Germany). The Invitrap® Spin Cell RNA Mini Kit (Stratec Molecular©, Germany) was used for ribonucleic acid (RNA) extraction.

Following pellet formation, 100 µl of Lyticase (SigmaAldrich, South Africa) was added to the yeast pellet to loosen it. The mixture was vortexed and incubated in an Orbital Shaker Incubator for 10-30 minutes at 30ºC. Next, 350 µl of Dithiothreitol (DTT)-containing lysis solution (Lasec, SA) was added to disrupt the yeast cell. The lysate was then transferred onto DNA-Binding Spin Filter, placed in a 2 ml Receiver Tube and incubated for 1 minute and centrifuged at 11,000 rpm for 2 minutes. Afterward, the deoxyribonucleic acid (DNA)-Binding Spin Filter was discarded.

250 µl of 96-100% ethanol was added to the RNA containing lysate and mixed thoroughly by pipetting up and down to adjust the RNA binding conditions. The RNA containing samples were transferred onto the RNA-RTA Spin Filter, incubated for 1 min and centrifuged at 11.000 rpm for 2 min. The flow-through was decanted and the RTA Receiver Tube was reused. Thereupon, the samples were washed using 600 µl wash buffer R1 and 700 µl wash buffer R2. After each washing step, the samples were centrifuged for 1 minute at 11.000 rpm. In the first washing step, the flow-through was decanted and the RNA-RTA Spin Filter was placed in a new RTA receiver tube. However, in the second washing step the RTA receiver tube was reused. The wash buffer R2 step was repeated.

The RNA-RTA Spin Filter was transferred into RNase-free Elution Tube and 40 - 100 µl of Elution Buffer R was pipetted directly onto the membrane of the RNA-RTA Spin Filter. The sample was Incubated for 2 min and centrifuged for 1 min at 11.000 rpm. The RNA-RTA Spin Filter was discarded and the eluted total RNA was placed immediately on ice.

4.5.3 Complementary DNA synthesis

The total RNA in all of the samples was converted into complementary DNA (cDNA) using iScript^{tm} cDNA synthesis kit (Bio-Rad Laboratories Inc.). The cocktail for the reaction was made in 1.5 ml eppendorf tube. The kit can only run 25 reactions, thus: the total volume of the cocktail was 250 µL, adequate for running 25 reactions. Consequently, the cocktail tube comprised of 100 µL of 5x iScript Reaction Mix, 25 µL of iScript Reverse Transcriptase and 125 µL of nuclease free water.

The total number of experimental reactions were 20, because of the limited number of reactions that can be run by the kit. Each treatment had 6 reactions in total, 3 for the target gene and 3 for the reference gene. On the other hand, the untreated sample had 2 reactions, 1 for the target

gene and 1 for the housekeeping gene. Initially, 10 µL of the cocktail was allocated in 20 wells in the 96 well plate, followed by adding 10 µL of the RNA template of each treatment to their respective wells. The total volume of each reaction was 20 µL. Next, the plate was sealed with a plate sealer to prevent contamination. Subsequently, the complete mix was incubated in RT-PCR (Bio-Rad Laboratories Inc.), according to manufacturer's instructions (Table 4-1).

Table 4-1: RT-PCR conditions for cDNA synthesis.

Cycling step	**Time and temperature**
Priming	5 minutes at 25ºC
Reverse Transcription	20 minutes at 46 ºC
Real Time (RT) inactivation	1 minute at 95 ºC

4.5.4 Primers

The oligonucleotide primers for the *Cg*CDR1 gene and actin1 (ACT1) reference gene (Inqaba Biotechnical Industries, Pretoria, SA), are listed in Table 4-2. Beforehand, the primers were blasted using National Center for Biotechnology Information (NCBI), to check their specificity. Next, they were diluted according to the manufacturer's instructions to 100 nM stock solution, using sterile water of 7.4 pH. Thereafter, they were further diluted with nuclease free water, to get the desired 500 nM concentration for the experiment.

Table 4-2: Forward and reverse primers of *Cg*CDR1 and ACT1.

Gene of interest	**Forward primer**	**Reverse primer**
***Cg*CDR 1** (Fonseca *et al*., 2014)	TTGTTGGTGTTCCTGGTGAA	ATGGACCATGCTGTTTGTGA
ACT 1 (Teste *et al*., 2009)	ATTATATGTTTAGAGGTTGCTGCTTTGG	CAATTCGTTGTAGAAGGTATGATGCC

4.5.5 Real-Time PCR

RT-PCR (CFX96 Real-Time PCR System; Bio-Rad), was used to determine the relative levels of *Cg*CDR1 transcripts in the samples, with ACT1 used as a reference candidal housekeeping

gene. SsoFast^{tm} EvaGreen® supermix (Bio-Rad, Laboratories Inc.), was used to record that amplification of the genes.

All components of SsoFast^{tm} EvaGreen® supermix was thawed at room temperature, mixed thoroughly by inverting the tube several times and centrifuged. Afterwards, two cocktails were made, one for the target gene and the other for the housekeeping gene. Accordingly, each cocktail comprised of 500 µL SsoFast^{tm} EvaGreen® supermix, 100 µL of forward primer, 100 µL of reverse primer and 200 µL of RNase free water. Next, 18 µL was transferred from the target gene cocktail and dispensed into 30 wells. Also, 18 µL was transferred from the reference gene cocktail and distributed into another 30 wells. Afterwards, 2 µL of cDNA template of each treatment was allocated into their corresponding target gene and reference gene wells. Next, 10 µL of 2.3 ng/µL standard gene, was aliquoted into a well containing 10 µL of SsoFast^{tm} EvaGreen® supermix. The total volume of each reaction was 20 µL. The standard gene was used to standardize the reaction. Negative controls (water), were included in the run. Thereupon, the plate was sealed with a plate sealer to prevent contamination.

The conditions of RT-PCR consisted of an initial cycle for enzyme activation at 95°C for 30 seconds. Followed by 40 cycles of denaturation and annealing at 95°C for 5 seconds and 60°C for 10 seconds, respectively (Table 4-3). The capturing of fluorescence was performed at the end of each cycle immediately following the denaturation and annealing step. All expression levels were normalized to *ACT1*. The relative quantification of gene expression was performed by RT-PCR following the Pfaffl method (Pfaffl 2001)

Table 4-3: The cycling conditions for RT-PCR.

cDNA			**Genomic DNA**		
Cycling step	**Temperature**	**Time**	**Cycles**	**Temperature**	**Time**
Enzyme activation	95-98ºC	30 seconds	1	98ºC	2 minutes
Denaturation	95-98ºC	1-5 seconds	30-40	98ºC	1-5 seconds
Annealing/Extension	60-65ºC	1-5 seconds		60-65ºC	1-5 seconds
Melt curve	65-95ºC	2-5 seconds/step	1	65-95ºC	2-5 seconds/step

Chapter 5: Data processing and analysis

The results were captured, then transferred to an Excel spreadsheet (Microsoft Corporation 2010, USA), to evaluate the effect of the treatments on the growth of *C. glabrata*. The data were expressed in Log CFU/mL and then analyzed using IBM SPSS® statistical software (version 25, IBM, USA).

The effects of the treatments were evaluated by assessing the growth of *C. glabrata* using non-parametric methods, specifically, descriptive statistics and Kruskal-Wallis one-way ANOVA test. The descriptive statistics was used to describe and summarize the data providing us with the mean and standard deviation for the growth of *C. glabrata* of each treatment per time. Kruskal-Wallis one-way ANOVA test was used to compare between the effects of the treatments on the growth of *C. glabrata*.

All statistical analysis for the gene expression were generated using CFX Maestro® software (Bio-Rad, Laboratories Inc.).

Chapter 6: Ethical approval

Ethical approval from the Biomedical Research Ethics Committee of the University of the Western Cape was obtained to conduct the research, according to a memorandum of understanding and ethical consideration in South Africa.

This was a laboratory-based study, there was no use of any human tissue during the experiment. The study was conducted in the Dental Research Laboratory (DRL) at the Faculty of Dentistry, University of the Western Cape, Tygerberg campus.

6.1 Conflict of interest

No conflict of interest. The providers of the materials had no participation in the study.

Chapter 7: Results

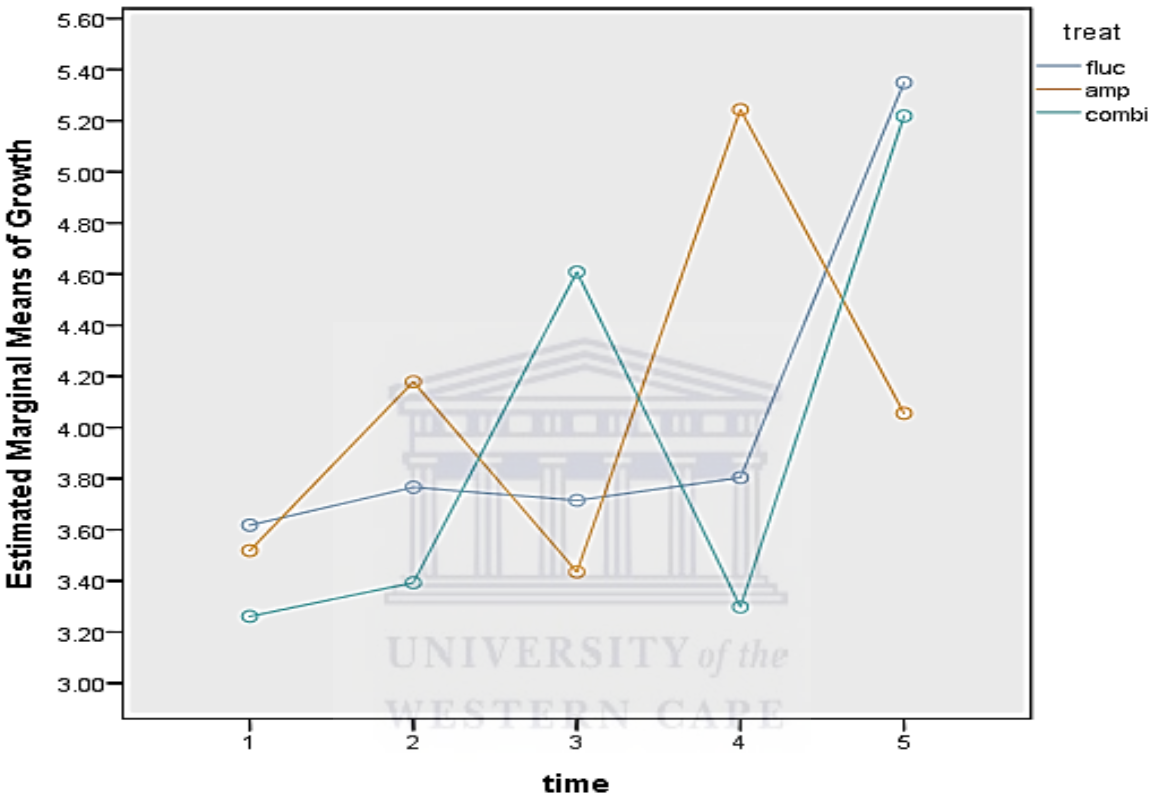
7.1 Effect of fluconazole, amphotericin B and combination therapy on the growth of *C. glabrata*

The effects of fluconazole, amphotericin B and the combination therapy on the growth of *C. glabrata* at each time interval were tested mutually and against the control conditions. The mean and standard deviation for the growth of *C. glabrata* on exposure to fluconazole per time is shown in Table 7-1.

Table 7-1: The descriptive statistics for the growth of *C. glabrata* when exposed to fluconazole over 24 hours

Growth Log_{10} (CFU/ml)					
Treatment	**Time**	**Mean**	**Std. Deviation**	**Minimum**	**Maximum**
Fluconazole	**30min**	3.5965	.35924	2.60	4.32
	2hrs	3.7646	.22047	3.08	4.11
	4hrs	3.7143	.31640	3.20	4.48
	6hrs	3.8422	.27801	3.20	4.35
	24hrs	5.4099	.49117	3.20	5.98
Amphotericin B	**30min**	3.5137	.46232	2.60	4.46
	2hrs	4.1777	.21261	3.68	4.65
	4hrs	3.4577	.19890	3.08	3.75
	6hrs	5.2683	.19655	4.88	5.63
	24hrs	3.9844	.80194	2.90	5.45
Combination therapy	**30min**	3.3644	.38151	2.60	4.05
	2hrs	3.3580	.29329	2.90	4.05
	4hrs	4.4705	.33858	3.68	4.85
	6hrs	3.3247	.30636	2.90	3.81
	24hrs	5.2172	.09222	5.03	5.41
Control	**30min**	6.2110	.19950	5.80	6.38
	2hrs	6.2110	.19950	5.80	6.38
	4hrs	6.2110	.19950	5.80	6.38
	6hrs	6.2110	.19950	5.80	6.38
	24hrs	6.2110	.19950	5.80	6.38

The mean growth of *C. glabrata* when exposed to fluconazole, amphotericin B and the combination therapy per time are presented (Figure 7- 1). The figure shows a paradoxical pattern of growth on exposure of *C. glabrata* to amphotericin B over time.



T1: 30min, **T2**: 2hrs, **T3**: 4hrs, **T4**: 6hrs, **T5**: 24hrs.
fluc: Fluconazole, **amp**: Amphotericin B, **combi**: Combination

Figure 7- 1: The growth of *C. glabrata* when exposed to fluconazole, amphotericin B and both medicines combined over time.

At 24 hours of exposure to fluconazole, amphotericin B and both medicines, the growth of *C. glabrata* appears more settled. The boxplot below shows how the three treatments compare at 24 hours and how they relate to no treatment (control) condition. Although the growth of *C. glabrata* seems most restricted by amphotericin B, the relative length of the box suggests less consistency than e.g. fluconazole (Figure 7- 2). The cases outside the whiskers are extremes.

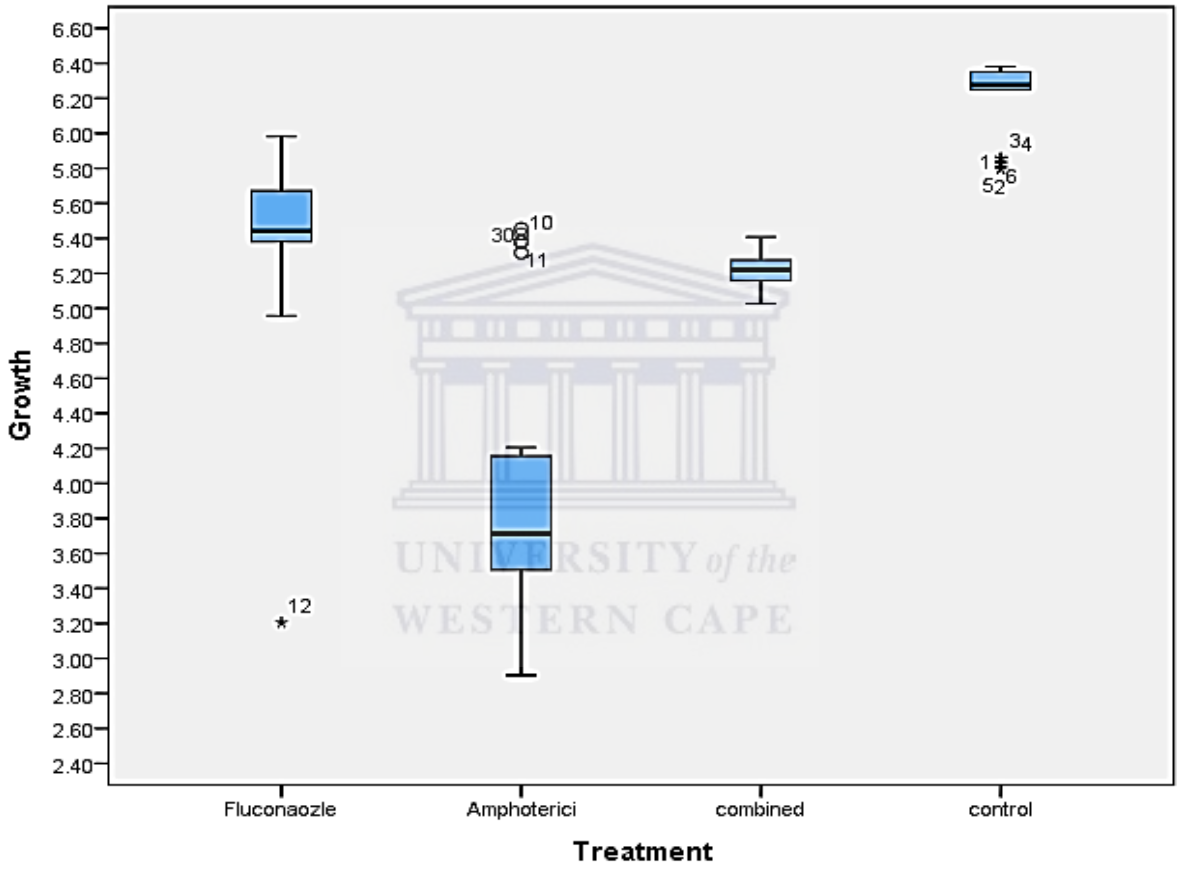


Figure 7- 2: Boxplot of the growth of *C. glabrata* when exposed to fluconazole, amphotericin B and both medicines combined and when not exposed to an antifungal.

A Kruskal-Wallis one-way ANOVA by ranks was used to test the differences between the treatments mutually and to the no treatment (control). Pairwise comparison of *C. glabrata* growth between the treatments and the growth of the control at 24hrs is shown in Table 7-2.

Table 7-2: Pairwise comparison between the treatments effect on the growths of *C. glabrata* and the control at 24hrs using a non-parametric test.

Pairwise Comparisons of Treatment					
Sample 1-Sample 2	**Test Statistic**	**Std. Error**	**Std. Test Statistic**	**Sig.**	**Adj. Sig.**
Amphotericin B-Combined	-18.804	8.911	-2.110	.035	.209
Amphotericin B-Fluconazole	41.329	8.985	4.600	.000	**.000**
Amphotericin B-Control	-77.954	8.911	-8.748	.000	**.000**
Combined-Fluconazole	22.526	8.831	2.551	.011	.065
Combined-Control	-59.150	8.756	-6.755	.000	**.000**
Fluconazole-Control	-36.624	8.831	-4.147	.000	**.000**
Each row tests the null hypothesis, that the Sample 1 and Sample 2 distributions are the same. Asymptotic significances (2-sided tests) are displayed. The significance level is .05.					

7.2 *Cg*CDR1 gene expression following treatment with Fluconazole, Amphotericin B and the combination therapy

The expression of *Cg*CDR1 was evaluated using relative quantitation method, calculated using control samples and reference targets. The data was analyzed using the normalized expression ($\Delta\Delta C_t$) test. The mean and standard error of the C_t of the samples that showed expression of the target gene is shown in Table 7-3.

Table 7-3: Shows the mean and standard error of the C_t of the samples that showed expression of the target gene.

Mean and Standard error Ct of the target gene			
Target	**Sample**	**Mean C_t**	**C_t SEM**
*Cg*CDR1	Fluconazole	28.18	4.16954
*Cg*CDR1	Combination	26.85	3.65440
*Cg*CDR1	Untreated	29.76	0.72876

A difference in the expression of *Cg*CDR1 among the samples was apparent. The samples treated with the combination therapy showed early expression of the target gene. While the samples treated with the Fluconazole showed late expression of the gene as compared to the combination (Figure 7-3).

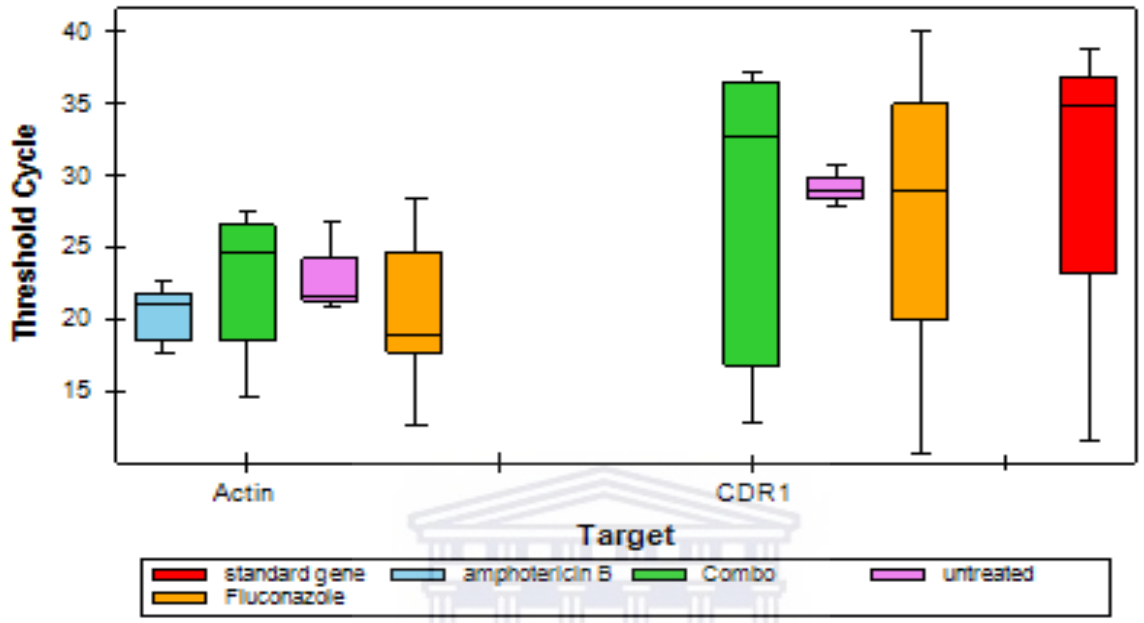


Figure 7-3: Box and Whisker plots demonstrates the median, distribution, maximum and minimum values of the *Cg*CDR1 and ACT1 gene expression in the samples.

The dot plot is used to show the number of samples per each treatment that exhibited expression of the target gene. 10 samples treated with the combination therapy showed expression of *Cg*CDR1, 6 samples treated with fluconazole and 3 untreated samples, demonstrated expression of the target gene. Also, Amphotericin B treated samples demonstrated no expression of the target gene. The number of samples expressing the target gene per each treatment is shown in (Figure 7-4).

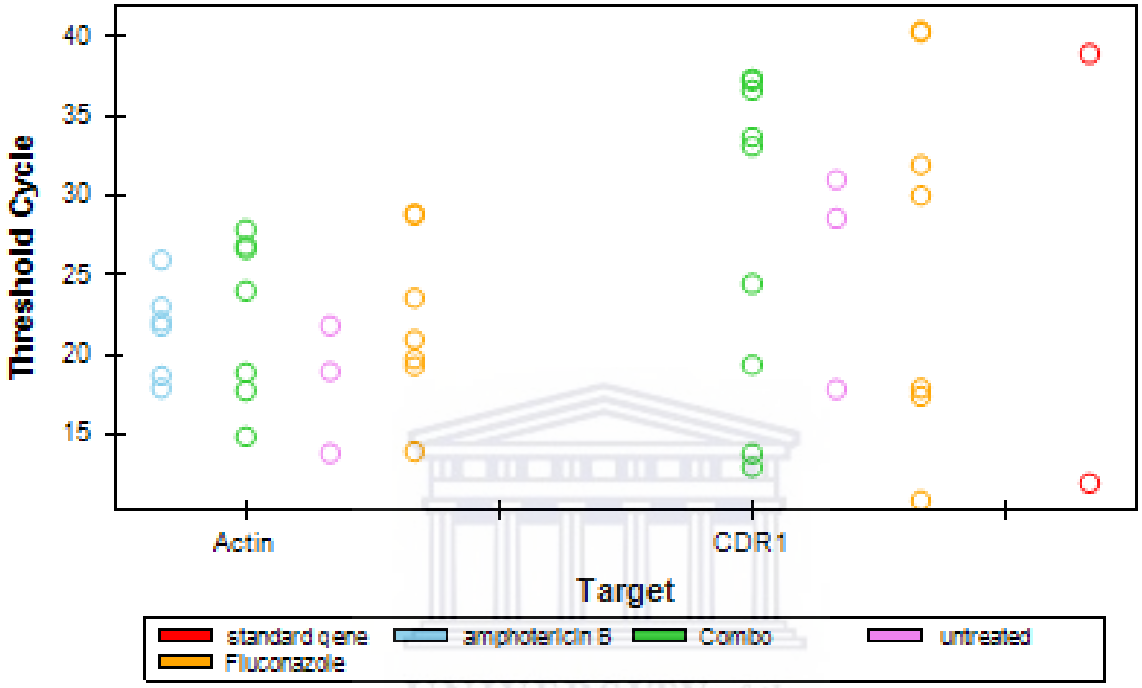


Figure 7-4: Dot plot demonstrates the samples that showed expression of the target and reference gene in each treatment.

Chapter 8: Discussion

The combination therapy was effective against *C. glabrata*, with a mean of growth lower than that of the control sample. However, the assessment of *Cg*CDR1 gene expression at 6 hours showed development of resistance to the combination therapy in the remaining cells of *C. glabrata*.

C. glabrata struggled at first to adapt to the environment, as indicated by the decline in mean Log CFU/mL at 30 minutes across the treatments, with the combination therapy being significantly more effective than fluconazole and amphotericin B alone (Figure 7- 1). This could be accredited to the different mechanisms of action of each of the antifungal agents, and the different adapting responses of *C. glabrata* to the environment of each drug (Kołaczkowska and Kołaczkowski 2016).

The combination therapy showed a consistently significant effect on the growth of *C glabrata* over time, even though there was an increase in Log CFU from 30 minutes to 6 hours, followed by a profuse growth observed at 24 hours (Figure 7- 1). At the first 6 hours the yeast managed to adapt to the stressed environment created by the antifungal combination, yet it was susceptible to the treatment. However, after 6 hours, some persister cells became active and were less susceptible to the killing effects of the drug. This could be due to the development of resistance, i.e. the persister cells were able to develop resistance over time, to ensure survival. Fidel *et al*., (1999) related the development of resistance over time to the haploid state of *C. glabrata*, which allows *C. glabrata* to rapidly adapt to the environment and enhances genome mutation to develop resistance (Kołaczkowska and Kołaczkowski 2016).

Interestingly, when the effect of the combination was compared to that of amphotericin B alone, no significant difference was noted after 24 hours. In contrast, the effect of amphotericin B was statistically better when compared to that of fluconazole (**P** < 0.05) (Table 7-2). This suggests that amphotericin B was capable of adding strength to the combination after 6 hours, at the time when fluconazole was showing a decrease in strength as a monotherapy (Figure 7-1). Thus, the antifungal effect of the combination therapy seemed to have an additive effect. In clinical practice, by using half the volume of amphotericin B in the combination therapy, avoidance of the side effects can be achieved. Also, the body might contribute to the elimination of the infection.

Reports on the effect of fluconazole and amphotericin B combinations against *Candida* species, are contradictory. Some reported the effect to be additive (Rex *et al*., 2003), while in other studies indifferent results were seen (Bachmann *et al*., 2003). Also, variability of the results within the same study was demonstrated by Lewis *et al*., (2002), who showed that exposure of *C. glabrata* to the combination of fluconazole and amphotericin B was indifferent by T-test and time kill method and additive by chequerboard method. These broad dissimilarities of results in studies of combination therapy, can often be ascribed to *candida* strain differences, medicine concentrations, method of evaluating the interaction between the drugs, and the standards used for interpretation. This emphasized the need for universal guidelines for testing of antifungal combinations before results from these studies can be widely correlated (Mukherjee *et al*., 2005).

To the best of our knowledge, this is the first study reporting on the effect of a combined fluconazole and amphotericin B on *Cg*CDR1 gene expression. It was interesting to observe that *Cg*CDR1 gene was expressed in 100% of the samples treated with the combination therapy, and 60% of the samples treated with fluconazole, while the samples treated with amphotericin B showed no expression of the target gene (Figure 7-4). Furthermore, the mean C_t (26.85) indicated that the target gene was expressed earlier in the samples treated with the combination than that treated with fluconazole only (28.18) (Table 7-3).

We suggest that at 6 hours, when the growth was at minimum, the dormant persister cells of *C. glabrata* survived and developed resistance in the combination therapy, through the expression of *Cg*CDR1 gene. Furthermore, we think that the steep decline of growth at 6 hours (Figure 7-1), could be due to *C. glabrata* having sacrificed most of its active cells to the combination therapy, to allow the persister cells to survive. Hence, we believe that the survival of the persister cells might have been due to any or all of the followings; emergence of resistance, presence of lesser number of competitors for nutrients, and/or evasion of other encountered stresses. This suggestion was supported by Nierman *et al*., (2015), their study indicated that the presence and survival of persister cells is not solely attributed to antifungal treatment, and can be attained by other stresses. Moreover, it could be that *C. glabrata* escaped the killing effect of amphotericin B by developing persister cells, which later resisted the effect of fluconazole by expressing *Cg*CDR1 gene. The interplay between persistence and resistance need further investigation. Is it possible that herein, the persistence of *C. glabrata* was the initial step towards resistance, or were these two mechanisms totally unrelated? Cohen *et al*.,

(2013), proposed that the persister cells can act as a reservoir of surviving cells from which resistant strains can develop. Furthermore, the stress responses that are active in persister cells are known to induce mutations, thus, could accelerate the development of resistant mutants (Cohen *et al*., 2013).

Gygax *et al*., (2011), reported that the higher the dose of fluconazole used to eradicate *C. glabrata* the higher is the expression of *Cg*CDR1. Subsequently, we developed the concept of relating the growth of *C. glabrata* to the level of expression of *Cg*CDR1 gene.

Similar to Gygax *et al*., (2011), herein, the ratio of fluconazole to persister cells (fluconazole: persister cells) increased with the decrease in the number of persister cells in the combination therapy, as compared to that of fluconazole monotherapy (6 hours; Figure 7- 1). Which means, the ratio of fluconazole concentration to the number of persister cells was low in the samples treated with fluconazole only, thus, low expression of the *Cg*CDR1 was evident. On the other hand, the ratio of fluconazole concentration to the number of persister cells was high in the sample treated with the combination therapy, hence, high expression of the *Cg*CDR1 was manifested.

In addition, the steepness of the growth slope from 6 hours to 24 hours (Figure 7- 1), could indicate that the persister cells of the combination therapy exhibited higher growth rate than that in fluconazole monotherapy. Keren *et al*., (2004), reported that the persister cells are commonly in a less active state, and manifest a slow rate of growth. Thus, we attributed this steepness of the growth slope to the development of resistance, through the expression of *Cg*CDR1. Also, the emergence of resistance allowed the persister colonies to replicate at a higher rate after 6 hours. This explains the profuse growth of cells observed at 24 hours. However, further studies are needed to link persistence with resistance and investigate the interplay between these two mechanisms. Subsequently, we concluded that, the presence of persister colonies in the samples, increased the possibility of *Cg*CDR1 gene expression, and the development of resistance led to increase in the growth rate afterwards.

The clinical importance of these findings is that the dose of fluconazole, whether used as a monotherapy or in a combination therapy, should be monitored to avoid the development of *C. glabrata* resistant strains.

Chapter 9: Conclusion, limitation and recommendation

9.1 Conclusion

The combination therapy showed significant reduction in the colony forming unit of *C. glabrata* especially at the first 6 hours, thus, it can be considered a plausible candidate to control *C. glabrata* growth.

The pronounced level of *Cg*CDR1 gene expression and the growth rate of *C. glabrata*, indicated that the yeast might have developed more resistance to fluconazole in the combination therapy than in fluconazole monotherapy, which allowed the yeast to proliferate at a higher rate after 6 hours. Hence, the development of resistance in the persister cells was considered to play a major role in the survival of *C. glabrata* in the combination therapy. However, the interaction between these two mechanisms needs further investigations.

9.2 Limitation

i. This is an *in vitro* study, where the conditions are controlled, thus, an *in vivo* study is needed.

ii. The concentrations of the drugs were fixed. Perhaps the results might differ with different concentrations of the drugs.

iii. The study was conducted with limited resources number of reagents for gene expression. Also, other genes need to be assessed.

9.3 Recommendations

i. Different concentrations of fluconazole and amphotericin B is recommended to be tested to further assess the efficacy of this combination.

ii. The mechanism of action between fluconazole and amphotericin B needs further investigations.

iii. The effect of the combination therapy on other resistance genes is an area that requires further investigations.

iv. The effect of the medications on the persister cells requires further investigations.

10. Reference

Abbes, S., Sellami, H., Michel-Nguyen, A., Ayadi, A. and Ranque, S., 2013. Interactions between copy number and expression level of genes involved in fluconazole resistance in *Candida glabrata*. *Frontiers in cellular and infection microbiology*, *3*, p.74.

Adler- Moore, J.P. and Proffitt, R.T., 2008. Amphotericin B lipid preparations: what are the differences? *Clinical Microbiology and Infection*, 14, pp.25-36.

Alexander, B.D., Johnson, M.D., Pfeiffer, C.D., Jiménez-Ortigosa, C., Catania, J., Booker, R., Castanheira, M., Messer, S.A., Perlin, D.S. and Pfaller, M.A., 2013. Increasing echinocandin resistance in *Candida glabrata*: clinical failure correlates with presence of FKS mutations and elevated minimum inhibitory concentrations. *Clinical infectious diseases*, *56*(12), pp.1724-1732.

Alves, I.A., Bandeira, L.A., Mario, D.A.N., Denardi, L.B., Neves, L.V., Santurio, J.M. and Alves, S.H., 2012. Effects of antifungal agents alone and in combination against *Candida glabrata* strains susceptible or resistant to fluconazole. *Mycopathologia*, *174*(3), pp.215-221.

Andrew, B., 2003. More common opportunistic infections; their symptoms, diagnosis, prophylaxis and treatment. Positive (+ ve), pp.1-10.

Azie, N., Neofytos, D., Pfaller, M., Meier-Kriesche, H.U., Quan, S.P. and Horn, D., 2012. The PATH (Prospective Antifungal Therapy) Alliance® registry and invasive fungal infections: update 2012. *Diagnostic microbiology and infectious disease*, *73*(4), pp.293-300.

Bachmann, S.P., Ramage, G., VandeWalle, K., Patterson, T.F., Wickes, B.L. and López-Ribot, J.L., 2003. Antifungal combinations against *Candida albicans* biofilms *in vitro*. *Antimicrobial agents and chemotherapy*, 47(11), pp.3657-3659.

Bagg, J., Sweeney, M.P., Lewis, M.A., Jackson, M.S., Coleman, D., Mosaid, A.A., Baxter, W., McEndrick, S. and McHugh, S., 2003. High prevalence of *non-albicans yeasts* and detection of anti-fungal resistance in the oral flora of patients with advanced cancer. *Palliative medicine*, *17*(6), pp.477-481.

Bennett, J.E., Izumikawa, K. and Marr, K.A., 2004. Mechanism of increased fluconazole resistance in *Candida glabrata* during prophylaxis. *Antimicrobial agents and chemotherapy*, *48*(5), pp.1773-1777.

Bolard, J. and Milhaud, J., 1996. Interaction of the anti-*Candida* amphotericin B (and other polyene antibiotics) with lipids. *Lipids of pathogenic fungi. CRC Press, Inc., Boca Raton, Fla*, pp.253-274.

Bouchara, J.P., Zouhair, R., Le Boudouil, S., Renier, G., Filmon, R., Chabasse, D., Hallet, J.N. and Defontaine, A., 2000. *In-vivo* selection of an azole-resistant petite mutant of *Candida glabrata*. *Journal of medical microbiology*, *49*(11), pp.977-984.

Brauner, A., Fridman, O., Gefen, O. and Balaban, N.Q., 2016. Distinguishing between resistance, tolerance and persistence to antibiotic treatment. *Nature Reviews Microbiology*, *14*(5), p.320.

Brun, S., Bergès, T., Poupard, P., Vauzelle-Moreau, C., Renier, G., Chabasse, D. and Bouchara, J.P., 2004. Mechanisms of azole resistance in petite mutants of *Candida glabrata*. *Antimicrobial agents and chemotherapy*, *48*(5), pp.1788-1796.

Bumgarner, R., 2013. Overview of DNA microarrays: types, applications, and their future. *Current protocols in molecular biology*, *101*(1), pp.22-1.

Byadarahally Raju, S. and Rajappa, S., 2011. Isolation and identification of *Candida* from the oral cavity. *ISRN dentistry*, *2011*.

Canuto, M.M., Rodero, F.G., de la Tabla Ducasse, V.O., Aguado, I.H., Gonzalez, C.M., Sevillano, A.S. and Hidalgo, A.M., 2000. Determinants for the development of oropharyngeal colonization or infection by fluconazole-resistant *Candida* strains in HIV-infected patients. *European Journal of Clinical Microbiology and Infectious Diseases*, *19*(8), pp.593-601.

Chapeland-Leclerc, F., Hennequin, C., Papon, N., Noël, T., Girard, A., Socié, G., Ribaud, P. and Lacroix, C., 2010. Acquisition of flucytosine, azole, and caspofungin resistance in *Candida glabrata* bloodstream isolates serially obtained from a hematopoietic stem cell transplant recipient. *Antimicrobial agents and chemotherapy*, *54*(3), pp.1360-1362.

Chapman, S.W., Sullivan, D.C. and Cleary, J.D., 2008. In search of the holy grail of antifungal therapy. Transactions of the American Clinical and Climatological Association, 119, p.197.

Clinical Laboratory Standards Institute (CLSI), 2008. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeast; Approved Standard-Third Edition. CLSI document M27-A3.

Coco, B.J., Bagg, J., Cross, L.J., Jose, A., Cross, J. and Ramage, G., 2008. Mixed *Candida albicans* and *Candida glabrata* populations associated with the pathogenesis of denture stomatitis. *Molecular Oral Microbiology*, *23*(5), pp.377-383.

Cohen, N.R., Lobritz, M.A. and Collins, J.J., 2013. Microbial persistence and the road to drug resistance. *Cell host & microbe*, *13*(6), pp.632-642.

Davies, A.N., Brailsford, S.R. and Beighton, D., 2006. Oral candidosis in patients with advanced cancer. *Oral oncology*, *42*(7), pp.698-702.

Denardi, L.B., Mario, D.A.N., de Loreto, É.S., Nogueira, C.W., Santurio, J.M. and Alves, S.H., 2013. Antifungal activities of diphenyl diselenide alone and in combination with fluconazole or amphotericin B against *Candida glabrata*. *Mycopathologia*, *176*(1-2), pp.165-169.

Dewsnup, D.H. and Stevens, D.A., 1994. Efficacy of oral amphotericin B in AIDS patients with thrush clinically resistant to fluconazole. *Journal of medical and veterinary mycology*, 32(5), pp.389-393.

Edition, A.S.N., 2012. CLSI document M07-A9. *Wayne, PA: Clinical and Laboratory Standards Institute*.

Enwuru, C.A., Ogunledun, A., Idika, N., Enwuru, N.V., Ogbonna, E., Aniedobe, M. and Adeiga, A., 2008. Fluconazole resistant opportunistic oro-pharyngeal *candida* and non-*candida* yeast-like isolates from HIV infected patients attending ARV clinics in Lagos, Nigeria. *African health sciences*, 8(3), pp.142-148.

Epstein, J.B., 1990. Antifungal therapy in oropharyngeal mycotic infections. *Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology*, *69*(1), pp.32-41.

Ferrari, S., Ischer, F., Calabrese, D., Posteraro, B., Sanguinetti, M., Fadda, G., Rohde, B., Bauser, C., Bader, O. and Sanglard, D., 2009. Gain of function mutations in CgPDR1 of *Candida glabrata* not only mediate antifungal resistance but also enhance virulence. *PLoS pathogens*, *5*(1), p.e1000268.

Ferrari, S., Sanguinetti, M., Torelli, R., Posteraro, B. and Sanglard, D., 2011. Contribution of *Cg*PDR1-regulated genes in enhanced virulence of azole-resistant *Candida glabrata*. *PloS one*, *6*(3), p.e17589.

Fidel, P.L., Vazquez, J.A. and Sobel, J.D., 1999. *Candida glabrata*: review of epidemiology, pathogenesis, and clinical disease with comparison to *C. albicans*. *Clinical microbiology reviews*, *12*(1), pp.80-96.

Fonseca, E., Silva, S., Rodrigues, C.F., Alves, C.T., Azeredo, J. and Henriques, M., 2014. Effects of fluconazole on *Candida glabrata* biofilms and its relationship with ABC transporter gene expression. *Biofouling*, *30*(4), pp.447-457.

Force, R.W. and Nahata, M.C., 1995. Salivary concentrations of ketoconazole and fluconazole: implications for drug efficacy in oropharyngeal and esophageal candidiasis. *Annals of Pharmacotherapy*, 29(1), pp.10-15.

Fryer, R.M., Randall, J., Yoshida, T., Hsiao, L.L., Blumenstock, J., Jensen, K.E., Dimofte, T., Jensen, R.V. and Gullans, S.R., 2002. Global analysis of gene expression: methods, interpretation, and pitfalls. *Nephron Experimental Nephrology*, *10*(2), pp.64-74.

Ghannoum, M.A., Fu, Y., Ibrahim, A.S., Mortara, L.A., Shafiq, M.C., Edwards, J.E. and Criddle, R.S., 1995. *In vitro* determination of optimal antifungal combinations against *Cryptococcus neoformans* and *Candida albicans*. *Antimicrobial agents and chemotherapy*, 39(11), pp.2459-2465.

Grim, S.A., Smith, K.M., Romanelli, F. and Ofotokun, I., 2002. Treatment of azole-resistant oropharyngeal candidiasis with topical amphotericin B. *Annals of Pharmacotherapy*, 36(9), pp.1383-1386.

Gygax, S.E., Vermitsky, J.P., Chadwick, S.G., Self, M.J., Mordechai, E. and Adelson, M.E., 2011. Method for determining azole resistance in *Candida glabrata*. *Medical Diagnostic Laboratories LLC. U.S. Patent* 8,021,846.

Hamill, R.J., 2013. Amphotericin B formulations: a comparative review of efficacy and toxicity. *Drugs*, 73(9), pp.919-934.

Johnson, M.D., MacDougall, C., Ostrosky-Zeichner, L., Perfect, J.R. and Rex, J.H., 2004. Combination antifungal therapy. *Antimicrobial agents and chemotherapy*, 48(3), pp.693-715.

Kadir, T., Pisiriciler, R., Akyüz, S., Yarat, A., Emekli, N. and Ipbüker, A., 2002. Mycological and cytological examination of oral candidal carriage in diabetic patients and non-diabetic control subjects: thorough analysis of local aetiologic and systemic factors. *Journal of oral rehabilitation*, *29*(5), pp.452-457.

Keren, I., Kaldalu, N., Spoering, A., Wang, Y. and Lewis, K., 2004. Persister cells and tolerance to antimicrobials. *FEMS microbiology letters*, *230*(1), pp.13-18.

Kiraz, N., Dag, I., Oz, Y., Yamac, M., Kiremitci, A. and Kasifoglu, N., 2010. Correlation between broth microdilution and disk diffusion methods for antifungal susceptibility testing of caspofungin, voriconazole, amphotericin B, itraconazole and fluconazole against *Candida glabrata*. *Journal of microbiological methods*, *82*(2), pp.136-140.

Kołaczkowska, A. and Kołaczkowski, M., 2016. Drug resistance mechanisms and their regulation in *non-albicans Candida* species. *Journal of Antimicrobial Chemotherapy*, *71*(6), pp.1438-1450.

Krogh-Madsen, M., Arendrup, M.C., Heslet, L. and Knudsen, J.D., 2006. Amphotericin B and caspofungin resistance in *Candida glabrata* isolates recovered from a critically ill patient. *Clinical infectious diseases*, *42*(7), pp.938-944.

Larsen, R.A., Bauer, M., Thomas, A.M. and Graybill, J.R., 2004. Amphotericin B and fluconazole, a potent combination therapy for cryptococcal meningitis. *Antimicrobial agents and chemotherapy*, *48*(3), pp.985-991.

Lewis, K., 2010. Persister cells. *Annual review of microbiology*, *64*, pp.357-372.

Lewis, R.E., Diekema, D.J., Messer, S.A., Pfaller, M.A. and Klepser, M.E., 2002. Comparison of Etest, chequerboard dilution and time–kill studies for the detection of synergy or antagonism between antifungal agents tested against *Candida* species. *Journal of Antimicrobial Chemotherapy*, 49(2), pp.345-351.

Li, P., Seneviratne, C.J., Alpi, E., Vizcaino, J.A. and Jin, L., 2015. Delicate metabolic control and coordinated stress response critically determine antifungal tolerance of Candida albicans biofilm persisters. *Antimicrobial agents and chemotherapy*, *59*(10), pp.6101-6112.

Lockhart, S.R., Joly, S., Vargas, K., Swails-Wenger, J., Enger, L. and Soll, D.R., 1999. Natural defenses against *Candida* colonization breakdown in the oral cavities of the elderly. *Journal of dental research*, *78*(4), pp.857-868.

Lopez, J., Dalle, F., Mantelin, P., Moiroux, P., Nierlich, A.C., Pacot, A., Cuisenier, B., Vagner, O. and Bonnin, A., 2001. Rapid identification of *Candida glabrata* based on trehalose and sucrose assimilation using Rosco diagnostic tablets. *Journal of clinical microbiology*, *39*(3), pp.1172-1174.

Luo, G. and Samaranayake, L.P., 2002. *Candida glabrata*, an emerging fungal pathogen, exhibits superior relative cell surface hydrophobicity and adhesion to denture acrylic surfaces compared with *Candida albicans*. *Apmis*, *110*(9), pp.601-610.

Luo, G., Samaranayake, L.P., Cheung, B.P.K. and Tang, G., 2004. Reverse transcriptase polymerase chain reaction (RT-PCR) detection of HLP gene expression in *Candida glabrata* and its possible role in *in vitro* haemolysin production. *Apmis*, *112*(4-5), pp.283-290.

Marcos-Arias, C., Eraso, E., Madariaga, L., Aguirre, J.M. and Quindós, G., 2011. Phospholipase and proteinase activities of *Candida* isolates from denture wearers. *Mycoses*, *54*(4).

Miyazaki, H., Miyazaki, Y., Geber, A., Parkinson, T., Hitchcock, C., Falconer, D.J., Ward, D.J., Marsden, K. and Bennett, J.E., 1998. Fluconazole Resistance Associated with Drug Efflux and Increased Transcription of a Drug Transporter Gene, PDH1, in *Candida glabrata*. *Antimicrobial Agents and Chemotherapy*, *42*(7), pp.1695-1701.

Mukherjee, P.K., Sheehan, D.J., Hitchcock, C.A. and Ghannoum, M.A., 2005. Combination treatment of invasive fungal infections. *Clinical microbiology reviews*, 18(1), pp.163-194.

Mullis, K.B., 1990. The unusual origin of the polymerase chain reaction. *Scientific American*, *262*(4), pp.56-65.

Muzyka, B.C., 2005. Oral fungal infections. *Dental Clinics*, *49*(1), pp.49-65.

Nierman, W.C., Yu, Y. and Losada, L., 2015. The *in vitro* antibiotic tolerant persister population in Burkholderia pseudomallei is altered by environmental factors. *Frontiers in microbiology*, *6*, p.1338.

Nijs, A., Cartuyvels, R., Mewis, A., Peeters, V., Rummens, J.L. and Magerman, K., 2003. Comparison and evaluation of Osiris and Sirscan 2000 antimicrobial susceptibility systems in the clinical microbiology laboratory. *Journal of clinical microbiology*, *41*(8), pp.3627-3630.

Nikawa, H., Nishimura, H., Makihira, S., Hamada, T., Sadamori, S. and Samaranayake, L.P., 2000. Effect of serum concentration on *Candida* biofilm formation on acrylic surfaces. *Mycoses*, *43*(3-4), pp.139-143.

Niimi, M., Nagai, Y., Niimi, K., Wada, S.I., Cannon, R.D., Uehara, Y. and Monk, B.C., 2002. Identification of two proteins induced by exposure of the pathogenic fungus *Candida glabrata* to fluconazole. *Journal of Chromatography B*, *782*(1-2), pp.245-252.

Niimi, M., Firth, N.A. and Cannon, R.D., 2010. Antifungal drug resistance of oral fungi. *Odontology*, *98*(1), pp.15-25.

Parihar, S., 2011. Oral candidiasis-A review.

Pappas, P.G., Kauffman, C.A., Andes, D., Benjamin Jr, D.K., Calandra, T.F., Edwards Jr, J.E., Filler, S.G., Fisher, J.F., Kullberg, B.J., Ostrosky-Zeichner, L. and Reboli, A.C., 2009. Clinical practice guidelines for the management of candidiasis: 2009 update by the Infectious Diseases Society of America. *Clinical infectious diseases*, pp.503-535.

Paul, S., Schmidt, J.A. and Moye-Rowley, W.S., 2011. Regulation of the CgPdr1 transcription factor from the pathogen *Candida glabrata*. *Eukaryotic cell*, *10*(2), pp.187-197.

Pfaller, M.A. and Diekema, D.J., 2007. Epidemiology of invasive candidiasis: a persistent public health problem. *Clinical microbiology reviews*, *20*(1), pp.133-163.

Pfaller, M.A., Sheehan, D.J. and Rex, J.H., 2004. Determination of fungicidal activities against yeasts and molds: lessons learned from bactericidal testing and the need for standardization. *Clinical Microbiology Reviews*, *17*(2), pp.268-280.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic acids research*, 29(9), pp.e45-e45.

Prasad, R., Gaur, N.A., Gaur, M. and Komath, S.S., 2006. Efflux pumps in drug resistance of *Candida*. *Infectious Disorders-Drug Targets (Formerly Current Drug Targets-Infectious Disorders)*, *6*(2), pp.69-83.

Ramage, G., Rajendran, R., Sherry, L. and Williams, C., 2012. Fungal biofilm resistance. *International journal of microbiology*, *2012*.

Redding, S.W., Kirkpatrick, W.R., Dib, O., Fothergill, A.W., Rinaldi, M.G. and Patterson, T.F., 2000. The epidemiology of *non-albicans Candida* in oropharyngeal candidiasis in HIV patients. *Special Care in Dentistry*, *20*(5), pp.178-181.

Redding, S.W., Kirkpatrick, W.R., Coco, B.J., Sadkowski, L., Fothergill, A.W., Rinaldi, M.G., Eng, T.Y. and Patterson, T.F., 2002. *Candida glabrata* oropharyngeal candidiasis in patients receiving radiation treatment for head and neck cancer. *Journal of clinical microbiology*, *40*(5), pp.1879-1881.

Redding, S.W., Dahiya, M.C., Kirkpatrick, W.R., Coco, B.J., Patterson, T.F., Fothergill, A.W., Rinaldi, M.G. and Thomas, C.R., 2004. *Candida glabrata* is an emerging cause of oropharyngeal candidiasis in patients receiving radiation for head and neck cancer. *Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology*, *97*(1), pp.47-52.

Rex, J.H., Pappas, P.G., Karchmer, A.W., Sobel, J., Edwards, J.E., Hadley, S., Brass, C., Vazquez, J.A., Chapman, S.W., Horowitz, H.W. and Zervos, M., 2003. A randomized and blinded multicenter trial of high-dose fluconazole plus placebo versus fluconazole plus amphotericin B as therapy for candidemia and its consequences in nonneutropenic subjects. *Clinical infectious diseases*, 36(10), pp.1221-1228.

Rodrigues, C.F., Silva, S. and Henriques, M., 2014. *Candida glabrata*: a review of its features and resistance. *European journal of clinical microbiology & infectious diseases*, *33*(5), pp.673-688.

Rodrigues, C.F. and Henriques, M., 2017. Oral mucositis caused by *Candida glabrata* biofilms: failure of the concomitant use of fluconazole and ascorbic acid. *Therapeutic advances in infectious disease*, *4*(1), pp.10-17.

Rogers, P.D., Vermitsky, J.P., Edlind, T.D. and Hilliard, G.M., 2006. Proteomic analysis of experimentally induced azole resistance in *Candida glabrata*. *Journal of Antimicrobial Chemotherapy*, *58*(2), pp.434-438.

Samaranayake, L.P., Fidel, P.L., Sweet, S.P., Teanpaisan, R., Coogan, M.M., Blignaut, E. and Wanzala, P., 2002. Fungal infections associated with HIV infection. *Oral diseases*, *8*, pp.151-160.

Sanati, H., Belanger, P.H., Fratti, R.A. and Ghannoum, M.A., 1996. Effect of voriconazole (UK-109,496) on the sterol biosynthesis of *Candida albicans* and *Candida krusei*. In *36th Interscience Conference on Antimicrobial Agents and Chemotherapy, New Orleans, Abstract C* (Vol. 65).

Sanati, H., Ramos, C.F., Bayer, A.S. and Ghannoum, M.A., 1997. Combination therapy with amphotericin B and fluconazole against invasive candidiasis in neutropenic-mouse and infective-endocarditis rabbit models. *Antimicrobial agents and chemotherapy*, *41*(6), pp.1345-1348.

Sanglard, D., Ischer, F., Calabrese, D., Majcherczyk, P.A. and Bille, J., 1999. The ATP binding cassette transporter Gene CgCDR1 from *Candida glabrata* is involved in the resistance of clinical isolates to azole antifungal agents. *Antimicrobial agents and chemotherapy*, *43*(11), pp.2753-2765.

Sanglard, D., Ischer, F. and Bille, J., 2001. Role of ATP-binding-cassette transporter genes in high-frequency acquisition of resistance to azole antifungals in *Candida glabrata*. *Antimicrobial Agents and Chemotherapy*, *45*(4), pp.1174-1183.

Sanglard, D., 2002. Current understanding of the modes of action and resistance mechanisms to conventional and emerging antifungal agents for treatment of *Candida* infections. *Candida and candidiasis*.

Sanguinetti, M., Posteraro, B., Fiori, B., Ranno, S., Torelli, R. and Fadda, G., 2005. Mechanisms of azole resistance in clinical isolates of *Candida glabrata* collected during a hospital survey of antifungal resistance. *Antimicrobial agents and chemotherapy*, *49*(2), pp.668-679.

Shahrokhi, S., Noorbakhsh, F. and Rezaie, S., 2017. Quantification of CDR1 Gene Expression in Fluconazole Resistant *Candida Glabrata* Strains Using Real-time PCR. *Iranian journal of public health*, *46*(8), p.1118.

Shen, Y.Z., Lu, H.Z. and Zhang, Y.X., 2010. Molecular mechanisms of fluconazole resistance in clinical isolates of *Candida glabrata*. *Zhonghua nei ke za zhi*, *49*(3), pp.245-249.

Silva, S., Negri, M., Henriques, M., Oliveira, R., Williams, D.W. and Azeredo, J., 2012. *Candida glabrata, Candida parapsilosis* and *Candida tropicalis*: biology, epidemiology, pathogenicity and antifungal resistance. *FEMS microbiology reviews*, *36*(2), pp.288-305.

Sutton, S. (2011). Accuracy of plate counts. *Journal of Validation Technology*, 17(3): 42-46.

Suzuki, T., Higgins, P.J. and Crawford, D.R., 2000. Control selection for RNA quantitation. *Biotechniques*, *29*(2), pp.332-337.

Szweda, P., Gucwa, K., Romanowska, E., Dzierz, K., Naumiuk, Ł., Brillowska-Da, A., Wojciechowska-Koszko, I. and Milewski, S., 2015. Mechanisms of azole resistance among clinical isolates of *Candida glabrata* in Poland. *Journal of medical microbiology*, *64*(6), pp.610-619.

Tsai, H.F., Krol, A.A., Sarti, K.E. and Bennett, J.E., 2006. *Candida glabrata* PDR1, a transcriptional regulator of a pleiotropic drug resistance network, mediates azole resistance in clinical isolates and petite mutants. *Antimicrobial agents and chemotherapy*, *50*(4), pp.1384-1392.

Taff, H.T., Nett, J.E., Zarnowski, R., Ross, K.M., Sanchez, H., Cain, M.T., Hamaker, J., Mitchell, A.P. and Andes, D.R., 2012. A *Candida* biofilm-induced pathway for matrix glucan delivery: implications for drug resistance. *PLoS pathogens*, *8*(8), p.e1002848.

Teste, M.A., Duquenne, M., François, J.M. and Parrou, J.L., 2009. Validation of reference genes for quantitative expression analysis by real-time RT-PCR in Saccharomyces cerevisiae. *BMC molecular biology*, *10*(1), p.99.

Valasek, M.A. and Repa, J.J., 2005. The power of real-time PCR. *Advances in physiology education*, *29*(3), pp.151-159.

Vale-Silva, L.A. and Sanglard, D., 2015. Tipping the balance both ways: drug resistance and virulence in *Candida glabrata*. *FEMS yeast research*, *15*(4).

Vandeputte, P., Tronchin, G., Bergès, T., Hennequin, C., Chabasse, D. and Bouchara, J.P., 2007. Reduced susceptibility to polyenes associated with a missense mutation in the ERG6 gene in a clinical isolate of *Candida glabrata* with pseudohyphal growth. *Antimicrobial agents and chemotherapy*, *51*(3), pp.982-990.

Vazquez, J.A., 2007. Diagnosing and managing oropharyngeal candidiasis. *Infections in Medicine*, 24(10), pp.427-436.

Vermitsky, J.P., Earhart, K.D., Smith, W.L., Homayouni, R., Edlind, T.D. and Rogers, P.D., 2006. Pdr1 regulates multidrug resistance in *Candida glabrata*: gene disruption and genome-wide expression studies. *Molecular microbiology*, *61*(3), pp.704-722.

Wada, S. I., Niimi, M., Niimi, K., Holmes, A. R., Monk, B. C., Cannon, R. D., et al., 2002. *Candida glabrata* ATP-binding cassette transporters CDR1p and PDH1p expressed in a Saccharomyces cerevisiae strain deficient in membrane transporters show phosphorylation-dependent pumping properties. *J. Biol. Chem.* 277, 46809–46821. doi: 10.1074/jbc.M207817200

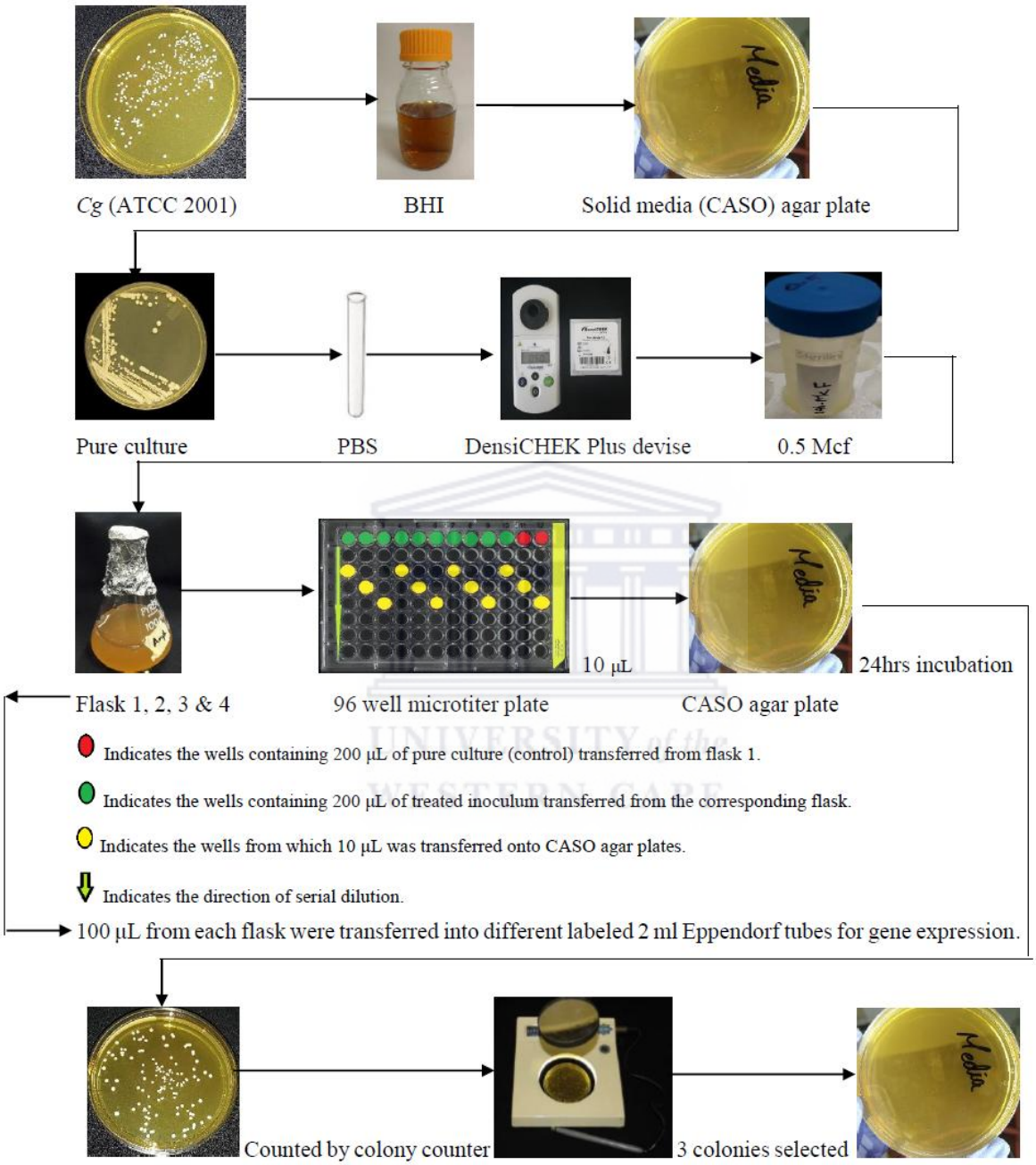
Williams, D.W. and Lewis, M.A.O., 2000. Oral Microbiology: Isolation and identification of *Candida* from the oral cavity. *Oral diseases*, *6*(1), pp.3-11.

Williams, D. and Lewis, M., 2011. Pathogenesis and treatment of oral candidosis. *Journal of oral microbiology*, *3*(1), p.5771.

Yoo, J.I., Choi, C.W., Lee, K.M. and Lee, Y.S., 2010. Gene expression and identification related to fluconazole resistance of *Candida glabrata* strains. *Osong public health and research perspectives*, *1*(1), pp.36-41.

11. Appendix

Appendix A: A diagram explaining the steps followed to run the experiment.



(30 CASO agar were used to grow the culture for each treatment and at each time interval.)

(9 CASO agar were used to grow the control culture at each time interval.)

- After counting, 3 colonies were selected and re-exposed to the treatment following the same procedure as the previous one.
- After the step of serial dilution, 3 plates were incubated for each treatment at each time interval.

Appendix B 1: Log10 CFU/ml of *C. glabrata* on re-exposure to fluconazole.

Growth of *Candida glabrata* when was re-exposed to Fluconaozle at 30 min in terms of Log10 (CFU/ml)		
Number of observation	CFU/ml	Log10(CFU/ml)
1	2500	3,40
2	2500	3,40
3	2500	3,40
Growth of *Candida glabrata* when exposed to Fluconaozle at 2 hours in terms of Log10 (CFU/ml)		
Number of observation	CFU/ml	Log10(CFU/ml)
1	3500	3,54
2	5500	3,74
3	6000	3,78
Growth of *Candida glabrata* when was re-exposed to Fluconaozle at 4 hours in terms of Log10 (CFU/ml)		
Number of observation	CFU/ml	Log10(CFU/ml)
1	20000	4,30
2	9500	3,98
3	26500	4,42
Growth of *Candida glabrata* when was re-exposed to Fluconaozle at 6 hours in terms of Log10 (CFU/ml)		
Number of observation	CFU/ml	Log10(CFU/ml)
1	13000	4,11
2	12500	4,10
3	8500	3,93
Growth of Candida glabrata when was re-exposed to Fluconaozle at 24 hours in terms of Log10 (CFU/ml)		
Number of observation	CFU/ml	Log10(CFU/ml)
1	100500	5,00
2	128500	5,11
3	121000	5,08

Appendix B 2: Log10 CFU/ml of *C. glabrata* on re-exposure to amphotericin B.

Growth of *Candida glabrata* when was re-exposed to amphotericin B at 30 minutes in terms of Log10 (CFU/ml)		
Number of observation	CFU/ml	Log10(CFU/ml)
1	3000	3,48
2	1500	3,18
3	4000	3,60
Growth of *Candida glabrata* when was re-exposed to amphotericin B at 2 hours in terms of Log10 (CFU/ml)		
Number of observation	CFU/ml	Log10(CFU/ml)
1	2000	3,30
2	2000	3,30
3	2000	3,30
Growth of *Candida glabrata* when was re-exposed to amphotericin B at 4 hours in terms of Log10 (CFU/ml)		
Number of observation	CFU/ml	Log10(CFU/ml)
1	11000	4,04
2	18000	4,26
3	17500	4,24
Growth of *Candida glabrata* when was re-exposed to amphotericin B at 6 hours in terms of Log10 (CFU/ml)		
Number of observation	CFU/ml	Log10(CFU/ml)
1	38500	4,59
2	12000	4,08
3	42000	4,62
Growth of *Candida glabrata* when was re-exposed to amphotericin B at 24 hours in terms of Log10 (CFU/ml)		
Number of observation	CFU/ml	Log10(CFU/ml)
1	150000	5,18
2	150000	5,18
3	150000	5,18

Appendix B 3: Log10 CFU/ml of *C. glabrata* on re-exposure to the combination therapy.

Growth of *Candida glabrata* when was re-exposed to the combination therapy at 30 mins in terms of Log10 (CFU/ml)		
Number of observation	CFU/ml	Log10(CFU/ml)
1	1500	3,18
2	4000	3,60
3	3000	3,48
Growth of *Candida glabrata* when was re-exposed to the combination therapy at 2 hrs in terms of Log10 (CFU/ml)		
Number of observation	CFU/ml	Log10(CFU/ml)
1	5500	3,74
2	6500	3,81
3	4000	3,60
Growth of *Candida glabrata* when exposed to the combination therapy at 4 hrs in terms of Log10 (CFU/ml)		
Number of observation	CFU/ml	Log10(CFU/ml)
1	4000	3,60
2	11000	4,04
3	8000	3,90
Growth of *Candida glabrata* when was re-exposed to the combination therapy at 6 hrs in terms of Log10 (CFU/ml)		
Number of observation	CFU/ml	Log10(CFU/ml)
1	17000	4,23
2	19000	4,28
3	7000	3,85
Growth of *Candida glabrata* when exposed to the combination therapy at 24 hrs in terms of Log10 (CFU/ml)		
Number of observation	CFU/ml	Log10(CFU/ml)
1	1500	3,18
2	1500	3,18
3	1500	3,18

Appendix B 4: Log10 CFU/ml of the control samples of *C. glabrata*.

Growth of control samples at 30 min of incubation		
Number of observation	CFU/ml	Log10(CFU/ml)
1	672000	5,83
2	627200	5,80
3	716800	5,86
Growth of control samples at 2 hours of incubation		
Number of observation	CFU/ml	Log10(CFU/ml)
6	1856000	6,27
7	1779200	6,25
8	1862400	6,27
Growth of control samples at 4 hours of incubation		
Number of observation	CFU/ml	Log10(CFU/ml)
11	2112000	6,32
12	2182400	6,34
13	2278400	6,36
Growth of control samples at 6 hours of incubation		
Number of observation	CFU/ml	Log10(CFU/ml)
16	2252800	6,35
17	2246400	6,35
18	1945600	6,29
Growth of control samples at 24 hours of incubation		
Number of observation	CFU/ml	Log10(CFU/ml)
21	2208000	6,34
22	2240000	6,35
23	2406400	6,38