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Candida species variability as seen through clinical covariates and drug susceptibility testing

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CANDIDA SPECIES VARIABILITY AS SEEN THROUGH CLINICAL COVARIATES AND DRUG SUSCEPTIBILITY TESTING

by

Jesse Lee Hollanbaugh

An Abstract

Of a thesis submitted in partial fulfillment
of the requirements for the Doctor of
Philosophy degree in Pharmacy
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Thesis Supervisor: Associate Professor Erika J. Ernst

ABSTRACT

With the recent emergence of candidemia as a significant cause of mortality in our health care system, clinicians must identify methods to minimize the sequelae of infection of this type in patients already burdened with serious underlying conditions. While well established as a major cause of blood stream infection (BSI), candidemia has been shown to have some of the highest rates of inappropriate therapy when compared to infections from all other sources. Rates of inappropriate therapy may be even higher for some of the less common and antifungal resistant non-*albicans* candidemia. Identifying those patients at risk for the development of these types of infections will help improve clinical outcomes. Antifungal activity is dependent both on species and agent, describing the unique susceptibility patterns between *Candida species* can help identify the appropriate therapy.

We performed a case-case-control study to identify clinical risk factors for the development of *Candida glabrata* candidemia compared to *Candida albicans* candidemia and an uninfected control using multivariate and logistic regression analysis. We observed that patients in the *C. glabrata* cohort were more likely to have gastrointestinal disorders and peripheral vascular disease than patients suffering from *C. albicans* BSIs. We also determined that when compared to the uninfected control group, patients with *C. glabrata* BSIs were more likely to have been prior colonized with *C. glabrata*, undergone dialysis, and have been catheterized with both arterial and urinary catheters. We concluded that patient exposure to unique clinical risk factors may be predictive of the development of future candidemia and may help distinguish between albicans versus non-*albicans* candidemia.

We performed a drug susceptibility study using time-kill methods with the echinocandin antifungal agents on *Candida parapsilosis* and two newly identified species of *Candida*, *C. orthopsilosis* and *C. metapsilosis*. The echinocandins as a group

displayed primarily fungistatic activity against the clinical isolates tested. However, we observed substantial variability in antifungal activity that varied by both the echinocandin used and *Candida* species analyzed. We concluded that this variability in activity that is both species and drug dependent should be considered when selecting the treatment of candidemia resulting from these non-*albicans* species.

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Graduate College
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CERTIFICATE OF APPROVAL

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PH.D. THESIS

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This is to certify that the Ph.D. thesis of

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To my family and friends, your unconditional love brought me through.

ABSTRACT

With the recent emergence of candidemia as a significant cause of mortality in our health care system, clinicians must identify methods to minimize the sequelae of infection of this type in patients already burdened with serious underlying conditions. While well established as a major cause of blood stream infection (BSI), candidemia has been shown to have some of the highest rates of inappropriate therapy when compared to infections from all other sources. Rates of inappropriate therapy may be even higher for some of the less common and antifungal resistant non-*albicans* candidemia. Identifying those patients at risk for the development of these types of infections will help improve clinical outcomes. Antifungal activity is dependent both on species and agent, describing the unique susceptibility patterns between *Candida species* can help identify the appropriate therapy.

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CHAPTER 1

INTRODUCTION

Candidemia

Over the past 20 years, fungal infections from the genus *Candida* have been a significant cause of human disease with dramatically increasing incidence (1, 2, 3, 4, 12). This is very troublesome among those who have been hospitalized with serious underlying diseases or those who may be immunocompromised (12). A recently published epidemiological study looking at the incidence of sepsis over a 22 year period in the United States found that fungal causes of sepsis have increased 207 % between the years 1979 and 2000 (5, 12). Numerous factors have contributed to this increasing frequency of *Candida* blood stream infections (BSI) in our health care system, probably the most relevant is the ever expanding population of immunocompromised patients that that is associated with underlying disease states such as AIDS, cancer or diabetes mellitus (7, 12). Other causes include the use of immunosuppressive drugs for chronic medical conditions or organ transplants, the use of central venous catheters, broad spectrum antimicrobial therapy and the extremes of age (7, 12).

Patients with infections caused by *Candida* have been reported to have high morbidity and mortality, increased lengths of hospital stays and higher costs of medical care when compared those that do not develop these infections (2, 11, 12, 13, 14, 30). Zaoutis et al. (14) published a case-control study of the epidemiology and outcomes with candidemia patients and reported that in adult patients' candidemia was associated with a 14.5 % increase in mortality, a mean 10.1 % increase in the total length of stay and a mean dollar increase in total hospital charges of $39,331. Another study published by Wilson et al. (4) examined the incidence and the first year cost of candidemia and

estimated a total annual financial burden of 1.7 billion in 1998 dollars for the treatment of candidiasis in the United States. In regards to patient mortality, Gudlaugsson et al. (11) published a retrospective case-control study that utilized a control group that matched the case patients on numerous clinically relevant factors (i.e., sex, age, medical conditions, procedures) in order to determine the direct impact of candidemia on patient mortality and found that once all other variables were matched and accounted for an attributable mortality of 49 % could be found (Figure1.1) (11).Clearly, the burden of candidemia on patients in the health care system requires that we do more to address this problem (12).

Treatment of Candidemia

With a reported attributable mortality of 49 % in patients with Candida BSIs, treatment should be delivered as soon as possible and be considered both safe and effective (11). Current guidelines recommend the use parenteral fluconazole for most cases of candidemia in nonneutropenic patients (65). The use of an echinocandin agent such as caspofungin, anidulafungin or micafungin is suggested to be the most appropriate choice of therapy for severely ill patients, patients who have had recent fluconazole exposure or are suffering from an infection due to *C. glabrata*. In neutropenic patients with candidemia or suspected candidemia treatment with an echinocandin is recommended (65).

Historically, the use of fluconazole has been the treatment of choice for all *Candida* BSIs. The use of fluconazole in the majority of patients with *C. albicans* candidemia has been an effective treatment strategy, as *C. albicans* isolates are almost always fluconazole susceptible (66, 67). However, *C. glabrata* BSIs while not as common as the *C. albicans* candidemia are associated with fluconazole resistance.

Resistance to fluconazole in *C. glabrata* isolates is believed to be associated with the upregulation of efflux pumps of the adenosine triphosphate-binding cassette (ABC) transporter family (66, 67). Prior use of fluconazole can lead to alteration of endogenous flora, enabling colonization and subsequent infection with potentially fluconazole resistant *C. glabrata* (66, 67). This issue of potential resistance in *C. glabrata* isolates is why fluconazole is not recommended in patients with prior fluconazole use or when a *C. glabrata* BSI is suspected.

In cases of suspected candidemia, the choice of the initial treatment should be based on clinical risk factors, serologic tests and culture data (65). In addition to lowering patient mortality, appropriate empiric antifungal treatment my help reduce the increased total health care costs and decrease total lengths of stay (12) associated with candidemia. The choice of appropriate empiric therapy will require the identification of unique clinical covariates that help distinguish those who are at high risk for candidemia and a reliable method for distinguishing between the *C. albicans* and non-albicans types candidemia (12).

Identification of Unique Risk Factors for Candidemia

Nosocomial candidemia is a treatable condition; however it has some of the highest rates of inappropriate therapy and hospital mortality of all etiologic agents of nosocomial sepsis (12, 15, 16, 17, 18, 19, 29). Appropriate antifungal therapy is typically defined as the use of a systemic antifungal drug which is active *in vitro* against a Candida isolate obtained from the patient and is dosed according the Infectious Diseases Society of America (IDSA) guidelines (29). Despite the many advances and wide availability of systemically active antifungal agents, failure to receive any initial treatment is the most common cause of inappropriate empiric antifungal therapy (15, 29). Klevay et al. (29)

reported the results of a retrospective case-control study of appropriate therapy and outcomes which compared a case group consisting of patients with *C. glabrata* BSIs with a matched control group consisting of patients with *C. albicans* BSIs. In this study they reported significantly higher 30-day crude mortality in patients receiving no treatment versus those that received any treatment (Figure 1.2). The reasons for the absence of appropriate antifungal therapy included: 1) death prior to blood culture turning positive, 2) removal of the central venous catheter felt to be adequate therapy, 3) resolution of fever prior to blood culture positivity felt to be an indication for withholding antifungal therapy, 4) repeat blood cultures negative, positive culture felt to be contaminant 5) withdrawal of aggressive care and removal of life support, 6) decision to await identification of yeast prior to starting therapy, and patient died prior to yeast identification and 7) no clear reason was given (29). The author concluded that further work is needed to develop improved rapid diagnostics or identify clinical risk factors that allow physicians to accurately predict those patients who would benefit from early empiric therapy (12, 15, 16, 17, 18, 19, 27).

The Role of Antifungal Susceptibility Testing

Antifungal drug activity on specific fungal isolates can be determined through a number of *in vitro* methods including the minimum inhibitory concentration (MIC), time-kill kinetic studies and minimum fungicidal concentration (MFC) (56). The MIC, or the lowest concentration of an antifungal that will inhibit the visible growth of an organism after an overnight incubation, is the most common laboratory measurement of activity of an antimicrobial against a fungal organism (56, 57). In most clinical settings, this is typically all that is needed in order to provide the patient an antifungal that is required to minimize the spread of an infecting organism from the site of infection (56). The use of

the MIC to guide therapy requires only that the host's immune system be working and achieves ultimate eradication of the organism (56). Interestingly, the majority of Candida species infections occur in patients with profoundly weakened or suppressed immune systems from various underlying medical conditions (56, 58, 59, 60). With this in mind, it would appear that the best therapeutic agent to be given to a patient with a known immune deficiency would be a drug that not only inhibits the growth of a fungal isolate, but one that kills it out right or in other words fungicidal (56). Time-kill studies of antifungal agents provide a more dynamic assessment of the rate and extent of killing of a fungal isolate and would appear to have more of a clinical value in selecting antifungal therapy for the immunocompromised (56). Time-kill studies were first performed with bacterial isolates in order to examine the killing of that isolate over time by one or more antimicrobial agents (56). The rate of killing of a fixed inoculum is determined by sampling a control which contains the organism and no drug and sampling combinations of the organism with differing concentrations of an antimicrobial at fixed intervals over a period of 24 hours and determining a survivor count (in CFU per milliliter). These survivor counts at fixed intervals are then plotted against one another to facilitate the comparison of the activity seen over time. In time-kill studies involving bacterial isolates, cidal or killing activity is usually defined as 99.9%, or 3-$\log_{10}$-unit reduction in CFU/ millimeter from the starting inoculums (56). Klepser et al. (47) adopted the similar criteria used for bacterial time-kill studies in order define antimicrobial activity in fungal isolates. He proposed a 99.9%, or 3-$\log_{10}$-unit reduction in CFU per milliliter from the starting inoculum that would define fungicidal activity and for fungistatic activity a $\leq$ 99.9%, or $\leq$ 3-$\log_{10}$-unit reduction in CFU per milliliter (47). This method of time-kill analysis is what is currently used in examining antifungal activity in the majority of research today.

Antifungal agents can display both fungistatic or fungicidal activity depending on the isolate tested (43). This would suggest that in order to clearly identify which

antifungal agents would be most appropriate for the immunocompromised patient one would want to assess whether we can expect to see fungistatic or fungicidal activity it expresses in the presence of the intended target fungal isolate.

The following chapters describe research carried out in order to further define relationships between clinical risk factors and the development of non-albicans candidemia and differentiate the type of candidemia based on the presence or absence of certain clinical risk factors. In addition we describe the activity of the echinocandin class of antifungal agents against *Candida parapsilosis* and the two recently described species of *Candida*, *C. orthopsilosis* and *C. metapsilosis*. The ultimate goal of this research is to help clinicians better identify who need is of empiric antifungal therapy and provide guidance in the appropriate antifungal selection.

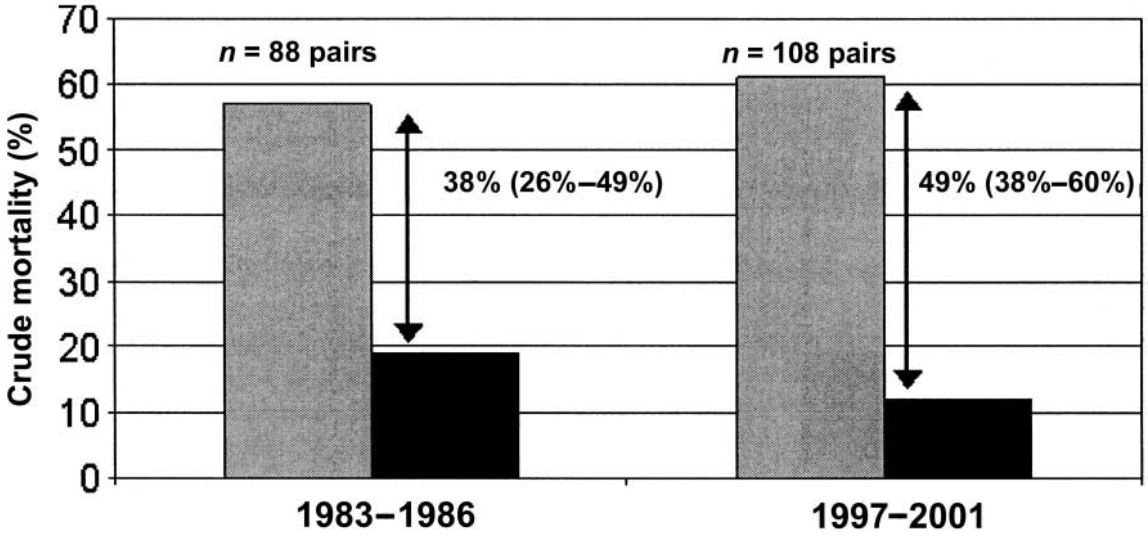

Figure 1.1 Comparison of the attributable mortality of nosocomial candidemia at the University of Iowa Hospitals. 95% Confidence intervals are given in the parentheses.

Source: Gudlaugsson O, Gillespie S, Lee K, Vande Berg J, Hu J, Messer S, Herwaldt L, Pfaller M, Diekema D. Attributable mortality of nosocomial candidemia, revisited. *Clin Infect Dis.* 2003 Nov 1; 37(9):1172-7.

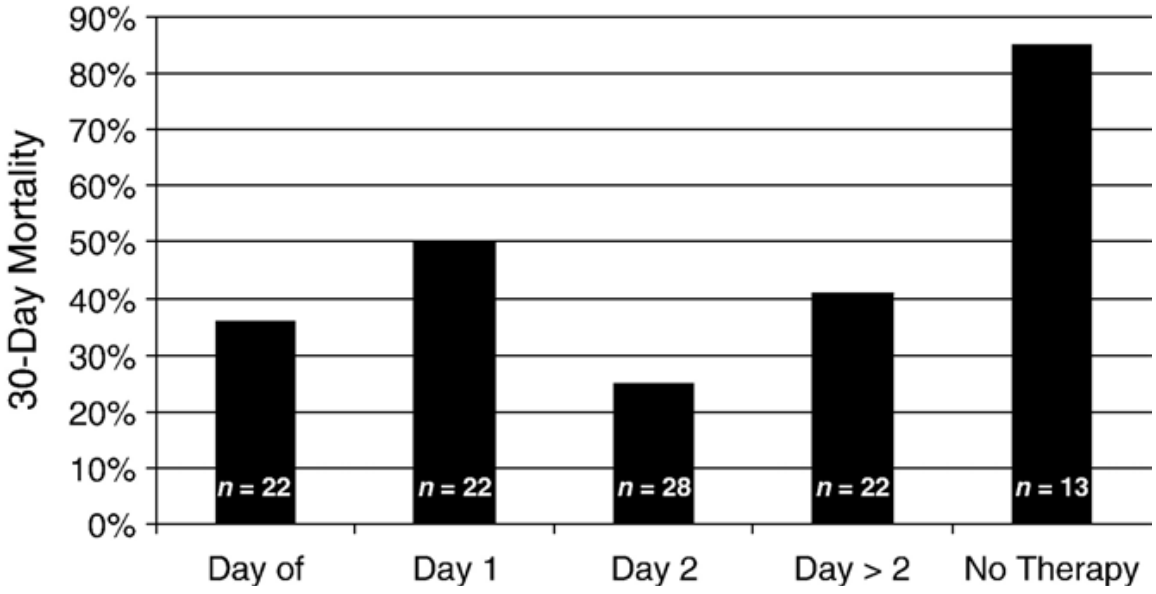

Figure 1.2 30-day mortality according to time of initiation of antifungal therapy (P=0.3).

Source: Klevay MJ, Ernst EJ, Hollanbaugh JL, Miller JG, Pfaller MA, Diekema DJ. Therapy and outcome of Candida glabrata versus Candida albicans bloodstream infection. *Diagn Microbiol Infect Dis.* 2008 Mar; 60(3):273-7.

CHAPTER 2

A CASE-CASE-CONTROL STUDY OF ANTIMICROBIAL USE AND OTHER RISK FACTORS FOR CANDIDEMIA CAUSED BY *CANDIDA GLABRATA* AND *CANDIDA ALBICANS* AT A TERTIARY CARE HOSPITAL.

Introduction

Candida species have emerged as the fourth most common cause of nosocomial bloodstream infection in the United States and are particularly prevalent in intensive care units (ICU) (6, 8, 9, 10). Previous investigators have reported *Candida species* are the third most common cause of all nosocomial infections, and directly responsible for as much as 10 % of all such infections (8, 10). Despite the introduction of multiple systemic antifungal agents with activity against *Candida species*, these infections continue to be associated with a high attributable mortality (11, 12). In addition to higher attributable mortality rates, nosocomial candidemia is also associated with increased lengths of stay in both the ICU and the hospital, resulting in higher overall health care costs (13).

Previous investigators have reported that it is common for the initiation of antifungal therapy to be delayed, and that delay in therapy beyond 12 hours after culture is associated with a higher patient mortality (15). Another study suggests that mortality increases incrementally with each day of delay in therapy for candidemia in patients treated with fluconazole (16). Clearly, early identification and treatment of candidemia is important in order to facilitate positive outcomes for hospitalized patients in regards to overall mortality and keeping health care associated costs down.

Although *Candida albicans* is the most common cause of nosocomial candidemia, the epidemiology of species causing candidemia is changing. *Candida glabrata* has

emerged as the 2nd most common cause of candidemia in hospitals in the United States (12, 22) and is less susceptible to fluconazoles and amphotericin B, than is the more common *Candida albicans* (12, 23). For this reason, patients with *C. glabrata* BSIs may be at higher risk for receipt of inappropriate therapy or adverse outcome compared to those with C. *albicans* BSIs. In order to ensure confidence in initiating safe and appropriate therapy, clinicians will need access to information that will help differentiate between patients who have or are at risk for *C. glabrata* BSIs compared to those who suffer from *C. albicans* BSIs. Currently, two approaches can help clinicians distinguish between *C. albicans* and non-*albicans* candidemia allowing for earlier and more accurate antifungal treatment. Rapid laboratory identification technology of *C. albicans* using a peptide nucleic acid-fluorescent in situ hybridization test (PNA FISH) is available and has been shown to help guide appropriate antifungal therapy, while keeping hospital costs down (24, 25). However this assay was reported to be not widely available and costly to implement (26). The second approach that may help guide empiric antifungal therapy is to examine clinical risk factors that can help distinguish *C. glabrata* infections from the more common *C. albicans* infections.

The purpose of this study was to identify risk factors for nosocomial C. *glabrata* infections in order to differentiate these patients from those infected with the more common *C. albicans*.

Materials and Methods

Setting: The University of Iowa Hospitals and Clinics (UIHC) is a 680-bed teaching hospital providing tertiary care for the state of Iowa. The UIHC is a level-one trauma center, has 128 intensive care unit beds, and supports active bone marrow transplant and solid organ transplant programs.

Study design: A matched retrospective cohort study was performed. All cases of *C. glabrata* BSI between July 1997 and December 2004 were identified from a database maintained in the Clinical Microbiology Laboratory at the UIHC. *C. glabrata* BSI was defined as at least one positive blood culture for *C. glabrata* during the study period. Patients with *C. albicans* BSI were selected from the database and matched 1:1 by closest date of hospital admission. Another group of uninfected patients were also selected as a control and matched 1:1 to *C. glabrata* cases by date of hospitalization. Data on antimicrobial therapy and potential risk factors were collected by retrospective chart review.

Microbiologic Methods: *Candida* species were identified using Vitek® and API® products or conventional methods, as appropriate, and antifungal susceptibility testing was performed with the reference broth microdilution method in accordance with CLSI standards (27). Interpretive criteria for fluconazole susceptibility were those published by the Clinical and Laboratory Standards Institute (CLSI). *C. parapsilosis* strain ATCC 22019 and *C. krusei* strains ATCC 6258 were tested with each run for quality control.

Antimicrobial Usage: Total antimicrobial use during admission for both the selected case and control patients were recorded. In order to facilitate comparison between groups in total antimicrobial use we standardize this value and utilizing the WHO standardized measure of drug consumption called the Defined Daily Dose (DDD) (28). DDDs were calculated for each antimicrobial used using the following equation:

Drug usage (DDDs) = (items issued X amount of drug per item) / (WHO DDD Measure)

Risk Factors: An itemized data collection sheet was used to gather all potential risk factors from a patient's medical record. Information collected included demography, date

of admission, reason for admission, admission unit, discharge date, patient mortality, time at risk, dates and results of all positive blood cultures, prior colonization, underlying chronic and acute illnesses. ICD-9 codes assigned to the patient, surgical procedures, central catheters, any other lines or tubes that were used in the treatment of that patient, total parenteral nutrition (TPN), antimicrobial use and source of bloodstream infection were also collected. Risk factors were assessed at the time of admission until a positive blood sample was obtained (i.e., time at risk)

Statistical Analysis: DDDs that were calculated for each antimicrobial class used by each patient were then compared between groups using multivariate and univariate analysis where appropriate. All antifungal use examined in this analysis of the antimicrobials is limited to use prior to first positive blood culture. Differences detected in antimicrobial use at a level of significance of a $P \leq 0.1$ were then dichotomized into "yes" or "no" variables and entered into further logistic regression analysis. Non-antimicrobial risk factors associated with candidemia due to *C. glabrata* versus both the *C. albicans* and the uninfected control groups were examined using chi-square or univariate analysis where appropriate. Variables that had a statistical significance of $P \leq 0.1$ were then entered into multivariate logistic regression analysis models along with statistically significant antimicrobial variables. Post hoc analysis between groups was done with the Tukey range test that identified differences between the groups compared using univariate and multivariate at a level of significance ≤ 0.05.

The Enter method, which enters all the variables into the model at the same time, was used to build a regression model to determine which variables were most strongly associated with blood stream infections due to *C. glabrata* compared to the C. *albicans* and the uninfected control group. A P-value of ≤ 0.05 was considered to be statistically significant in the logistic regression analysis. Statistical analysis was performed using SPSS version 13.0 for Windows (Chicago, IL).

Results

Demographics and Clinical Characteristics: Fifty-five patients had *C. glabrata* BSI identified during the study period. Due to difficulty in matching one study patient with a *C. albicans* control patient, this patient was excluded, leaving 54 patients in the *C. albicans* group. Seven of the *C. glabrata* patients had polymicrobial bloodstream infection episodes with other *Candida* species (*C. albicans* (6) and *C. parapsilosis* (1)), and one *C. albicans* patient also had *C. lusitaniae* isolated from the blood. Fifty-four uninfected controls matched 1:1 to the date of admission of *C. glabrata* patients were also included in the study. Non-antimicrobial risk factors that differed between groups ($P \leq 0.10$) included age, time at risk, prior colonization, chronic renal failure, dialysis, diabetes mellitus, other liver disease, peripheral vascular disease, infection, acute renal failure, mechanical ventilation, cardiac arrhythmia, GI bleed, central catheter, arterial catheter, urinary catheter, Ngogtube and total parenteral nutrition (Table 2.1). These variables were entered into a multivariate logistic regression analysis along with the antimicrobial risk factors identified in a univariate or multivariate analysis.

Multivariate Analysis of Antimicrobial Risk Factors: The results of the analysis describing the association between DDDs calculated for each individual antimicrobial agent from the *C. glabrata*, *C. albicans* and uninfected groups is presented in Table 2.2. The mean vancomycin DDD calculated for both the *C. glabrata* group (4.56) and the C. *albicans* group (4.92) was statistically significant from the mean DDD calculated for vancomycin use in the uninfected control group (1.67). The mean metronidazole DDD and calculated for the *C. glabrata* group (3.19) and the mean carbapenam DDD calculated for *C. glabrata* group (0.62) while not statistically significant, demonstrated a trend towards difference when compared to the uninfected controls and met the $P \leq 0.10$ criteria to be included into logistic regression analysis along with vancomycin use and the

non-antimicrobial risk factors identified. Of note, antifungal DDDs calculated for each of the case and control groups prior to first positive blood culture did not differ significantly from one another and all antimicrobial use between C. *glabrata* and *C. albicans* case groups did not differ significantly.

Multivariate Logistic Regression: The results of the multivariable regression analysis are shown in Tables 2.3, 2.4 and 2.5. For *C. glabrata* candidemia, time at risk, prior colonized, dialysis, arterial catheter, urinary catheter and vancomycin met the criteria ($P < 0.05$) in the logistic regression model to be risk factors when compared to the uninfected controls (Table 2.3). In contrast the frequency of GI disorders and peripheral vascular disease were significantly higher in *C. glabrata than* compared to *C. albicans* candidemia (Table 2.4). In the final logistic regression analysis the *C. albicans* group was compared to the uninfected control. Age, prior colonized, arterial and urinary catheters were included in the final model demonstrating the required level of significance (Table 2.5).

Discussion

C. glabrata has emerged as an important nosocomial pathogen in the United States (22, 23). The reduced susceptibility of *C. glabrata* to azoles and amphotericin B poses a challenge in the selection of appropriate empiric antifungal therapy for patients with candidemia prior to availability of species identification or antifungal susceptibility results. In an earlier published study using the same patient population and cases as our study, it was postulated that patients with *C. glabrata* BSI may be less likely than those with *C. albicans* BSIs to receive appropriate therapy, or perhaps have greater delays to initiation of appropriate therapy (29).

This difference was not detected in the receipt of appropriate antifungal therapy dependent on *Candida species* (29). Patients infected with *C. glabrata* were as likely to receive appropriate antifungal therapy as those infected with *C. albicans* (29). This was thought to demonstrate physicians' familiarity with the variable fluconazole susceptibility patterns of *C. glabrata*. (29) Empiric therapy once yeast was identified appeared to be guided by the microbiology of previous Candida colonization and prior azole exposure, leading to more patients with *C. glabrata* being started on an echinocandin or high-dose fluconazole therapy. However, among the patients suffering from candidemia there was a significant number of individuals who were not treated at all (29). Among this group, 30-day mortality was significantly higher than those that received any treatment without regard to the timing (29). It was concluded that the identification of other species–specific risk factors may provide clinicians with additional guidance in choosing empiric antifungal therapy to avoid not treating those at risk (29).

In this previous study, patients receiving at least seven days of appropriate antifungal therapy following bloodstream clearance had a lower mortality at 30 days compared to those that did not. This is consistent with the findings of Almirante et al, who found a decrease in mortality in candidemic patients receiving at least five days of any antifungal (30). Likewise, Morgan et al found lower attributable mortality rates in candidemic patients receiving at least seven days of antifungal therapy than those who received inadequate therapy (31).

Delays in antifungal therapy until blood cultures are positive for yeast is common. In a single center cohort of 157 candidemic patients, 134 patients did not receive empiric antifungal therapy prior to culture positivity. In the nine patients who were initiated on therapy within 12 hours of culture, mortality was significantly lower than in those initiated on therapy after 12 hours (15). Gary et al (16) reported 192 candidemic patients from four centers and found an incremental increase in mortality for each day of delay in the initiation of fluconazole (16). Timing to initiation of treatment in the study published

by Klevay et al. (29) did not demonstrate this incremental increase in mortality, which may be a reflection of the severity of illness at the time of candidemia in these patients. The absence of antifungal therapy had more of an impact on mortality than did timing of therapy, as demonstrated by 85 % mortality in untreated patients (29).

Over the past two decades, treatment of all episodes of candidemia has been recommended, and formal guidelines for therapy were first published in 2000. Although *Candida* BSI rarely clears with removal of an infected central venous catheter without antifungal therapy, it is difficult to predict who develops complications of hematogenous seeding of *Candida*. The morbidity and risk of these sequelae outweigh the risk of antifungal therapy, especially with the development of multiple newer antifungal agents with improved side effect profiles compared to amphotericin B deoxycholate (32). In most instances of untreated candidemia that have been reported, patients have either died by the time yeast is identified in blood cultures or palliative care has been instituted (33, 55). This was consistent with the results of the study published by Klevay et al. (29), and this paper also reported other reasons for lack of therapy that included: 1) physician attributing the positive blood culture to contamination, 2) the removal of an infected central venous catheter was thought to be sufficient, 3) the patient appeared to be clinically improved without therapy, or 4) physicians awaiting identification of the yeast prior to treating infection (29).

Though these cases may represent a minority of candidemic patients, there appears to be an opportunity for the education of physicians that isolation of *Candida* from blood cultures never should be considered a contaminant and that catheter removal without antifungal therapy is a gamble with a high risk of failure. In the case of apparent clinical improvement without therapy, we should be concerned that the patient will have hematogenously seeded a distant site and therefore be at risk for developing complications of candidemia. Lastly, most cases of yeast isolated in hospitalized patients will most likely be a member of the *Candida* group (34); therefore empiric therapy based

on clinical risk factors or advanced identification technology should be initiated once these results becomes available.

Further evidence supports the idea that in order to reduce the number of untreated patients we must include preemptive or earlier empiric therapy (35). Piarroux et al. (54) reported results of a successful preemptive strategy in surgical intensive care unit patients at a single center with the use of a *Candida* colonization index. Patients with a corrected colonization index of ≥0.4 (number of surveillance cultures of oropharynx, gastric, tracheal aspirate, urine, and rectum with heavy growth of *Candida* per number of cultures obtained) received preemptive therapy with full dose fluconazole and had a lower incidence of invasive *Candida* infection compared to a retrospective cohort of controls (3.8% vs. 7%, P=0.03). The incidence of SICU-acquired *Candida* infection decreased to 0.0 % from 2.2 % in controls (P<0.001). The generalizability of these results and cost-effectiveness of such an approach are yet to be determined.

In this study we attempted to determine if antimicrobial use or clinical covariates unique to individual patients could be identified to help predict who would be at risk for candidemia from either *C. glabrata* or *C. albicans*. We found many variables (Table 2.1 and Table 2.2) through univariate and multivariate analysis that were statistically associated with candidemia from both *C. albicans* and *C. glabrata.* We then applied a logistic regression model to identify which were most likely to be predictors of infection. The risk for *C. albicans* BSIs have been well established and not changed much over the last few decades. However, attempting to distinguish between *C. albicans* and the other potentially drug resistant non-albicans species would be of significant value in clinical practice. In this study, the clinical covariates we identified and included in our final model to predict those patients at risk for *C. glabrata* compared to uninfected patients included the variables time at risk, prior colonized, dialysis, arterial catheter, urinary catheter and vancomycin (Table 2.3). We reported the resulting Odds ratio's of the logistic regression analysis, and though they attained a level of significance≤0.05 and

where included in the final model, some of these were associated with large confidence intervals which might be attributable to the small sample size. Our finding that the antimicrobial vancomycin is a potential risk factor for *C. glabrata* is consistent with Lin et al who in a previous study looking to identify risk factors for non-albicans BSIs found that the antimicrobials vancomycin and piperacillin-tazobactam were strongly correlated ($P<0.05$) to non-*albicans* BSI, which in that study included those infected by *C. glabrata* and *C. krusei*. Clinical covariates found to be associated with non-albicans candidemia in that same study included total parenteral nutrition, intubation, central venous catheter, and liver disease (52). We found through logistic regression analysis, that differences in potential risk factors for *C. glabrata* versus *C. albicans* included the variables other GI disorders and peripheral vascular disease. Many studies have been published in recent years identifying risk factors for candidemia with findings similar to what our group reported and the results of this study supports the conclusion that differences in exposure to various clinical covariates may be predictive of candidemia (12). Here however we were able to determine risk factors that differed between the *Candida species* studied in this analysis and that potentially may help clinicians differentiate between them. This finding contrasts with a study done by Shorr et al. (53) who found no difference between clinical factors between patients infected with non-*albicans* species and those infected with *C. albicans*. The study done by Shorr et al. (53) was similar to ours in design, also had comparable numbers and only defined the presence or absence of a risk factor. However, variables selected for inclusion into that study were not entirely the same as those selected for the one reported here (53). Recognizing that both the sample size and the single center design of our study may have limited ability to completely assess the risk of non-*albicans* BSI due to *C. glabrata*. It is interesting to note we were able to identify differences in exposure to some variables that have been reported as risk factors for candidemia in previous works. It is widely believed that previous antifungal therapy can be predictive of the more resistant forms of candidemia, which in this study would

include those infected with *C. glabrata* (12). Interestingly, no differences in antifungal use prior to the first positive blood culture were detected.

Conclusions

From a clinical standpoint, the results of this study would suggest that exposure to both specific antimicrobial and non-antimicrobial factors may be predictive either directly or indirectly for the subsequent development of *C. glabrata* BSI. In this study, 30-day mortality for *C. glabrata* case patients was roughly 43 % compared to the 28 % seen in the *C. albicans* group. This could be a reflection of how sick these patients were, as we did not detect a difference in inappropriate therapy (29). Differences in appropriate therapy between groups in this population were not observed and were reported in an earlier publication (29). This is in contrast to another paper that did detect differences in the receipts of appropriate initial therapy between patients with *C. albicans* BSIs and *C. glabrata* BSIs (52). Given that lack of antifungal therapy has a significant impact on patient mortality; strategies are needed to more rapidly identify cases of candidemia and facilitate both appropriate and timely therapy. Further work needs to be done in order to develop improved rapid diagnostic technology that is both widely available and cost effective for healthcare institutions, newer clinical approaches to identify patients at great risk for the development of BSIs from non-albicans *Candida* in order to provide appropriate empiric therapy if needed.

We were able to identify unique clinical risk factors that distinguished patients with *C. glabrata* BSIs from patients with *C. albicans* BSIs and those who are uninfected with candidemia. Most studies done of this type compare non-*albicans* candidemia versus *albicans* candidemia, we compared *C. glabrata* candidemia to *C. albicans* candidemia.

Table 2.1 Demographic and clinical characteristics of case and control groups.

Characeristics	*C. glabrata* n=54	*C. albicans* n=54	Uninfected n=54	P
Male (%)	40	57	56	0.12
Mean Age in Years, (SD)	55(19)[#]	41(27.5)[$]	53(19.8)	0.003
Age Range in Years	12-83[#]	0-88[$]	2-82	0.003
Time at Risk in days (SD)	12.5(13)[#]	20.59(20.3)[$]	13.02(13.1)	0.013
Prior colonization	22[#*]	27[$]	1	<0.001
COPD	10	13	15	0.52
Congestive heart failure	8	10	5	0.38
Chronic renal failure	8[#]*	2	2	0.039
Dialysis	13*	6	3	0.015
Diabetes mellitus	17	8	10	0.088
Cirrhosis	4	7	2	0.2
Other Liver Disease	12[*]	9[$]	1	0.006
Other GI Disorders	23[#*]	9	8	0.001
Leukemia	3	6	3	0.6
Lymphoma	7	2	6	0.21
Chemotherapy	21	15	13	0.22
Bone Marrow Transplant	7	3	6	0.41
Coronary artery disease	22	16	17	0.43
Cerebrovascular disease	3	4	2	0.7
Peripheral vascular disease	6[#*]	1	1	0.037
Burn	0[*]	3[$]	0	0.047
Acute renal failure	25[*]	17[$]	2	<0.001
Mechanical ventilation	32[*]	35[$]	5	<0.001
Cardiac arrhythmia	8[*]	8[$]	0	0.012
GI Bleed	18[*]	10	5	0.008
Central catheter	50[*]	51[$]	23	<0.001
Peripheral IV	54	50	50	0.2
Arterial Catheter	32[*]	29[$]	2	<0.001
Urinary catheter	32[*]	33[$]	1	<0.001

Ngogtube	28[*]	35[$]	5	<0.001
Total Parenteral Nutrition	38[*]	37[$]	9	<0.001
30-day Mortality (%)	23 (43)[*]	15 (28)[$]	3 (0.1)	<0.001

[#] Significance P≤0.05 *C. glabrata* versus *C. albicans*
[*] Significance P≤0.05 *C. glabrata* versus Uninfected
[$] Significance P≤0.05 *C. albicans* versus Uninfected

Table 2.2 Mean (SD) Defined Daily Doses of antimicrobials used in cases and controls.

Antimicrobial	*C. glabrata*	*C. albicans*	Uninfected	
	Mean (SD)	Mean(SD)	Mean(SD)	P
Penicillins	1.02(5.26)	0.44(1.62)	1.23(8.2)	0.75
Penicillinase resistant penicillins	0.84(4.58)	0.2(1)	0.08(0.61)	0.3
Extended spectrum penicillins	0.4(1.46)	0.32(1.28)	0.18(0.98)	0.63
Beta lactamase penicillins	5.4(12.22)	5.56(10)	3.2(9.27)	0.44
Vancomycin	4.56(6.64)	4.92(7.08)	1.67(4.51)	0.013
Aminoglycosides	1.41(3.42)	2.23(6.46)	1.2(4.77)	0.54
1st generation cepalosporins	0.68(2.4)	0.63(1.67)	0.52(1.53)	0.91
2nd generation cepalosporins	0.36(1.2)	0.54(1.76)	0.22(0.95)	0.48
3rd generation cepalosporins	0.31(1.25)	0.86(2.89)	0.64(2.17)	0.43
4th generation cepalosporins	1.77(4.6)	1.69(6.1)	2.12(5.94)	0.91
Quinolones	3.11(5.52)	4.48(7.59)	2.99(5.59)	0.4
Metronidazole	3.19(5.15)	2.63(4.53)	1.09(4.62)	0.065
Bactrim	0.25(1.07)	0.34(1.58)	0.95(4.66)	0.4
Macrolides	0.69(1.68)	0.63(1.47)	1.3(4.71)	0.5
Clindamycin	1.15(3.26)	0.43(1.7)	0.75(3.14)	0.4
Carbapenems	0.62(2.11)	0.34(1.4)	0	0.09
Itraconazole	1.94(8.14)	0.02(0.14)	0	0.11
Fluconazole	1.15(3.5)	1.69(5.72)	0.81(4.54)	0.62
Caspofungin	0.03(0.19)	0	0	0.37
Amphotericin B	1.05(4)	0.12(0.54)	0.85(3.73)	0.27
Voriconazole	1.83(9.9)	0.09(0.68)	0.06(0.41)	0.19

Abbreviations: SD, standard deviation.

Table 2.3 Risk Factors for *C. glabrata* compared to an uninfected control group determined by logistic regression analysis.

Predictor	Odds ratio (95 % CI)	P
	C. glabrata	
Time at risk	0.89 (0.8-1)	0.06
Prior colonize	222 (5-9118)	0.004
Dialysis	306 (0.99-94373)	0.05
Arterial catheter	94 (1.6-5727)	0.03
Urinary catheter	429 (6.1-30089)	0.005
Vancomycin	21.2 (0.9-497.1)	0.06

Table 2.4 Risk Factors for *C. albicans* compared to *C. glabrata* determined by logistic regression analysis.

Predictor	Odds ratio (95 % CI) *C. albicans*	P
Other GI disorders	0.2 (0.05-0.76)	0.017
Peripheral vascular disease	7.62E-10 (7.62E-10-7.62E-10)	<0.01

Table 2.5 Risk Factors for *C. albicans* compared to an uninfected control group determined by logistic regression analysis.

Predictor	Odds ratio (95 % CI) *C. albicans*	P
Age	0.94 (0.84-1.05)	0.06
Prior colonize	154.3 (4.2-5680)	0.006
Arterial catheter	42 (0.71-2486)	0.07
Urinary catheter	679.1 (9.6- 47804.9)	0.003

CHAPTER 3

IN VITRO ACTIVITY OF THE ECHINOCANDIN ANTIFUNGAL AGENTS AGAINST ISOLATES OF *CANDIDA PARAPSILOSIS, CANDIDA ORTHOPSILOSIS* AND *CANDIDA METAPSILOSIS* BY TIME-KILL METHODS.

Introduction

Candida parapsilosis ranks among the five most common causes of fungal nosocomial infections in the world and has been ranked as high as the third among all causes of fungal nosocomial infections in North America. (12, 61) While the place of *C. parapsilosis* among the top five causes of nosocomial fungal infection has remained unchanged over the last 10 years, the frequency of its isolation has increased according to epidemiologic studies from 1.2- 3.1 % over that period (12, 61). Previous work has demonstrated that drug activity and susceptibility on the basis of time-kill methodology varies by the *Candida* species isolate and drug concentration used in the study (43, 63). Knowing what species and the type of activity a particular antimicrobial agent will have against that species is important in guiding the most appropriate drug therapy.

C. parapsilosis Groups II and III have been recently proposed to be replaced with the species designations *C. orthopsilosis* and *C. metapsilosis* (36). Replacing one species designation with three separate designations should be expected to have a profound impact on how clinicians handle antimicrobial treatment if any of these 3 species were to be identified; knowing that drug susceptibility is species dependent. Previous studies looking at time-kill analysis did not distinguish the group (I, II or III) of *C. parapsilosis* tested and it has been reported through retrospective analysis using DNA-based methods

that around 10% of all isolates formerly identified as *C. parapsilosis* were actually *C. orthopsilosis* and *C. metapsilosis* (37,62).

Caspofungin, anidulafungin and micafungin are echinocandin antifungal agents. As a novel class of antifungal agents; their mechanism of action involves the inhibition of 1, 3-beta-D-glucan synthase, which along with chitin is important in providing the integrity and shape of the fungal cell (38, 39, 40, 41, 42). The echinocandins antifungal agents are also reported to have a broad spectrum of activity against fungal pathogens and are similar to one another with respect to their *in vitro* activity against a variety of *Candida species*, with micafungin and anidulafungin having similar minimum inhibitory concentrations (MICs) that are lower than the MIC of caspofungin. Overall the echinocandins are suggested to display decreased activity against *C. parapsilosis*; and data on their activity against *C. orthopsilosis* and *C. metapsilosis* determined by time-kill methods has not been described in detail. What has been described is that the MICs of the echinocandins are highest against *C. parapsilosis*; though it is not known whether this will affect clinical outcomes (38). Szabo et al. (45) recently described the activity of the azole antifungals and amphotericin B against *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis* isolates and reported differences in activity based on the antifungal and species (45). This is consistent with another publication by Varga et al. (46), who described the activity of caspofungin against a small number of *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis* isolates (46). Among the three species, variability was seen in the type of activity observed and was dependent on the species and concentration of caspofungin used (46). Though these studies add to our understanding of the activity of various antifungal agents to these newly described *Candida species*, more work needs to be done to fully describe and understand the activity of the echinocandins.

The purpose of this study will be to utilize time-kill methods in order to describe the activity of the echinocandin antifungal agents against isolates of *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis*. We will also examine and report how the MICs of

these isolates correlated by the time-kill activity of each echinocandin, with their respective log reduction at 24 hours.

Materials and Methods

Test Isolates: Fourteen *C. parapsilosis*, 12 *C. orthopsilosis* and 11 *C. metapsilosis* isolates were obtained from the ARTEMIS global antifungal surveillance program, the SENTRY antimicrobial surveillance study and the SCOPE program.

MIC Determination: The minimum inhibitory concentration (MIC) of each antifungal was determined against test isolates by using broth microdilution techniques as described by the Clinical Laboratory Standards Institute (CLSI) (47). The MIC was defined as the lowest concentration at which there was a visually prominent reduction in growth (approximately 50%) relative to the drug-free growth control after 24 H of incubation.

Time-kill Studies: Time-kill studies were conducted as previously described in papers published by Ernst et al and Klepser et al, using starting inoculums of $1x10^5$-$5x10^5$ cells/ml (43, 47). The echinocandin concentrations tested ranged from 0.25 to 8 times the MIC. Commercial grade powder caspofungin (Merck lot # 06880), anidulafungin (Pfizer lot # 0AFKT) and micafungin (Astellas lot # 0170) were obtained and diluted according to package insert and stored frozen at -70 ° C in 1ml aliquots until utilized. Analytical grade powder of caspofungin (Merck lot # VEK 0090) and anidulafungin (Pfizer lot # 0003) were obtained and diluted into concentrations containing 100 mcg/ml and stored

frozen at -70 ° C in 1ml aliquots until utilized. Test tubes were incubated with agitation in the dark at 35 °C. At time points 0, 2, 4, 8 and 24 h, samples (100µL) were removed and serially diluted in sterile water. One 30 µL aliquot was placed on potato dextrose agar for colony count determination after incubation for 24 to 48 hours. Individual time-kill plots (log_{10}CFU/ml vs. time) were constructed for each isolate tested. Using a 30 µL sampling volume, the lower limit of quantitation was 100 CFU/ml for each isolate. The log reduction or log growth for each isolate at each multiple of the MIC was determined at 24 hours using the following equation Log 1-(starting inoculums – inoculums at 24 hours ÷ starting inoculums). The mean log growth or reduction in CFU from starting inoculums for each concentration tested (multiple of MIC) at 24 hours was also calculated. Fungicidal activity was defined as a ≥99.9 % or 3-log_{10}-unit decrease in CFU/ml from the starting inoculums. No inhibition or growth occurred when the inoculums at 24 hours exceeded the starting inoculums. The relationship between MIC values and log change in CFU/ml at 24 hours was evaluated by linear regression. As a positive control of our time-kill methods American Type Culture Collection (ATCC) strain, ATCC 90028 was analyzed by our methods.

Results

Susceptibility: The *C. parapsilosis* MICs ranged from 0.12-1 mcg/ml for caspofungin with a median MIC value calculated at 0.5 mcg/ml, a range of 1-4 mcg/ml for anidulafungin with a median MIC value calculated at 2 mcg/ml and a range of 0.5-2 mcg/ml with micafungin with a median MIC value of 1 mcg/ml. (Table 3.1) The *Candida*

orthopsilosis MICs ranged from ranged from 0.03-0.5 mcg/ml for caspofungin with a median value calculated at 0.12 mcg/ml, a range of 0.5-2 mcg/ml for anidulafungin with a median MIC value of 1 mcg/ml and a range of 0.25-1 mcg/ml for micafungin with a median MIC value calculated at 0.375 mcg/ml. (Table 3.2) The *Candida metapsilosis* MICs ranged from 0.06-1 mcg/ml for caspofungin with a median MIC value calculated at 0.12 mcg/ml, a range of 0.25-1 mcg/ml for anidulafungin with a median MIC value calculated at 0.5 mcg/ml and a range of 0.25-0.5 for micafungin with a median MIC of 0.25 mcg/ml. (Table 3.3)

Time-kill: The mean log change (SD) at 24 hours for all isolates at all the multiples of the MIC were calculated for the three *Candida species* against each antifungal agent and presented in Table 3.4. Caspofungin displayed primarily fungistatic activity at concentrations at or above the MIC against *C. parapsilosis* in 13 of the 14 isolates tested. Minimal activity was seen at concentrations below the MIC and no activity was observed a single isolate of *C. parapsilosis* when challenged with caspofungin. A representative time- kill curve for caspofungin activity against a clinical isolate of *C.parapsilosis* is displayed in Figure 3.1. Caspofungin displayed fungistatic activity against 6 of the 12 *C. orthopsilosis* isolates at concentrations exceeding the MIC. Little to no activity was seen in the other 6 *C. orthopsilosis* isolates. Figure 3.2 displays a representative time-kill curve for caspofungin activity against a clinical isolate of *C. orthopsilosis*. Caspofungin displayed no inhibitory activity against all but 3 of the 11 isolates of *C. metapsilosis* tested and only at concentrations exceeding the MIC. Figure 3.3 displays a representative time-kill curve for caspofungin activity against a clinical isolate of *C. metapsilosis*.

Anidulafungin displayed fungistatic activity against all of isolates the isolates tested from all three species of Candida. Figures 3.4, 3.5 and 3.6 display the representative time-kill curves of anidulafungin activity against clinical isolates of *C. parapsilosis, C. orthopsilosis* and C. *metapsilosis.* Anidulafungin concentrations from 0.25 times the MIC to 8 times the MIC caused an average net log reduction in growth of *C. parapsilosis* isolates when compared to the starting incoculum. This average net log reduction in growth was also seen at concentrations exceeding 0.5 times the MIC with *C. orthopsilosis* and 2 times the MIC with *C. metapsilosis.*

Micafungin displayed primarily fungistatic activity at concentrations exceeding 2 times the MIC in 11 of the 12 *C. parapsilosis* isolates tested. Minimal activity was seen at concentrations below the MIC and no activity was observed a single isolate of C. parapsilosis. A representative time- kill curve for micafungin activity against a clinical isolate of *C.parapsilosis* is displayed in Figure 3.7. Micafungin displayed no inhibitory activity against all but 1 of the 6 isolates of *C. orthopsilosis* tested and only at a concentration exceeding 8 times the MIC. In this single isolate that fungistatic activity was seen at a concentration of micafungin 8 times the MIC. Figure 3.8 displays a representative time-kill curve for micafungin activity against a clinical isolate of *C. orthopsilosis*. Micafungin displayed fungistatic activity in all three of the *C. metapsilosis* isolates tested at concentrations exceeding 2 times the MIC. Figure 3.9 displays a representative time-kill curve for micafungin activity against a clinical isolate of *C. metapsilosis.*

At the highest concentration tested (8 times the MIC) the mean reduction in CFU/ml at 24 hours was slightly lower for caspofungin, anidulafungin and micafungin compared to the mean log reduction seen at 2 times and 4 times the MIC in the *C. parapsilosis* isolates. This decrease in activity was also seen with anidulafungin in the *C. orthopsilosis* isolates, where the mean reduction in CFU/ml at the highest concentration

tested at 24 hours was slightly lower than the mean reduction seen 2 times and 4 times the MIC.

The mean log change and the standard deviation at 24 hours for all isolates at all the multiples of the MIC were calculated for the three *Candida species* against the antifungal agents caspofungin and anidulafungin using analytical grade powder are presented in Table 3.5. Caspofungin displayed fungistatic activity against all of isolates tested from all three species of *Candida*. Figures 3.10, 3.11 and 3.12 display the representative time-kill curves of caspofungin activity against clinical isolates of *C. parapsilosis*, *C. orthopsilosis* and C. *metapsilosis* respectively. Caspofungin concentrations exceeding 2 times the MIC caused an average net log reduction in growth of all *C. parapsilosis*, *C. orthopsilosis* and C. *metapsilosis* isolates when compared to the starting inoculum.

Anidulafungin displayed primarily fungistatic activity against all of the isolates tested from all three species of *Candida*. Figures 3.13, 3.14 and 3.15 display the representative time-kill curves of caspofungin activity against clinical isolates of *C. parapsilosis*, *C. orthopsilosis* and C. *metapsilosis* respectively. Anidulafungin concentrations exceeding 1 times the MIC caused an average net log reduction in growth of all *C. parapsilosis*, *C. orthopsilosis* and C. *metapsilosis* isolates when compared to the starting inoculum.

MIC Correlation with Log Change: The relationship between log change at 8 times the MIC at 24 hours was explored for each isolate with each drug used in this study and displayed variation by species and drug (Figures 3.16, 3.17, 3.18). Caspofungin displayed no apparent increase in activity with increasing MICs and there did not appear to be a relationship between MIC and log change. Anidulafungin displayed less activity with increasing MICs with the *C. parapsilosis* and *C. orthopsilosis* isolates, though no

relationship was seen between MICs and the *C. metapsilosis* isolates. Micafungin displayed no apparent increase in activity with increase in MIC in the *C. parapsilosis* and *C. orthopsilosis* isolates. Micafungin's relationship with between log change and MIC in the *C. metapsilosis* isolates could not be established do to the limited number of isolates.

Using the analytical grade caspofungin and anidulafungin displayed no relationship between MIC and log change between for all isolates tested, in any antifungal *Candida species* combination.

Discussion

The echinocandin classes of antifungal agents are often considered to be a class of antifungal which display predominately fungicidal activity against Candida species. However, from the results of this study these agents are primarily fungistatic and in some instances display little to no inhibitory activity (38, 39, 40, 41, 42). This is consistent with the findings of Varga et al. (46) that reported a primarily fungistatic activity with caspofungin against a small number of *C. parapsilosis* and *C. orthopsilosis* isolates using the same methodology (46). Fungicidal activity was reported with caspofungin against *C. metapsilosis* in the study by Varga et al. (46), but only at 16 and 32 times the MIC (46).

Caspofungin time-kill curve studies demonstrated antifungal activity which was exclusively fungistatic or displayed a ≤99.9 % reduction in CFU/ml when compared to the starting inoculum. This fungistatic activity was primarily seen in the *C. parapsilosis* isolates, with little to no activity seen with the *C. orthopsilosis* and *C. metapsilosis* isolates. Anidulafungin time-kill curve studies demonstrated antifungal activity which was primarily fungistatic and was active against all three species of isolates tested. Micafungin time-kill curve studies demonstrated antifungal activity which was mostly

fungistatic or displayed a ≤99.9 % reduction in CFU/ml when compared to the starting inoculum. This fungistatic activity seen with micafungin was seen with the *C. parapsilosis* and *C. metapsilosis* isolates, with little or no activity observed with the *C. orthopsilosis* isolates. It did appear from the mean log change calculated at the 8 times the MIC during the 24 mark that differences in the relative activity of the three antifungal agents was displayed. Anidulafungin was the most active agent against *C. parapsilosis*, followed by caspofungin and then micafungin. Anidulafungin was also the most active antifungal agent against *C. orthopsilosis,* followed by caspofungin with micafungin displaying no activity. Anidulafungin was again the most active agent against *C. metapsilosis*, followed by micafungin with caspofungin demonstrating no activity against these isolates. The overall results of our time-kill study consistent with what had been previously stated in earlier papers, and that is drug susceptibility is both dependent on the specific species being examined and on the particular drug being used (43, 46, 63).

The lack of activity with caspofungin in some of the isolates, specifically against the *C. metapsilosis* caused us to reconsider the stability of the commercial powder used in this assay. The echinocandins used in this analysis were made into stock solutions with the appropriate diluents according to packaging instructions supplied by the respective drug manufacturers and were stored frozen at -70 ° C in the dark until used. We believe that stability of the echinocandin commercial powder used in this analysis did not affect the results of this study. With the isolates that showed no activity according to time-kill methodology, we would suspect that if stability was an issue we would identify activity early on in the analysis and than a decrease in activity over time as the antifungal degraded. We identified a consistent lack of activity at all time points we sampled. The positive control that we tested displayed the appropriate and expected activity using or methodology and stock solutions of the echinocandins. A separate analysis was conducted, selecting isolates used in the previous study and using analytical grade caspofungin and anidulafungin powder. Interestingly, though fewer isolates were tested

we observed greater activity overall in the time-kill studies. Once again anidulafungin was the most active against all the *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis* isolates demonstrating fungistatic activity which was consistent with the results of our earlier analysis. Caspofungin while not as active as anidulafungin against the isolates did display greater activity with the analytical powder as compared to the commercial powder against all three species. This increase in activity was most apparent against *C. metapsilosis*, where in the earlier analysis we observed no activity. With the isolates of the three species of *Candida* that were selected and analyzed using the analytical grade powder, the results were consistent with previously published data regarding the activity of caspofungin against *C. parapsilosis* isolates (46).

As time kill studies are frequently an extension of the MIC test, one would assume that a relationship between the MIC and the results of the time-kill study should be observed. Isolates with relatively high MIC values should be the most resistant to therapy and those with low MIC values should respond best to treatment. Based on the results of this study, echinocandin activity by time-kill was not related to the MIC and in some cases appeared to be negatively correlated. The results of the MIC and time-kill correlation test suggests that isolates with higher MICs actually responded better to the *in vitro* antifungal treatment then those isolates with lower MIC values. One possibility is that the difference in starting inoculums could at least be in part responsible for this lack of correlation between these two tests. The standard CLSI susceptibility testing method for MICs for fungal isolates recommends a starting inoculum of 1×10^3 to 5×10^3 CFU/ml, where as the starting inoculum recommended for time-kill studies is 1×10^5 to 5×10^5 CFU/ml (27). This lower starting inoculum could account for lower MIC estimates then one would infer from the time-kill tests using a higher starting inoculum.

The CLSI have designated breakpoints based on the MIC that that define *Candida species* as susceptible to echinocandins if the *in vitro* MIC obtained is ≤ 2 mcg/ml and resistant at a value of ≥ 2 mcg/ml. In this study all isolates tested had *in vitro* MICs ≤ 2

mcg/ml and fungistatic activity was observed dependent on the species and antifungal used. The only exception to this was 1 isolate of *C. parapsilosis* that had a reported MIC of 4 mcg/ml to anidulafungin. However, in this case fungistatic activity was still observed in this isolate when challenged with anidulafungin suggesting that the MIC may not be related to the rate and extent of activity of the echinocandins against this species.

Caspofungin has been shown in previous studies to display what is called a paradoxical effect against some *Candida species* (48, 49, 50). Paradoxical effect can be defined as a decreased activity of an antifungal agent or growth that occurs at concentrations well above the minimum fungicidal concentration (MFC) and MIC (48, 48, 50). It was observed that the *C. parapsilosis* isolates displayed the greatest activity or log reduction at concentrations of the echinocandins 4 times the MIC. And lower activity was observed at concentrations of the echinocandins 8 times the MIC. This decreased level of activity beyond a maximum level of activity may be suggestive of paradoxical phenomenon, however what is typically described in the literature is no activity and growth beyond a concentration known to be fungicidal. In these isolates we only observed a modest decrease in activity.

Conclusion

Overall the results of this study demonstrate that the echinocandin antifungal agents display primarily fungistatic activity against *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis*. However this activity does differ by drug and species. This may be particularly the case with *C. metapsilosis* which displayed no activity when challenged with the caspofungin commercial powder. The results of this study highlight the importance of the species identification of the C*andida* being treated. The apparent lack

of correlation between MIC and reduction in CFU/ml by time-kill methods is also concerning.

This work displays the fungistatic activity of the echinocandin antifungal agents on large number of clinical isolates that include *C. parapsilosis* and the newly identified *C. orthopsilosis* and *C. metapsilosis* and adds a large body of work to rapidly expanding area of research.

Table 3.1 Minimum inhibitory concentration (mcg/ml) for *Candida parapsilosis.*

	Candida parapsilosis		
	Capsofungin MIC	Anidulafungin MIC	Micafungin MIC
	0.5	2	0.5
	0.25	1	2
	0.5	1	0.5
	0.5	2	0.5
	0.25	1	2
	0.25	2	2
	0.12	4	1
	1	1	1
	0.5	2	1
	0.5	2	1
	1		1
	0.5		1
	0.5		
	0.5		
Median	0.5	2	1
Range	0.12-1	1-4	0.5-2

Table 3.2 Minimum inhibitory concentration (mcg/ml) for *Candida orthopsilosis*.

	Candida orthopsilosis		
	Capsofungin MIC	Anidulafungin MIC	Micafungin MIC
	0.12	2	1
	0.25	2	0.5
	0.25	1	0.25
	0.25	1	0.5
	0.12	2	0.25
	0.25	0.5	0.25
	0.12	2	
	0.06	1	
	0.5	1	
	0.06		
	0.06		
	0.03		
Median	0.12	1	0.375
Range	0.03-0.5	0.5-2	0.25-1

Table 3.3 Minimum inhibitory concentration (mcg/ml) for *Candida metapsilosis.*

	Candida metapsilosis		
	Capsofungin MIC	Anidulafungin MIC	Micafungin MIC
	0.12	0.25	0.5
	0.12	0.5	0.25
	0.12	0.25	0.25
	0.12	1	
	0.06	0.5	
	0.06	2	
	0.12	0.5	
	0.12	0.25	
	1	0.5	
	0.12		
	0.12		
Median	0.12	0.5	0.25
Range	0.06-1	0.25-1	0.25-0.5

Table 3.4 Time-kill activity results: log change at 24 hours. (Commercial Powder)

Drug	Organism		0.25X	0.5X	1X	2X	4X	8X
Caspofungin	*C. parapsilosis*	(n=14)	0.96(0.8)	-0.02(0.92)	-0.83(0.87)	-1.43(1.02)	-1.37(0.83)	-1.1(0.74)
	C.orthopsilosis	(n=12)	1.63(1.09)	1.96(0.25)	1.47(1.31)	1.28(0.91)	0.48(0.93)	-0.19(1.13)
	C. metapsilosis	(n=11)	1.5(0.67)	1.54(0.76)	0.75(1)	0.68(1.27)	0.73(1.19)	0.67(1.43)
Anidulafungin	*C. parapsilosis*	(n=10)	-0.01(0.96)	-0.41(0.59)	-0.97(0.59)	-1.51(0.92)	-1.86(0.96)	-1.18(0.88)
	C.orthopsilosis	(n=9)	0.31(0.97)	-0.47(1.02)	-1.5(1.02)	-1.78(1.07)	-1.66(1.35)	-1.33(0.98)
	C. metapsilosis	(n=9)	0.52(0.86)	0.85(0.35)	2.4(0.35)	-1.4(1.18)	-1.55(1.03)	-2.12(0.77)
Micafungin	*C. parapsilosis*	(n=12)	1.2(0.52)	0.17(0.83)	0.24(1.58)	-1.03(1.81)	-1.2(1.55)	-0.9(1.13)
	C.orthopsilosis	(n=6)	0.67(1.16)	0.86(0.73)	0.93(1.33)	1.14(0.36)	1.12(1.59)	0.77(0.82)
	C. metapsilosis	(n=3)	1.64(0.29)	0.54(0.66)	0.47	-0.33(1.13)	-2.1	-0.46(2.67)

Note: Mean log growth or reduction in CFU (Standard Deviation) from starting inoculums for each concentration tested (multiple of MIC) at 24 hours.

Table 3.5 Time-kill activity results: log change at 24 hours. (Analytical Powder)

Drug	Organism		0.25X	0.5X	1x	2X	4x	8x
Caspofungin	*C. parapsilosis*	(n=2)	1.6(0.16)	1.12(0.82)	0.28(0.52)	-1(0.1)	-1.5(0.6)	-1.69(0.1)
	C.orthopsilosis	(n=4)	1.78(0.32)	1.68(0.22)	0.94(0.82)	-0.2(0.85)	-1.14(0.83)	-1.42(0.84)
	C. metapsilosis	(n=2)	1.98(0.08)	0.8(0.74)	0.37(0.16)	-0.18(0.47)	-1.24(0.59)	-1.35(0.45)
Anidulafungin	*C. parapsilosis*	(n=2)	0.35(0)	0(0.51)	-0.86(1.07)	-2.62(0.42)	-2.62(0.26)	-2.71(0.29)
	C.orthopsilosis	(n=2)	0.95(0.67)	0.41(1.68)	-1.23(1.44)	-2.05(0.92)	-1.19	-2.29(0.58)
	C. metapsilosis	(n=2)	1.18(0.79)	0.22(0.04)	-0.26(0.45)	-1.03(0.78)	-1.45(0.13)	-2.12(0.34)

Note: Mean log growth or reduction in CFU (Standard Deviation) from starting inoculums for each concentration tested (multiple of MIC) at 24 hours.

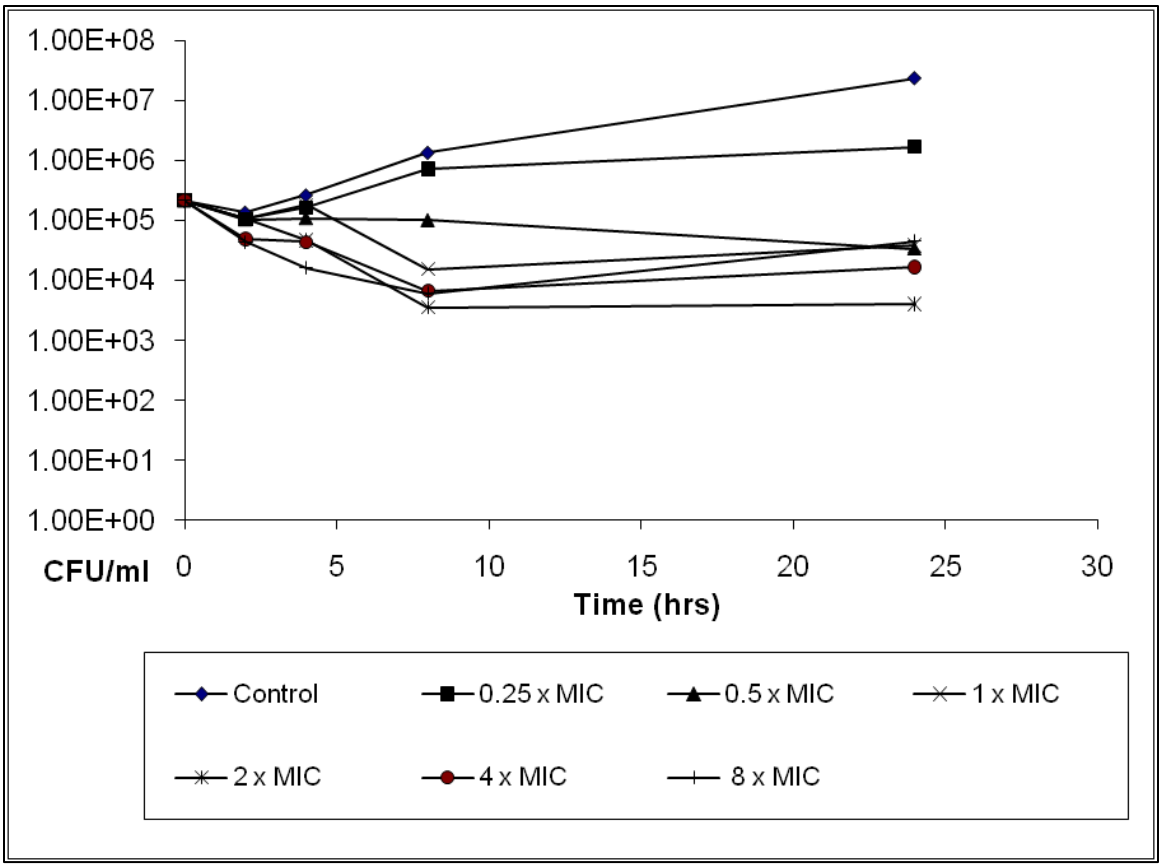

Figure 3.1 Representative time-kill curve for caspofungin activity against *Candida parapsilosis*. (Commercial Powder)

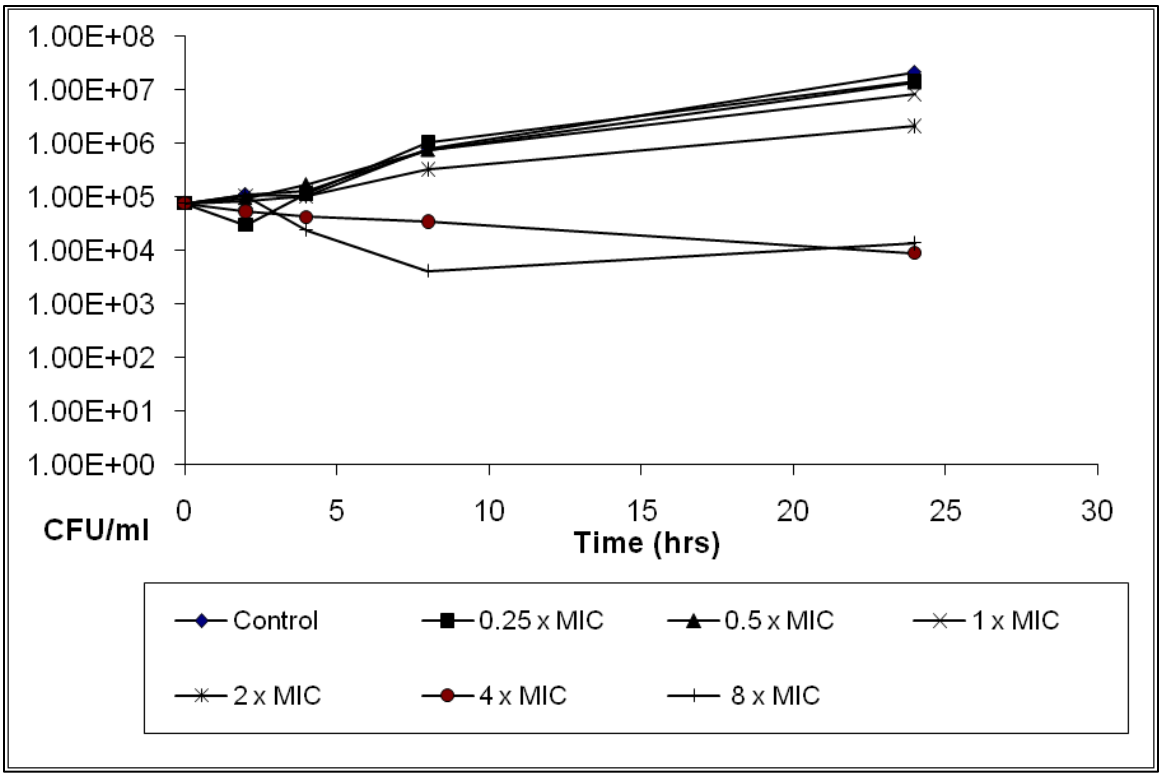

Figure 3.2 Representative time-kill curve for caspofungin activity against *Candida orthopsilosis*. (Commercial Powder)

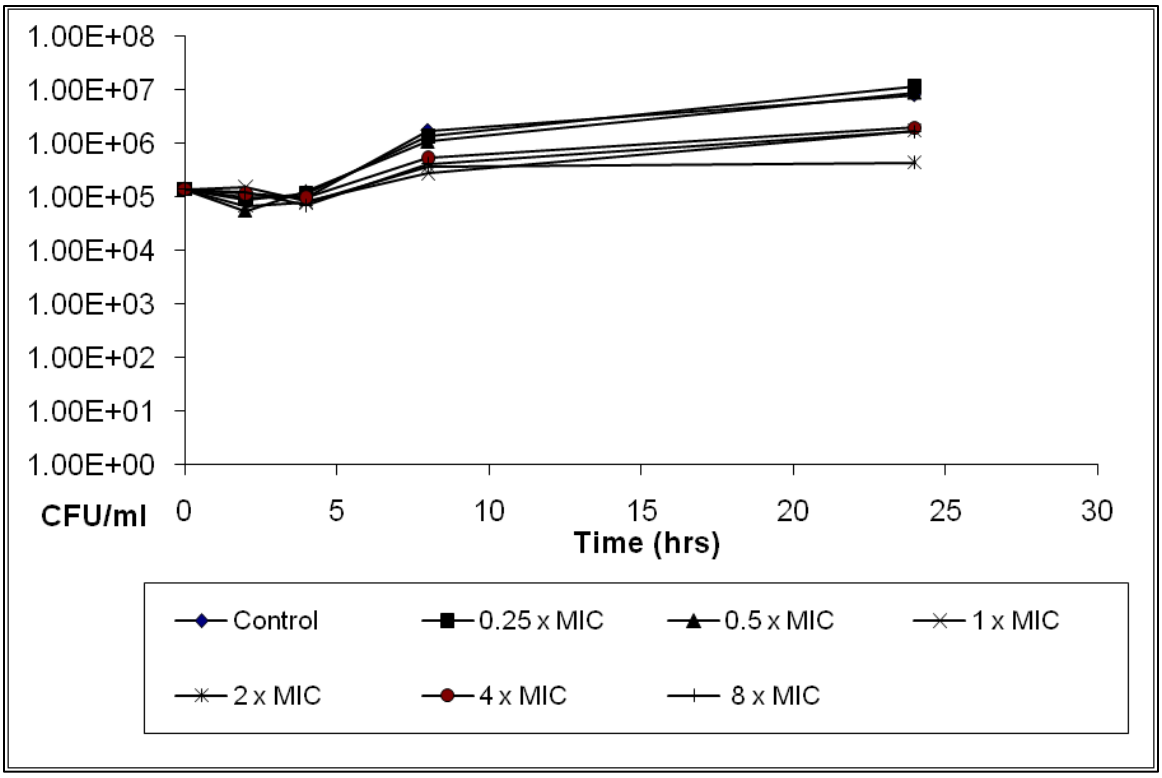

Figure 3.3: Representative time-kill curve for caspofungin activity against *Candida metapsilosis*. (Commercial Powder)

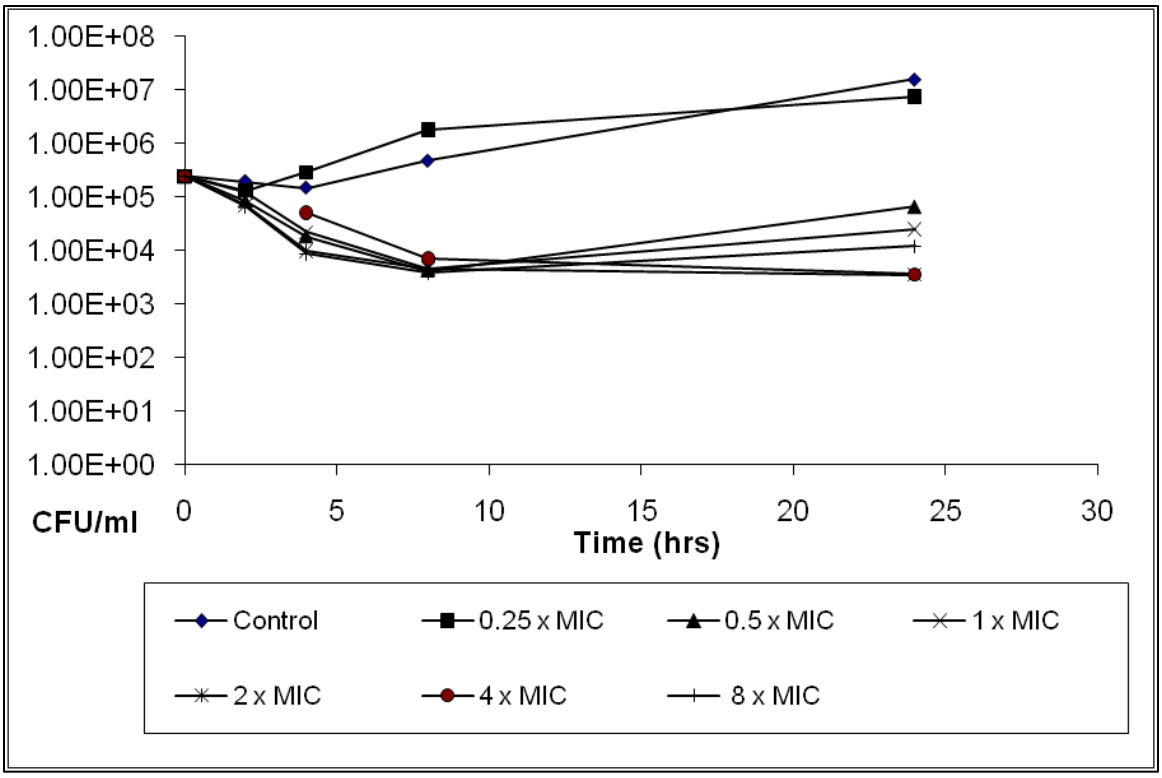

Figure 3.4 Representative time-kill curve for anidulafungin activity against *Candida parapsilosis*. (Commercial Powder)

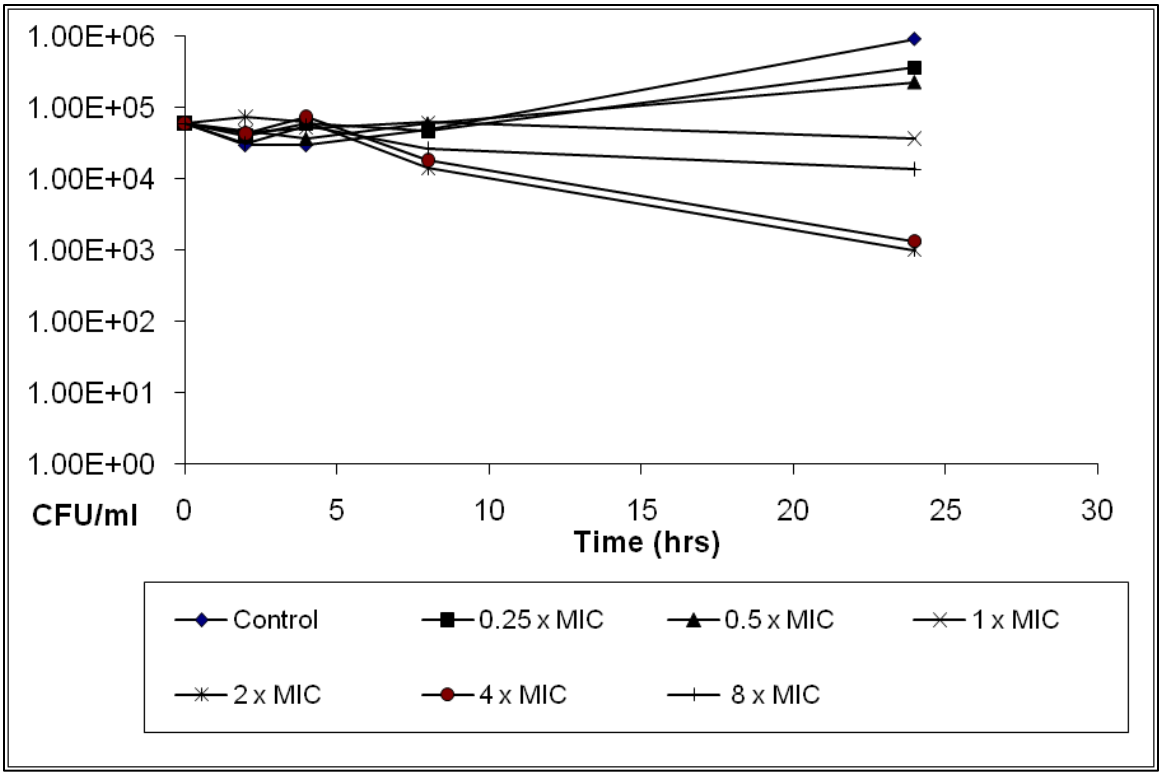

Figure 3.5 Representative time-kill curve for anidulafungin activity against *Candida orthopsilosis*. (Commercial Powder)

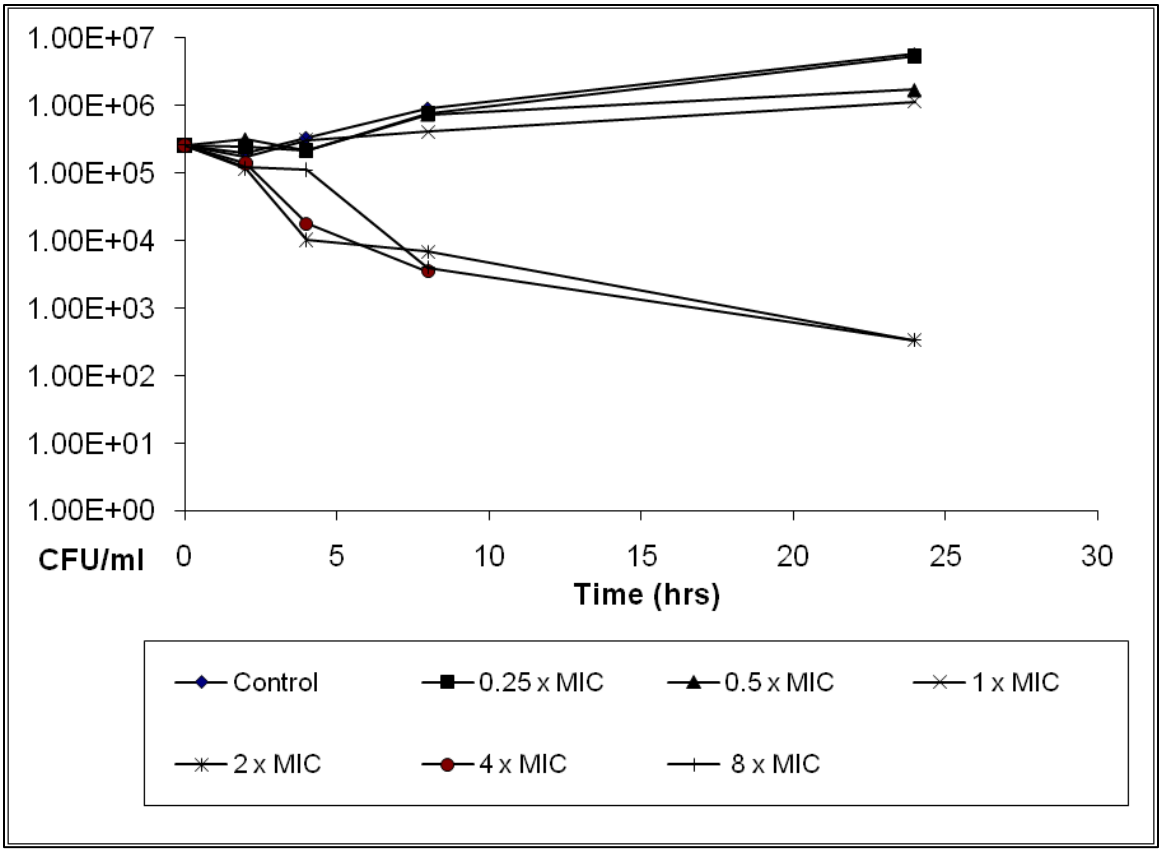

Figure 3.6 Representative time-kill curve for anidulafungin activity against *Candida metapsilosis*. (Commercial Powder)

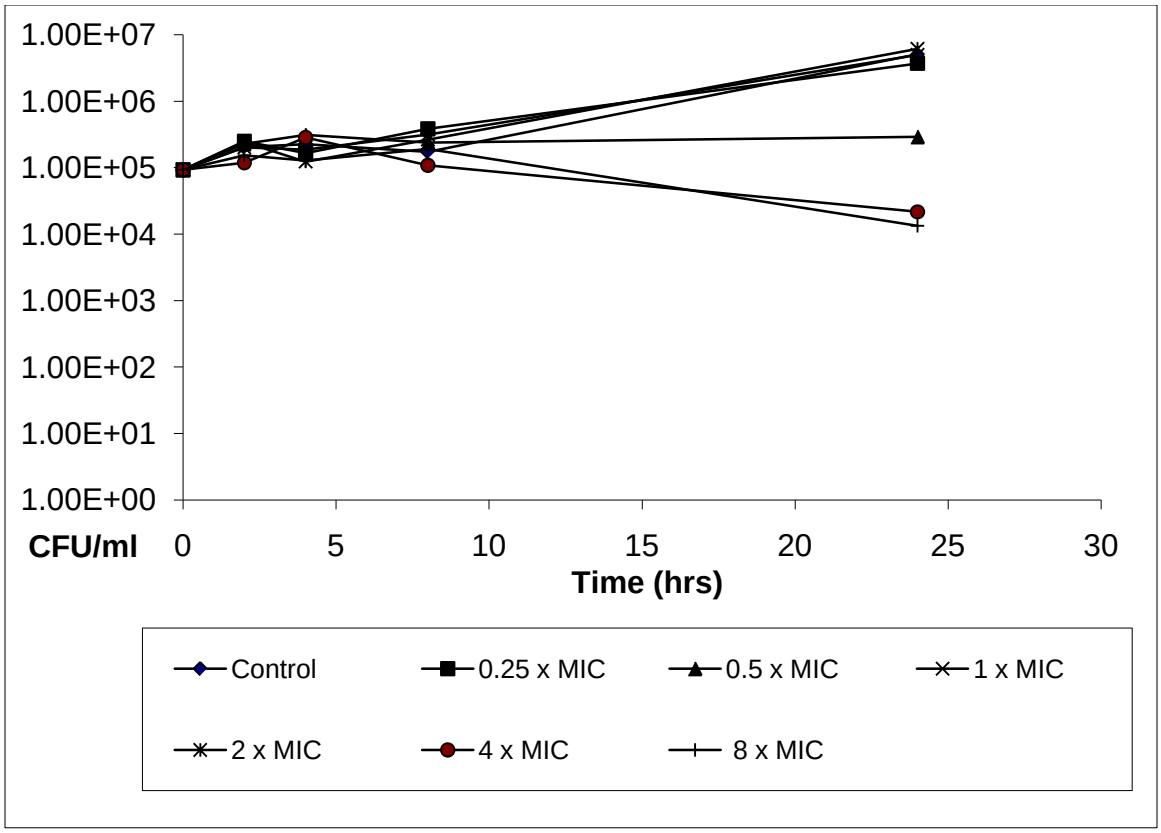

Figure 3.7 Representative time-kill curve for micafungin activity against *Candida parapsilosis*. (Commercial Powder)

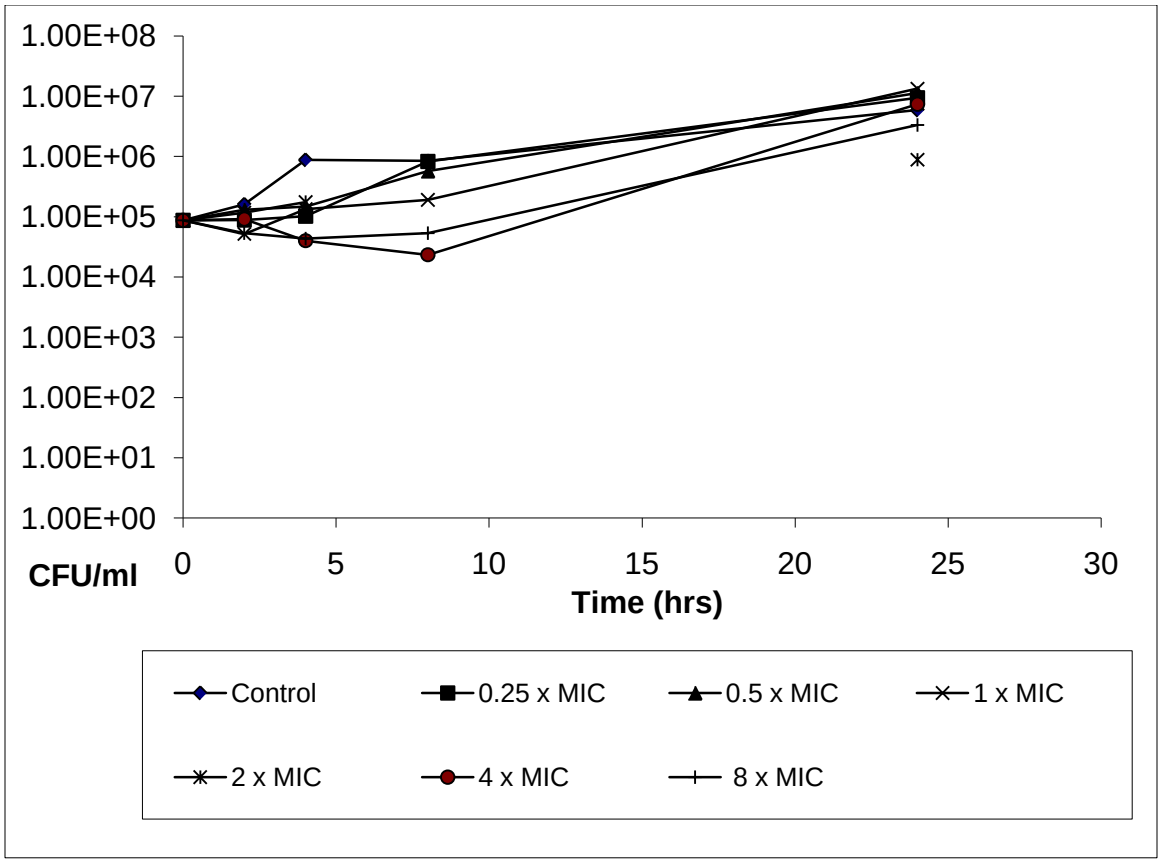

Figure 3.8 Representative time-kill curve for micafungin activity against *Candida orthopsilosis*. (Commercial Powder)

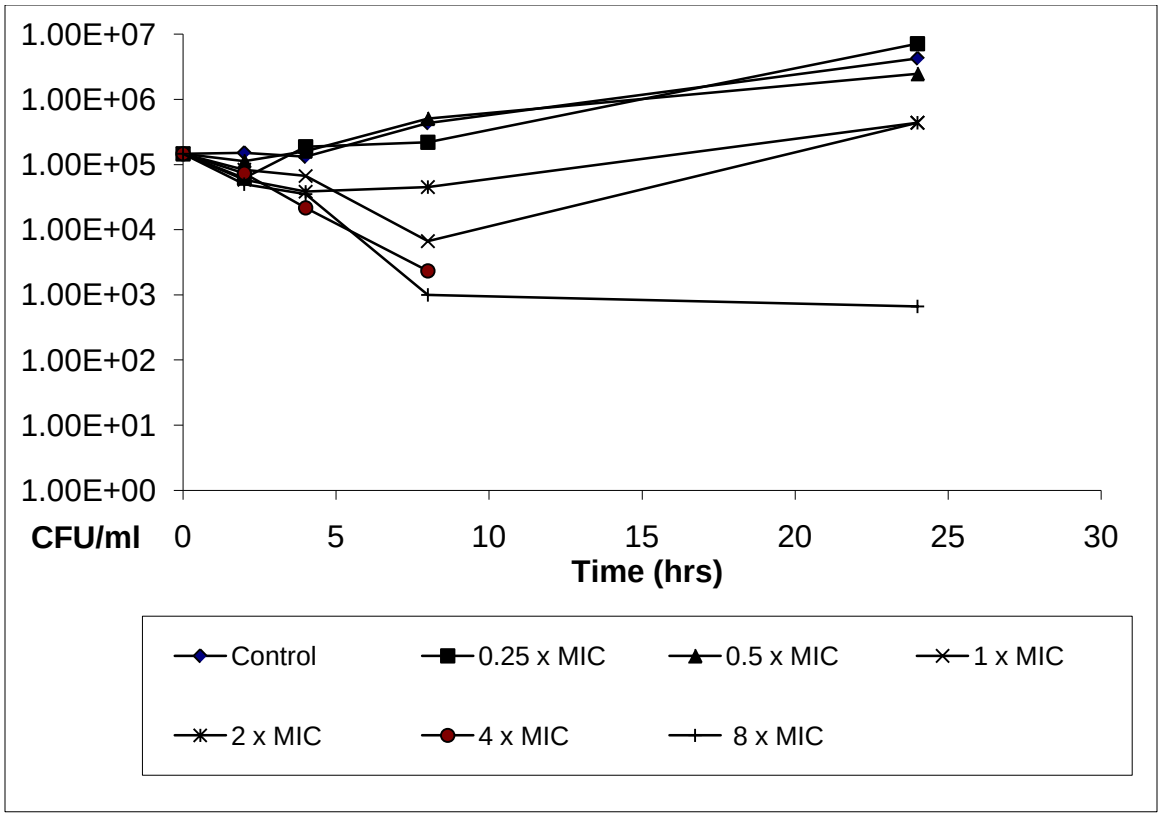

Figure 3.9 Representative time-kill curve for micafungin activity against *Candida metapsilosis*. (Commercial Powder)

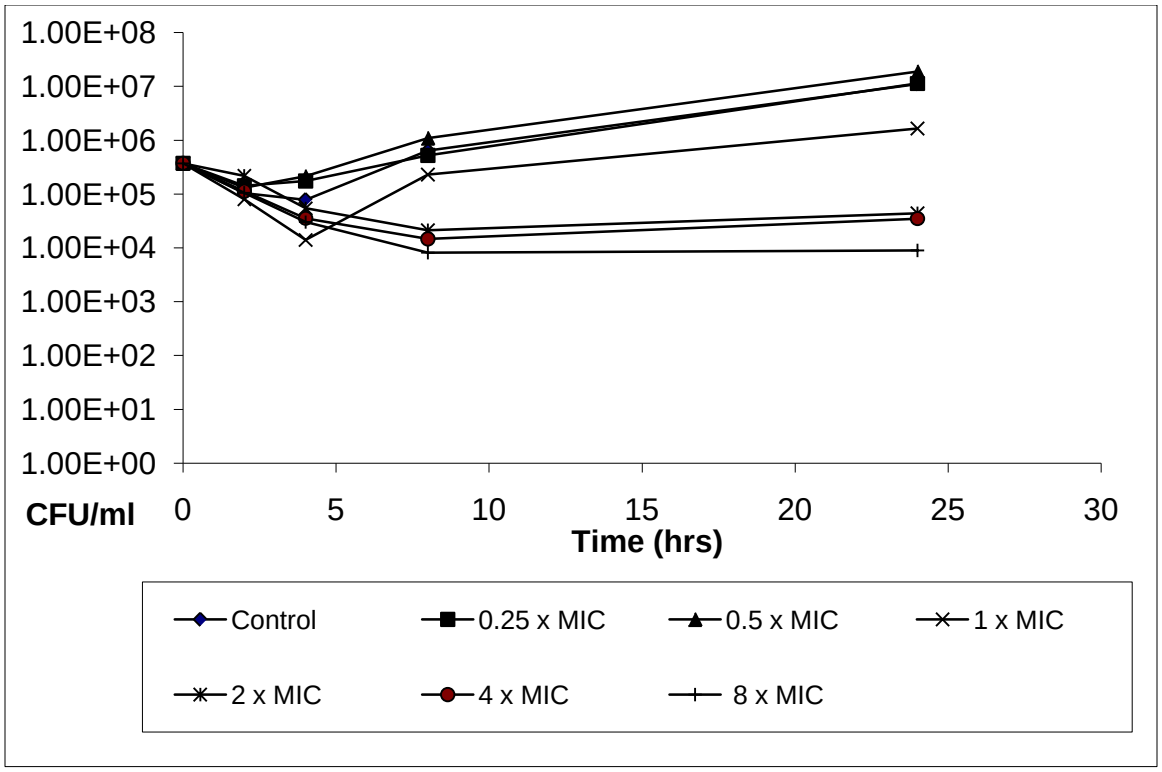

Figure 3.10 Representative time-kill curve for caspofungin activity against *Candida parapsilosis* (Analytical Grade Powder).

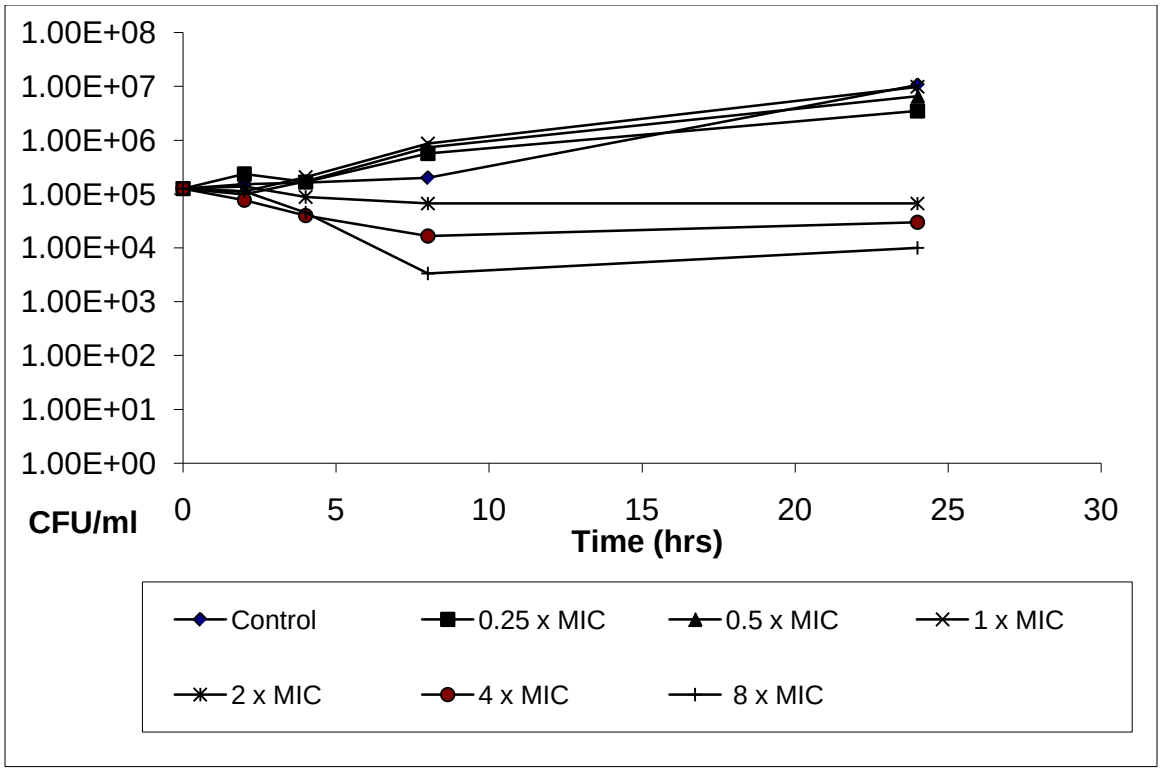

Figure 3.11 Representative time-kill curve for caspofungin activity against *Candida orthopsilosis* (Analytical Grade Powder).

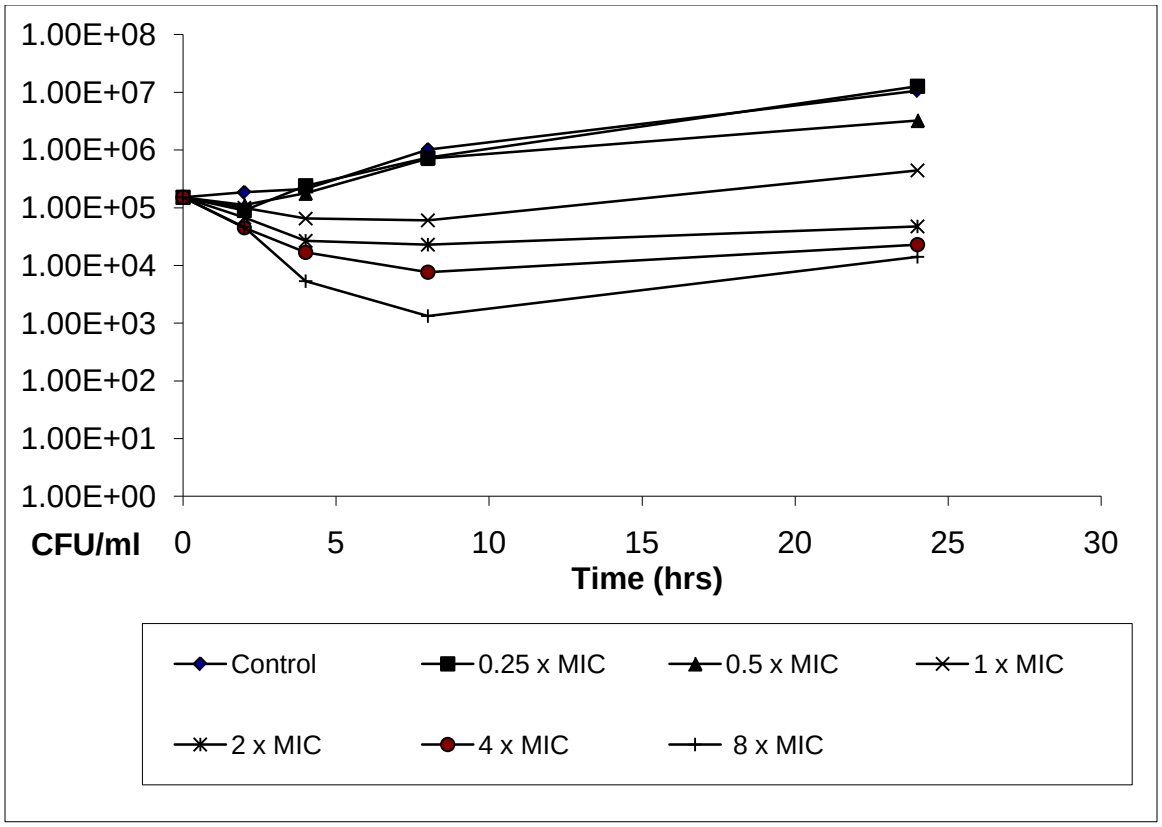

Figure 3.12 Representative time-kill curve for caspofungin activity against *Candida metapsilosis* (Analytical Grade Powder).

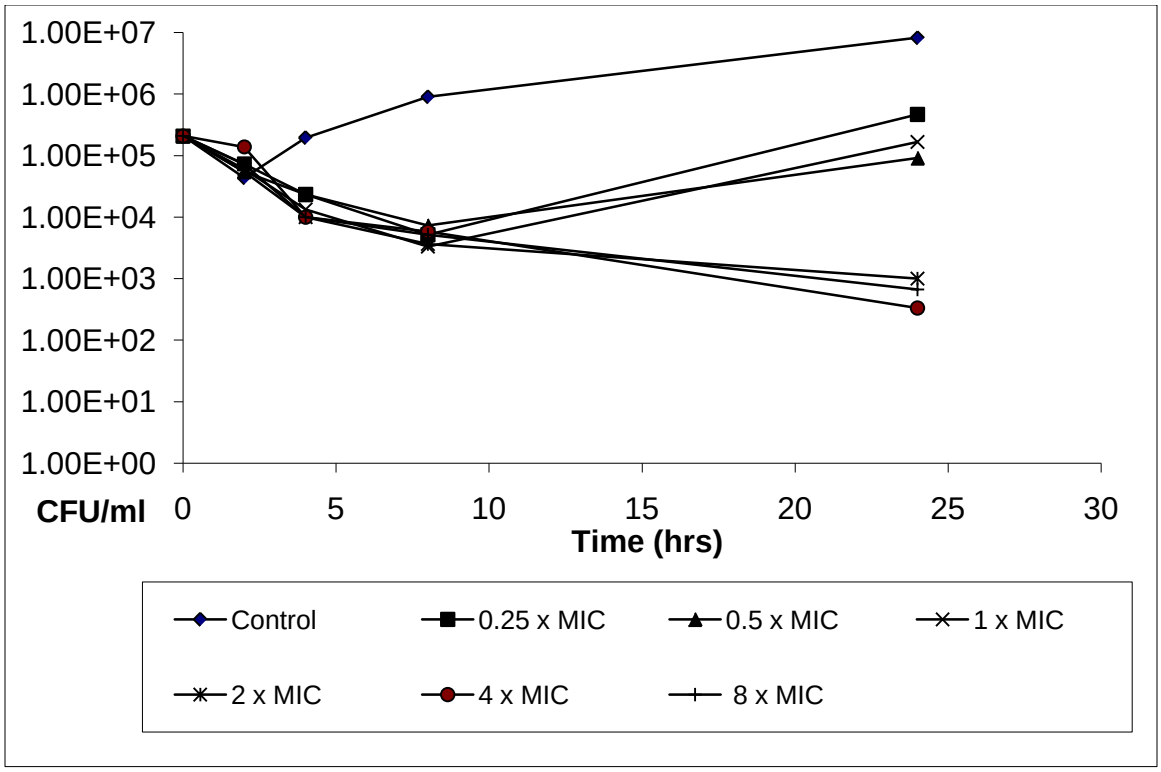

Figure 3.13 Representative time-kill curve for anidulafungin activity against *Candida parapsilosis* (Analytical Grade Powder).

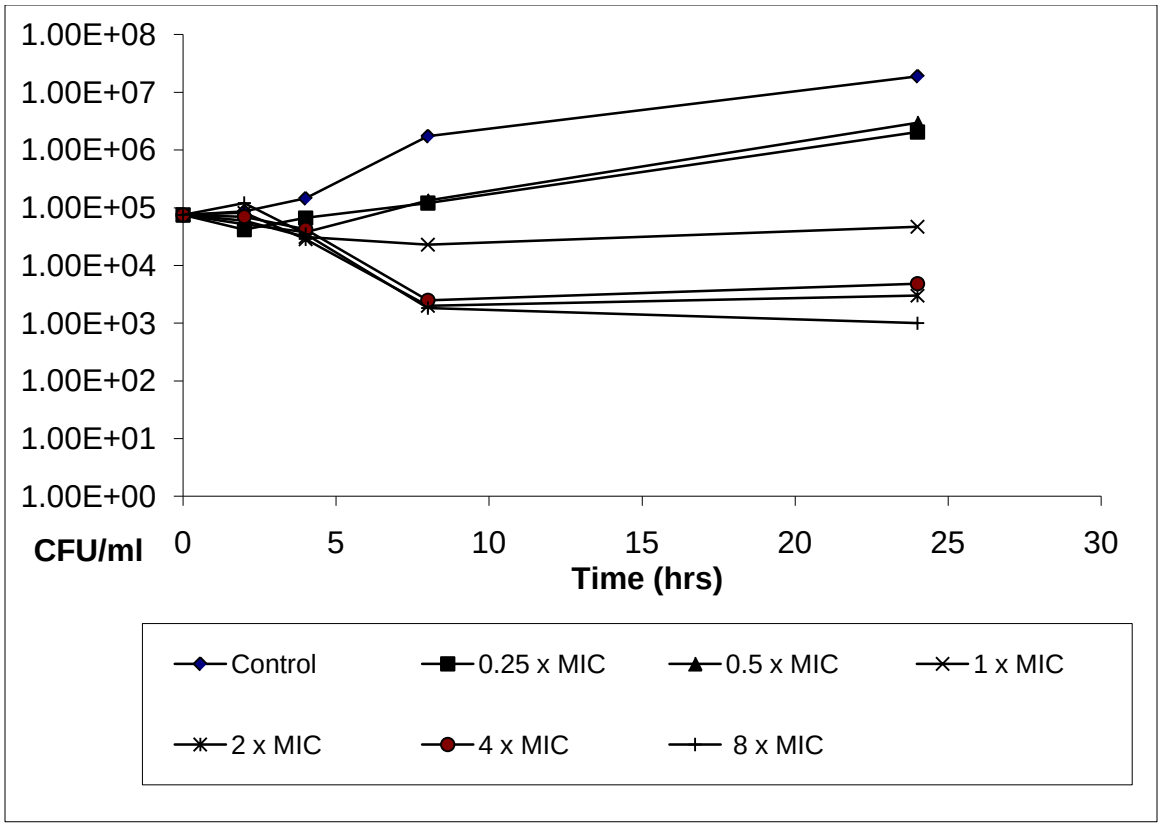

Figure 3.14 Representative time-kill curve for anidulafungin activity against *Candida orthopsilosis* (Analytical Grade Powder).

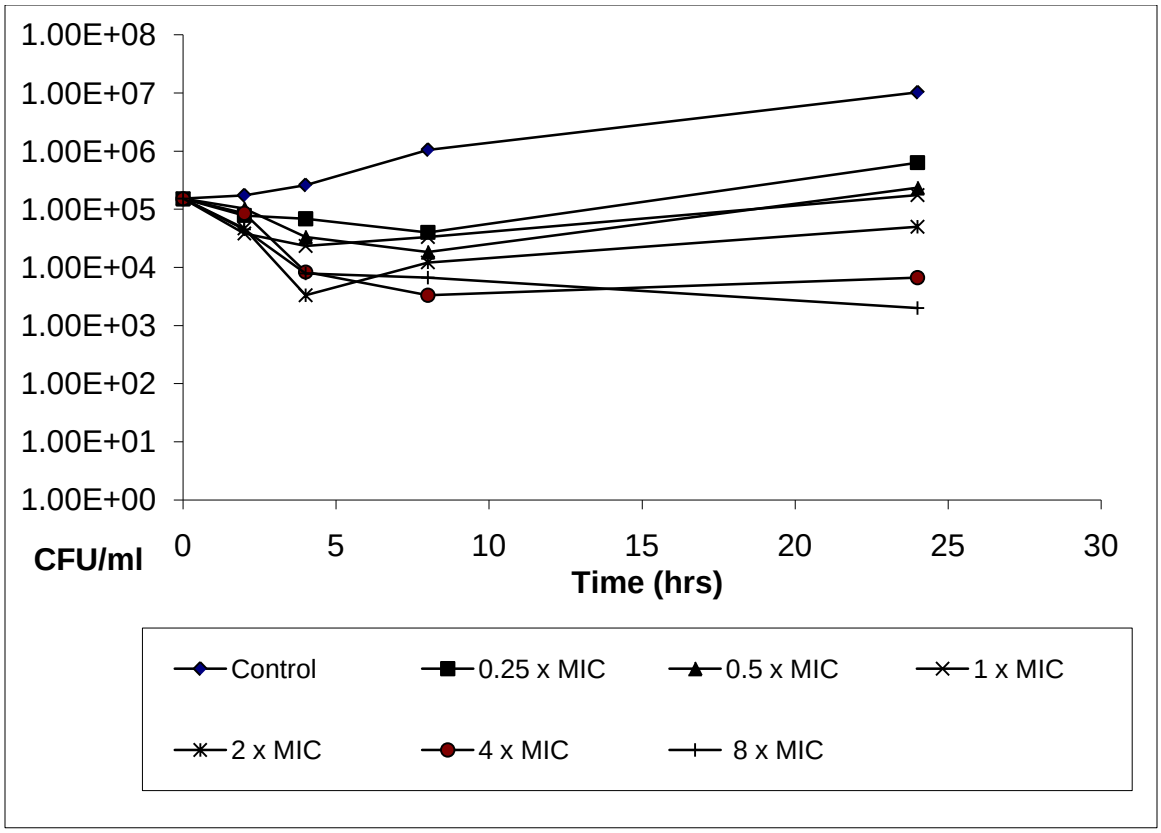

Figure 3.15 Representative time-kill curve for anidulafungin activity against *Candida metapsilosis* (Analytical Grade Powder).

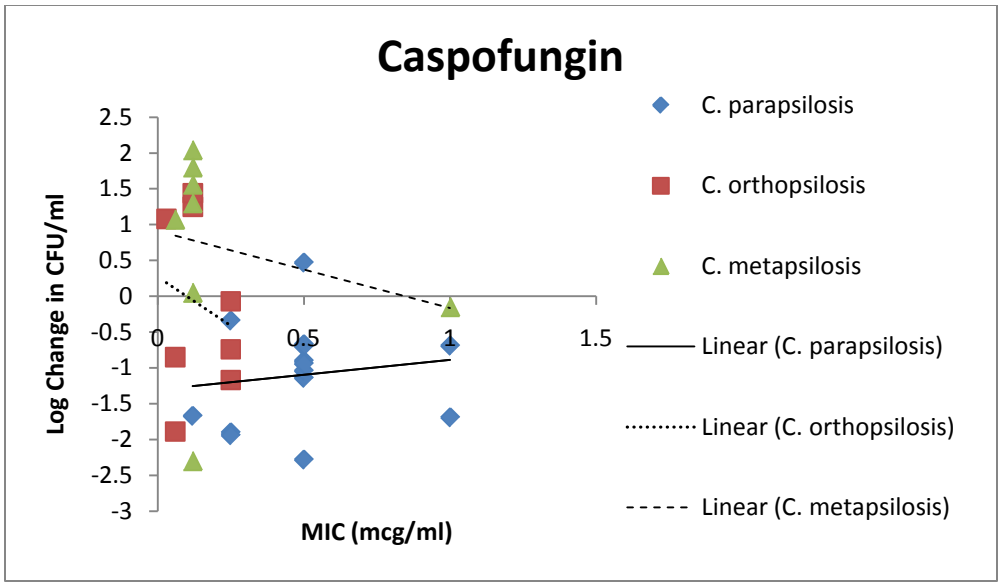

Figure 3.16 Relationship between log changes at 8 x the MIC at 24 hours with caspofungin. (Commercial Powder)

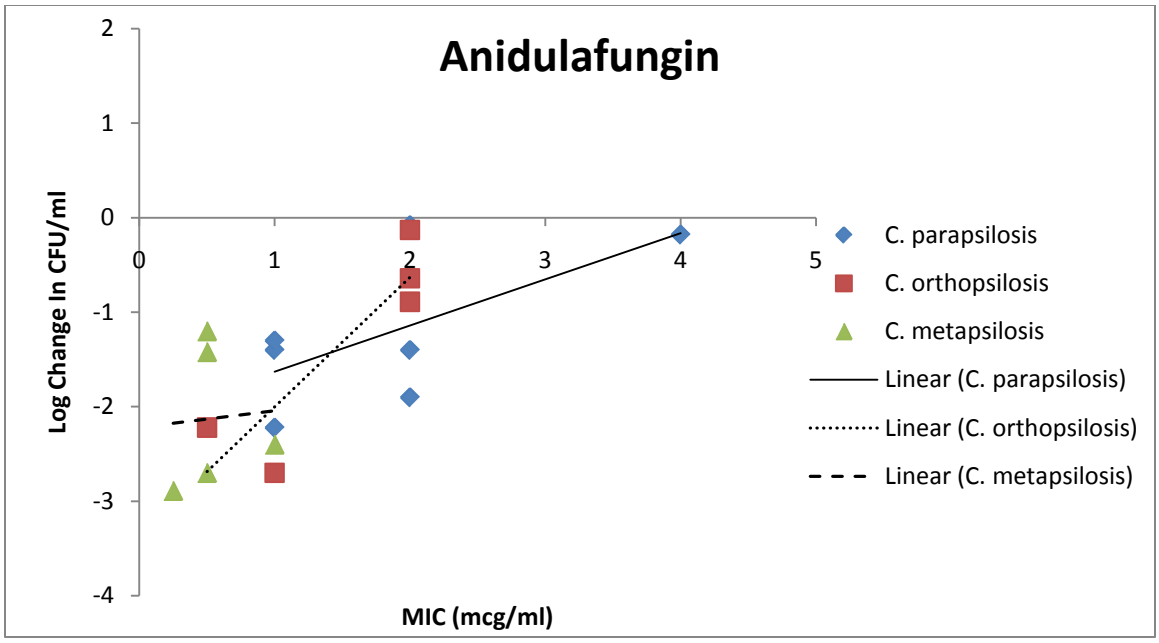

Figure 3.17 Relationship between log changes at 8 x the MIC at 24 hours with anidulafungin. (Commercial Powder)

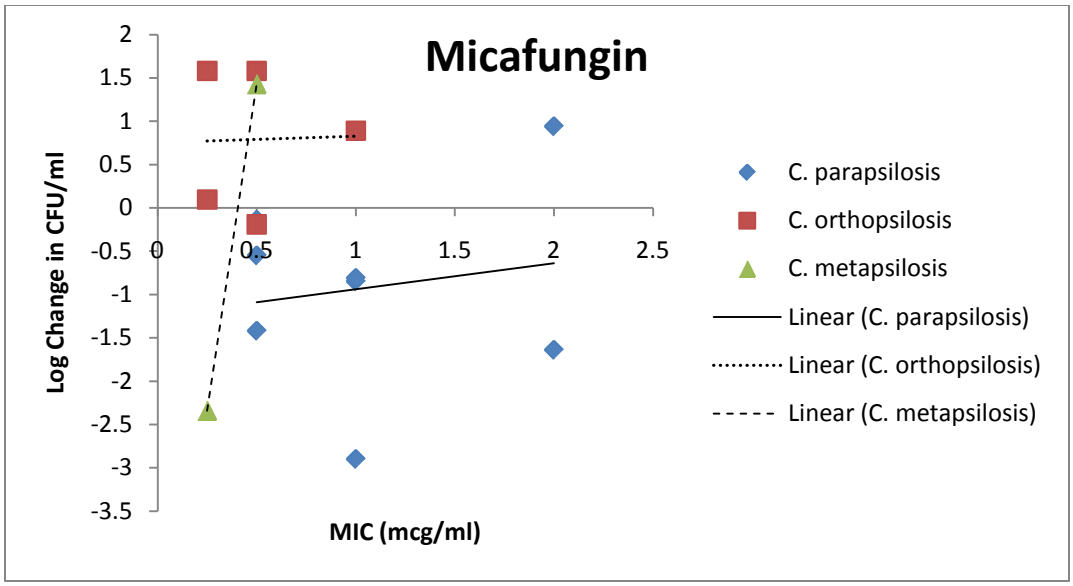

Figure 3.18 Relationship between log changes at 8 x the MIC at 24 hours with micafungin. (Commercial Powder)

CHAPTER 4

SUMMARY AND CONCLUSIONS

With the relatively recent emergence of candidemia as a major cause of human disease and mortality in our health care system, clinicians must identify ways to minimize this additional burden on individuals who are already suffering from serious underlying medical conditions (12). The growing population of those who are immunocompromised, the increasing use of broad spectrum antibiotics, metabolic disorders such as diabetes mellitus, the growing population of the very young or the very old and the use of immunosuppressive chemotherapy has all been implicated in facilitating this increasing incidence of fungal infections (12).

Blood stream infections resulting from Candida species have been shown to have some the highest rates of in appropriate therapy among all BSIs from any other source (12, 29). Identification of those patients who may be at increased risk for the development of nosocomial candidemia can be used to guide early empiric therapy. Early treatment with the appropriate therapy should maximize the likelihood of reducing unnecessary patient mortality and reduce costs associated with health care.

Substantial variability in the susceptibility of fungal isolates to antifungal agents has been well established (43, 63). The identification of the specific species of *candida* that requires antifungal management is of the utmost importance given the high rates of resistance and lower levels of activity some therapeutic agents have against fungal pathogens.

The results of our own research have identified some patient specific clinical factors that may be of help in the early identification of patients at risk for candidemia. In CHAPTER 2, we presented the results of a case-case-control study done at a single health care center. We observed that patients in the *C. glabrata* cohort were more likely to have

gastrointestinal disorders and peripheral vascular disease than patients suffering from *C. albicans* BSIs. We also determined that when compared to the uninfected control group, patients with *C. glabrata* BSIs were more likely to have been prior colonized with *C. glabrata,* undergone dialysis, and have been catheterized with both arterial and urinary catheters. We concluded that patient exposure to unique clinical risk factors may be predictive of the development of future candidemia and may help distinguish between albicans versus non-*albicans* candidemia.

Though larger, multicenter studies are required to validate these results they do add to a growing body of work aimed at improving patient outcomes.

In CHAPTER 3, we performed a drug susceptibility study using time-kill methods with the echinocandin antifungal agents on *Candida parapsilosis* and two newly identified species of *Candida, C. orthopsilosis* and *C. metapsilosis*. The echinocandins as a group displayed primarily fungistatic activity against the clinical isolates tested. However, we observed substantial variability in antifungal activity that varied by both the echinocandin used and *Candida* species analyzed. We concluded that this variability in activity that is both species and drug dependent should be considered when selecting the treatment of candidemia resulting from these non-*albicans* species.

. This variability if confirmed by further studies may affect which antifungal agents will ultimately be used to treat such infections.

Overall, the described research if confirmed either prospectively or retrospectively will help to improve clinical outcomes of antifungal therapy and reduce patient mortality through the timely initiation of appropriate antifungal therapy (i.e. identification of patient specific risk factors for the different types Candidemia and/or describing the activity of specific antifungal agents to specific species of Candidemia).

APPENDIX

PEER REVIEWED PUBLICATIONS AND MANUSCRIPTS BY

JESSE L. HOLLANBAUGH

1. Klevay MJ, Ernst EJ, Hollanbaugh JL, Miller JG, Pfaller MA, Diekema DJ. Therapy and outcome of Candida glabrata versus Candida albicans bloodstream infection. *Diagn Microbiol Infect Dis*. 2008 Mar; 60(3):273-7.

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