BIOFILM PRODUCTION AND SUSCEPTIBILITY AMONG *CANDIDA ALBICANS* ISOLATES FROM VARIOUS CLINICAL SITES

by

Ellen Grace Press

Bachelor of Science in Microbiology, University of Michigan 2009

Submitted to the Graduate Faculty of

the School of Pharmacy in partial fulfillment

of the requirements for the degree of

Master of Science

University of Pittsburgh

2014

UNIVERSITY OF PITTSBURGH

SCHOOL OF PHARMACY

This thesis was presented

by

Ellen Grace Press

It was defended on

October 27, 2014

and approved by

Cornelius J. Clancy, MD; School of Medicine, Infectious Diseases

Kerry M. Empey, PharmD, PhD; Department of Pharmacy and Therapeutics

Thesis Directors:

Samuel M. Poloyac, PharmD, PhD; Department of Pharmaceutical Sciences

Ryan K. Shields, PharmD, MS; School of Medicine, Infectious Diseases

BIOFILM PRODUCTION AND SUSCEPTIBILITY AMONG *CANDIDA ALBICANS* ISOLATES FROM VARIOUS CLINICAL SITES

Ellen Grace Press, B.S.

University of Pittsburgh, 2014

Candida albicans exists as a commensal in healthy adults but is one of the most common causes of fungal infections in the United States. Candida is known to form biofilms (highly organized networks of cells adherent to a surface) on foreign devices and host tissues; infections associated with these structures are associated with increased virulence and drug resistance. However, the *in vitro* methods of growth and quantification used to assess these characteristics are poorly standardized. *In vitro* studies suggest minor alterations in growth conditions can drastically affect resultant structures. This project seeks to determine the best methods for biofilm growth and analysis. Utilizing these methods, this study examines whether biofilm production of clinical *Candida albicans* isolates varies based on conditions of clinical collection, namely the presence or absence of a urinary or bloodstream catheter at time collection and clinical collection site. Additionally, the relationship between extent of biofilm production and antifungal susceptibility will be examined. Eighteen bloodstream (n=10) or urine (n=8) clinical isolates, with (n=9) and without (n=9) a catheter present, will be exposed to urinary catheters and allowed to grow. Resultant biofilm will be quantified using four reported methods: biomass by crystal violet and dry weight, and metabolic activity of free-floating (planktonic) and adherent (sessile) cells, separately. Sessile bioactivity was the most reliable of tested methods, and dry weight was the least. Methods of quantification did not correlate well. Based on reproducibility and correlation, crystal violet and sessile metabolic activity, used together, provide a good indication as to the

extent of biofilm production of clinical isolates. Biofilm production did not vary for isolates based on catheter presence or clinical site at time of collection, suggesting biofilm is capable of forming under many clinical conditions. Antifungal susceptibility testing of adherent biofilms showed increased minimum inhibitory concentrations to amphotericin B and fluconazole, with minor increases for caspofungin. There was no difference in drug susceptibility by catheter association or collection site. Biofilm susceptibility is warranted in the clinic; however, quantification methods described here are both labor- and time-consuming. Future studies are needed to develop new methods of quantification.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

1.0 INTRODUCTION

1.1 CANDIDA BIOFILMS

In humans, Candida exists mainly as a commensal yeast. Asymptomatic colonization can occur throughout the human body – from the oral cavity to the gastrointestinal tract, and even on the skin. Disruption of host defenses may contribute to Candida transitioning from a commensal lifestyle to a pathogenic one; these potential disruptions include: long term antimicrobial therapy, placement of a medical devices, and surgical procedures [1].

Candida is one of many organisms able to form biofilms, or highly organized networks of cells which grow adherent to a biotic or abiotic surface. Within these structures, two populations of phenotypically and genetically distinct cells exist; those which grow attached to a given surface are called sessile cells while unattached, free-floating cells in the surrounding environment are called planktonic cells [2, 3]. *Candida albicans*, a dimorphic yeast, is the most common of the five main species of Candida that cause infection in humans and was the focus of this study. Filamentation, a process unique to the species *C. albicans*, is not explicitly required for biofilm formation. However, this differentiation allows for more complex, physically stable structures form [4-6]. During biofilm formation, dividing cells attach to a surface. They begin to produce the extracellular matrix (ECM), a complex protective covering. The ECM is made up of a variety of substances, both secreted by growing cells and trapped in the structure from the

surrounding environment. These may include: carbohydrates, lipids, trapped cell fragments, and even viable viral particles [7, 8]. The matrix serves, most importantly, as a layer of protection, masking cellular immune trigger molecules present on cellular membranes and functioning to prohibit antifungals from accessing growing cells by physical blockage and/or drug entrapment [9, 10].

1.2 CLINICAL RELEVANCE

The lifestyle of biofilm production by Candida is a clinically relevant concern as production has been linked to increased virulence and drug resistance [11-13]. Biofilm growth is associated with a variety of indwelling devices, including catheters, shunts, contact lenses, and dentures [14, 15]. In cases of infection, *Candida spp*. can be isolated from various sites throughout the human body, including normally sterile sites such as the bloodstream or urine. In fact, Candida species are the fourth leading cause of bloodstream infections in United States hospitals, with *C. albicans* accounting for over half of invasive candidiasis cases, and *C. albicans* alone is the third most common cause of catheter associated urinary tract infections [16, 17]. Unfortunately, increased use of medical devices is related to an increased occurrence of complications. Indwelling devices are very common in the intensive care unit, where a recent study reports the worldwide intensive care unit prevalence of candidemia (Candida present in the bloodstream) to be nearly 7% [18]. In a clinical setting, if a device is known to be infected, removal is recommended and known to be effective in many cases. This is not always feasible considering possible device locations [19, 20]. If the device itself is not the source of the infection, reseeding and regrowth on the device may occur.

Knowing the likely decreased susceptibility of Candida biofilm growth, confirming presence on a device is important to determine appropriate treatment, especially in instances where device removal is problematic or reseeding is likely. Many mechanisms of antifungal resistance in *C. albicans* biofilms have been reported, including the up-regulation of drug efflux pumps and altered genetic expression [12, 21]. This study will assess if the extent of biofilm production by a clinical isolates is related to increased resistance *in vitro.* Current methods of clinical susceptibility testing used to guide antifungal therapy are based on planktonic cell susceptibility. Biofilm structures consist of sessile (adherent) cells which behave differently than surrounding planktonic (free-floating) cells. *In vitro* studies show that these populations react differently to antifungal challenge [12, 22, 23] and current susceptibility methods are incapable of appropriately accounting for sessile cell populations.

There are three main classes of antifungal drugs in use today. The polyene amphotericin B (AMB) is broadly applicable, and there are few reports of resistance. However, renal toxicity limits clinical use. It functions by binding ergosterol in the fungal cell wall and presumably causes pore formation and cell death [24]. Fluconazole (FLUC), a member of the fungistatic azole class, functions by blocking synthesis of the same cell wall component, ergosterol, and it is widely prescribed for invasive candidiasis. High levels of resistance to azoles of Candida biofilms have been reported for nearly 20 years [25]. The newest class of fungicidal agents are the echinocandins (anidulafungin, caspofungin (CSP), and micafungin). These agents, which inhibit synthesis of specific cell wall glucans, are highly effective against Candida infections and highly tolerable by patients. Limited reports of clinical resistance exist, often related to a specific genetic mutation [26]. Paradoxical growth (significant growth above the MIC) in the presence of

high concentrations of caspofungin is seen in *in vitro* testing, though the clinical relevance remains unclear [27].

1.3 TECHNICAL HURDLES

A discord exists between clinical susceptibility testing and the environment in which Candida live *in vivo*. Simple, standardized methods for growth, quantification and susceptibility testing of both sessile biofilm Candida cells are needed to address this gap. Few studies have attempted to look at clinically relevant Candida biofilm susceptibility patterns, due in part to the complexities of *in vitro* biofilm growth and quantification. Over time, many different methods for the determination of Candida biofilm biomass and bioactivity have been published [22, 28-30]. Biomass is most often established by crystal violet staining or dry weight measurement, and bioactivity is determined by the enzymatic reduction of XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) into formazan dye.

It is known that even small manipulations in protocols can drastically change results [31, 32]. Different media, substrates, temperatures, incubations periods, and/or Candida strains lead to the formation of phenotypically different structures. For example, one study shows that the irregular substrate polymethacrylate is capable of supporting *Candida albicans* biofilms 25-30 microns high. In the same study, organism exposed to smooth silicone elastomer resulted in biofilm over ten times as high (450 microns) [2]. On the contrary, Teflon, a very smooth surface was shown by another group to be much less capable of supporting biofilm growth as compared to irregular surfaces [31]. Beyond those of substrate irregularity, other alterations can be made:

ranging from basic growth in a 96 well culture plate to complicated and expensive systems which mimic blood or urine flow through a vessel [22, 33-35].

Testing susceptibility introduces an additional layer of complexity. Susceptibility is dependent on structure, growth conditions affect structural changes based on conditions [36]. Specifically, it is well recognized that different substrates result in biofilms of varying mass, strength, and activity [31, 36]. One recent study looked at the susceptibility of 115 clinical isolates, but biofilms were grown on polystyrene, a material not commonly used in medical devices [37]. Length of incubation following adherence and time of drug exposure, generally an additional 24 or 48 hours, are not often consistent. As biofilms age and mature, tolerance increases, which has been linked to increased metabolic activity [2]. Therefore, quantification and susceptibility of biofilm will vary based duration of the *in vitro* growth period. It is unclear how the observed changes in tolerance over time *in vitro* connect to the "age" of a biofilm within a patient. Taken together, selecting the appropriate *in vitro* conditions is a critical step in developing a biofilm assay.

In this study, Candida isolates were collected from either the human bloodstream or urine – two distinct environments. The experimental conditions *in vitro* were standardized across isolates to detect differences in biofilm production. Importantly, there are differences between the bloodstream and urine, such as pH and access to viable nutrients, which may affect an organism's ability to produce biofilm. Moreover, immune cells and products are more often present in the bloodstream. Organisms capable of developing biofilms in spite of the immune responses found in the more nutrient-rich bloodstream seem likely to have an increased fitness in comparison to organisms which have had to adapt to life in the harsher environment of the urine.

1.4 PURPOSE

Additional *in vitro* research is needed to address the current limitations of biofilm quantification and antifungal susceptibility testing of biofilm-associated Candida. Until these gaps in knowledge are addressed, the clinical impact of biofilm production cannot be clearly understood.

For this study, clinical *Candida albicans* were collected from two common sites of infection, the bloodstream and the urine. These sites are often associated with indwelling catheters, known to be associated with biofilms. This study seeks to identify if detectable differences of biofilm production exist in organisms themselves rather than simply environmental conditions. Isolates propagated in the blood and urine – comparable to media differences for *in vitro* studies, may have adapted to growth under their respective *in vivo* environments to the point that *in vitro* testing is capable of discerning this.

Specifically, I hypothesize that clinical *Candida albicans* isolates collected through an indwelling catheter will have an increased ability to form biofilm due to their exposure to a device *in vivo*. Additionally, I hypothesize that biofilm production will be lower among isolates collected from the urine as they will have adapted to a less nutritionally robust environment. In other words, isolates in the urine may be more dependent on obtaining nutrients rather than altering lifestyle to aid survival, while bloodstream isolates have decreased pressure to perform basic cellular functions and can therefore adapt in other ways, including an increased production of biofilm. Furthermore, I expect that increased biofilm production will be associated with decreased antifungal susceptibility.

Three objectives are proposed to test these hypotheses. First, identification of reproducible methods of biofilm growth and quantification on a clinically relevant substrate must

be established. Second, biofilm production by catheter association and collection site will be compared. Thirdly, the relationship between biofilm production and sessile antifungal susceptibility will be assessed. As described here, these objects are within the scope of a Master of Science thesis project.

2.0 EXPERIMENTAL DESIGN

2.1 ISOLATES

Eighteen clinical *Candida albicans* isolates from the bloodstream (n=10) or urine (n=8) were collected from unique patients at the University of Pittsburgh Medical Center between 2010 and 2013, as a part of routine care. Nine isolates were collected from patients without indwelling catheters at the site of collection. Nine were collected from patients through a bloodstream or urinary catheter which had been in place for ≥ 3 days. These will be referred to as "catheter associated" throughout further analyses. Isolates were stored at -80°C in yeast peptone dextrose (YPD) broth containing 20% glycerol. Prior to each set of experiments, organisms were subcultured on Sabouraud dextrose agar overnight, and a single colony was chosen to inoculate YPD at 35°C overnight, with agitation. Inoculums were standardized using a spectrophotometer and diluted in Roswell Park Medical Institute (RPMI) medium at pH=7.0 supplemented with 2% glucose to a final concentration of $1x10^6$ cfu/ml prior to catheter adherence.

2.2 BIOFILM GROWTH ON A CLINICAL SUBSTRATE

Literature review and preliminary tests were performed to determine the best substrate for use in this study. Of four tested substrates (three types of urinary catheters and an intravenous needle),

Bard® urinary catheters were chosen for their clinical applicability and ability to readily support Candida biofilm, as well as for availability and low cost. After the first set of quantification experiments were completed, the catheter used became unavailable. Additional quantification experiments and sessile susceptibility testing were completed on a Bard® urinary catheter with 28% larger surface area, per one centimeter segment. Composition was identical. Methods of preparation, growth, and quantification of biofilm were the same for both sets of experiments. Owing to the aforementioned idiosyncrasies in measuring biofilm production, results for the two sizes of catheter will be presented separately. Biofilms were grown according to established methods, with minor adaptations - as standardized one centimeter long catheter segments were used throughout [22, 28, 29].

Figure 1 shows the process of growth and quantification that was applied for all experiments. Segments were sterilized by autoclave and incubated at 37°C overnight with inactivated fetal bovine serum (FBS) prior to inoculation with *C. albicans*. Serum preconditioning has been shown to reduce electrical and hydrophobic interactions between Candida cells and substrate, more consistent with *in vivo* conditions [31]. The use of 37°C throughout also more closely mimics *in vivo* conditions. Serum was aspirated and segments were rinsed twice with sterile water to remove residual FBS. Each segment was transferred to a sterile 24-well culture plate, and 1.5 ml of organism at $1x10^6$ cfu/ml was added to entirely cover the segment. *C. albicans* was allowed to adhere for 90 minutes at 37°C on a rocking table. Media and organism were immediately aspirated, and segments were washed twice with room temperature phosphate buffered saline (PBS) at pH=7.4 to remove non-adherent cells. Catheter pieces were moved to a fresh 24-well plate containing 1.5ml fresh, room temperature RPMI per

well. Plates were arranged in Ziploc bags, to avoid over-evaporation of media, and incubated for 48 hours at 37°C on a rocker.

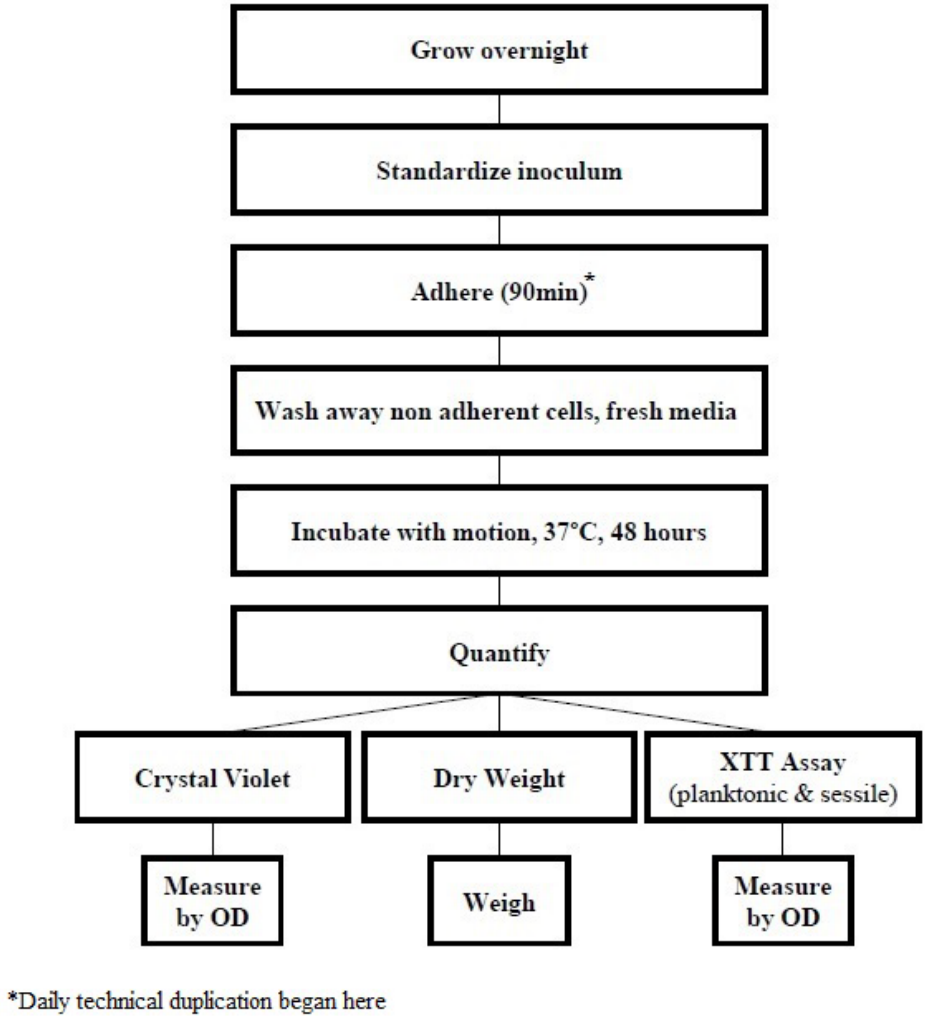

Figure 1: Growth and Quantification of Biofilms. From a single isolated colony, each of the 18 isolates were grown and quantified as shown above. The entire process was completed twice (once on each narrow and wide segments).

2.3 QUANTIFICATION

Each isolate was added to two separate catheter segments for each method of biomass production and two for measurement of metabolic activity (planktonic and sessile activity were performed on the same catheter segment). Following the 48 hour growth period, plates were removed from the incubator and each structure was quantified (Figure 2).

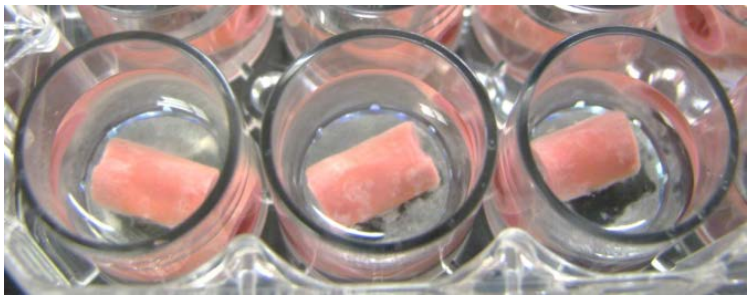

Figure 2: *Candida albicans* Biofilm Growth on a Catheter Segment. Off-white *Candida albicans* biofilm growth on one centimeter segments of red rubber catheter is visible to the naked eye after 48 hours.

2.3.1 Biomass

Biofilm mass was determined using two commonly referenced methods [29, 37]. The first utilizes crystal violet staining to determine the presence of biofilm material, living and non-living, on the catheter surface [38]. For this project, after the 48 hour growth period, planktonic cells were aspirated after the 48 hour growth period and segments were carefully transferred to a sterile 48-well culture plate. One milliliter of 1% crystal violet in PBS was added, ensuring full submersion of the catheter. Stain was allowed to penetrate for 45 minutes at room temperature, then vacuumed (Figure 3, Panel A). Segments were rinsed twice with sterile water to remove

excess stain. To release attached stain (Figure 3, Panel B), 1ml of 70% ethanol/10% isopropanol was added for 15 minutes.

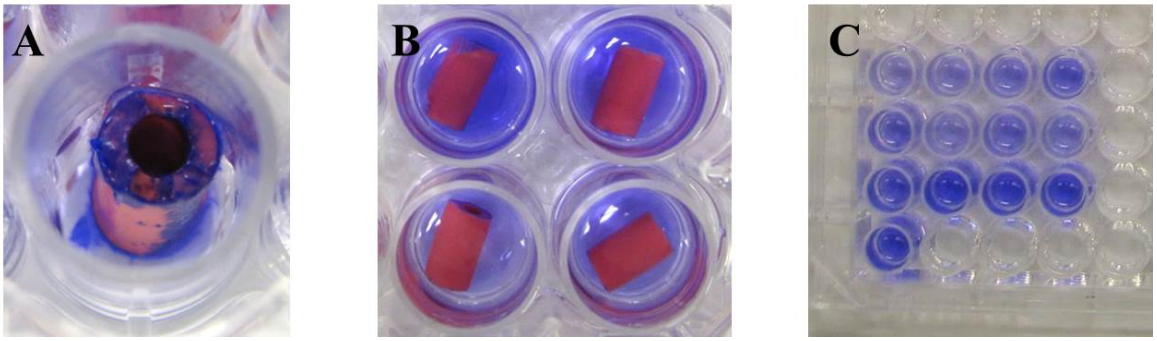

Figure 3: Crystal Violet Quantification of Sessile Biomass. Panel A shows the purple staining of the biofilm growing adherent to the pink catheter segment. Panel B shows the release of dye which is then transferred to a fresh 96 well plate for OD readings, as shown in panel C.

From each catheter containing well, 200µl of released dye were moved to a 96 well plate (Figure 3, Panel C), in duplicate, and optical density (OD) was measured at a wavelength of 490 nanometers (nm). Special care was taken not to disturb biofilm structure, which would interfere with OD readings. OD of the alcohol mixture alone was also measured, and readings were adjusted to remove this background signal.

The second method of biomass determination measures dry weight. Standard methods of dry weight determination involve removal by scraping or sonication and collection by centrifugation [29]. Multiple movements of these small flakes of cellular material are required throughout this process, which is undesirable as accuracy is often sacrificed as sample is often lost. Therefore, in this study, assessment of dry weight was significantly modified to ensure inclusion of biofilm structure present on the interior lumen or the catheter and to remove any acknowledged inaccuracies introduced by scraping methods. Here, pre-growth catheter weight

was subtracted from dried, post-growth weight to determine net biofilm weight. Planktonic cells were removed and catheter segments were rinsed twice with sterile water. Liquid was aspirated and the plate lid was vented to allow segments to dry overnight.

2.3.2 Metabolic Activity

Metabolic activity was determined for planktonic and sessile cells separately using a well-established enzymatic XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) reduction assay [22, 39, 40]. The XTT assay determines metabolic activity based on cellular production of NADPH which reduces the tetrazolium ring of XTT to form an orange formazan dye. Increased OD readings correspond to a greater color change, indicative of a higher metabolic activity. XTT was suspended in PBS at 2mg/ml and filter sterilized, in batch. XTT is light-sensitive, and unstable once suspended, therefore aliquots were frozen at -80°C, protected from light, for no more than three months. Appropriate volumes were thawed and diluted to 0.5mg/ml with PBS on the day of each experiment, and 1µl of 10mM menadione was added per 10ml of enzyme to accelerate the enzymatic reaction (as described in previous reports) [30].

To determine baseline planktonic metabolic activity, overnight cultures of each organism were standardized to $1x10^6$ cfu/ml, and 100µl of organism was transferred to a 96 well culture plate, in duplicate. Each well then received 100µl of prepared enzyme (final concentration 0.25mg/ml XTT, 0.5µM menadione). To control for the turbidity caused by cellular growth, control wells for each organism were made by adding 100µl of organism and 100µl of PBS. Plates were wrapped in aluminum foil, to protect the enzyme from light, and statically incubated at 37°C for 2-3hours. Wells of XTT alone were used as to confirm no light activation occurred. Colorimetric change, which correlates to metabolic activity, was measured by OD at 490nm.

Background readings of organism plus PBS (no enzyme) were subtracted from active well values to adjust for cell interference.

After 48 hours, biofilm metabolic activity was measured for both planktonic and sessile growth. Five hundred microliters of planktonic cells were carefully transferred to a fresh plate, and the remaining planktonic cells were aspirated. Catheter segments were rinsed twice with sterile water and transferred to a fresh plate, to avoid testing activity of cells growing adherent to the polystyrene plate. To ensure similar concentrations of enzyme for both catheter and planktonic tests, 500µl of sterile, room temperature PBS was added to catheter containing wells. All wells received 500µl of prepared enzyme (final concentration 0.25mg/ml XTT, 0.5µM menadione). Plates were wrapped in aluminum foil, to protect the enzyme from light, and statically incubated at 37°C for 2-3hours. Wells of XTT alone were used as control to ensure there was no light activation or enzyme contamination. Colorimetric change, which correlates to metabolic activity, was measured by OD at 490nm by carefully transferring 200µl of enzyme to a fresh 96 well plate (Figure 4).

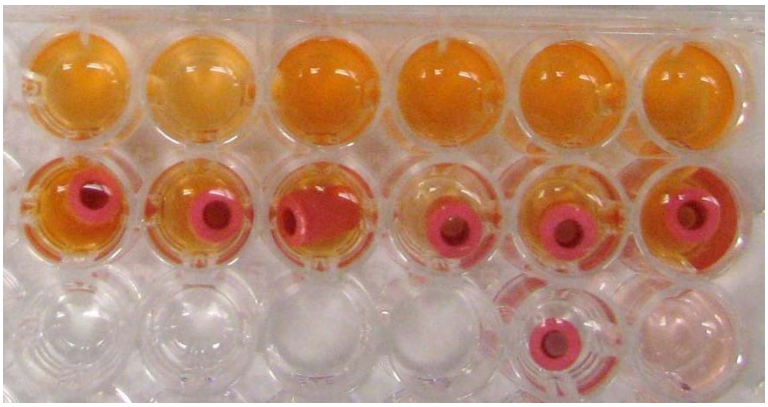

Figure 4: Bioactivity Quantification by XTT Assay. The top row contains planktonic cells corresponding to the catheter in the middle row. The bottom row contains controls. Three isolates are tested here, each in duplicate.

2.4 SUSCEPTIBILITY TESTING

2.4.1 Standard Planktonic Measurement

Minimum inhibitory concentrations (MICs) were determined against AMB, CSP, and FLUC according to the Clinical and Laboratory Standards Institute (CLSI) methods [41]. Briefly, a 1.0 x 10^3 cells/ml inoculum in RPMI, was exposed to doubling dilutions of AMB (0.015-16µg/ml), CSP (0.015-16µg/ml) and FLUC (0.06-64µg/ml) for 24 hours at 35°C. MIC endpoints were read, visually, at 50% inhibition of control (no drug) for CSP and FLUC and 100% inhibition for AMB, as defined by the CLSI.

2.4.2 Sessile Cell Measurement

For each isolate, 24 segments of wide catheter were prepared and biofilm was grown for 48 hours, as described above. Following the biofilm growth period, planktonic cells were aspirated and washed once with sterile, room temperature PBS. Eight segments were used for each drug. Segments were transferred to a sterile 48-well plate containing RPMI with doubling dilutions of drug ranging from 0.12-8µg/ml for AMB and CSP and 8-512µg/ml for fluconazole. Higher ranges were used for sessile testing than for planktonic as decreased susceptibility of biofilm populations was expected. RPMI alone served as control. Plates were incubated for an additional 24 hours at 35°C to mimic conditions of planktonic MIC testing as described by the CLSI [41]. Endpoints were determined visually in a manner similar to that for standard MICs. MICs were read as a 50% reduction of control (no drug) metabolic activity for CSP and FLUC and 100% inhibition for AMB.

In this study, drug was used as treatment rather than prevention. Therefore, measurements of biomass would not accurately evaluate the effect of drug on structures. Living cells must be distinguished from dead cells. The XTT assay as performed on catheter segments is capable of determining susceptibility. There are numerous reports in the literature describing metabolic activity as a measure of biofilm drug response [22, 28, 42]. Again, 200µl of enzyme was transferred to a fresh 96 well plate, taking special care to not remove planktonic cells or biofilm structure which would interfere with OD readings (Figure 5).

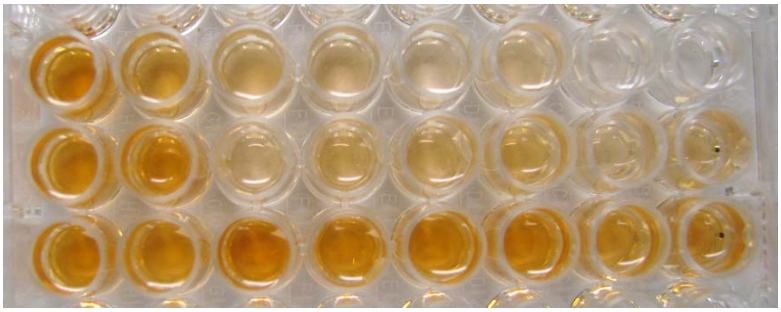

Figure 5: XTT Results for Determination of Sessile Minimum Inhibitory Concentrations. A susceptibility test of one isolate to amphotericin B (top row), caspofungin (middle row), and fluconazole (bottom row). The left most well contains no drug, and dilutions of drug begin in column two and double as wells move to the right.

2.5 STATISTICAL ANALYSIS

Analysis was performed using GraphPad Prism5 software. Continuous variables were compared by the Mann-Whitney U test, and correlations were assessed using the non-parametric Spearman correlation coefficient. These tests are appropriate for a small sample size that is not normally distributed. Fisher's exact test was used to analyze presence of paradoxical growth. Significance was set at $P < 0.05$. Intraclass correlation coefficients were calculated in SAS, version 9.4, with the help of the University of Pittsburgh Statistical Consulting Center.

3.0 RESULTS

3.1 PRELIMINARY TESTING

Baseline metabolic activity for isolates in their planktonic state was compared to ensure any variation found in biofilm metabolic activity was specific to biofilm production. Baseline activity of isolates (in their planktonic state) did not vary by collection site (Figure 6, left) or association with an indwelling catheter (Figure 6, right). There was one isolate which showed significantly higher production.

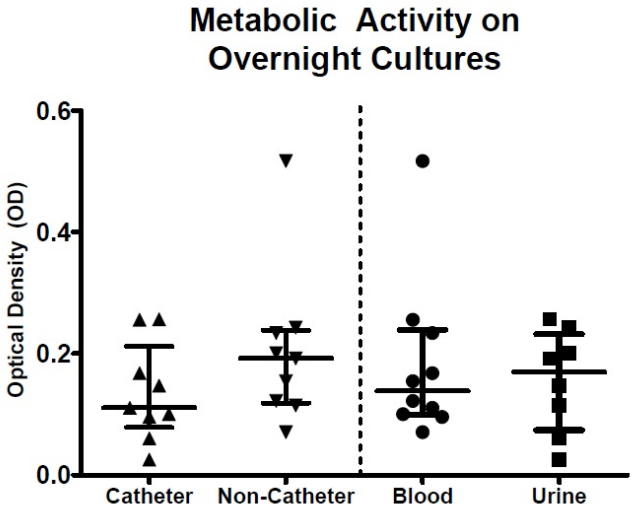

Figure 6: Metabolic activity of *Candida albicans*. Metabolic activity of isolates did not vary at baseline.

Use of a clinically relevant substrate was desired. Four clinical substrates were tested for their ability to support *Candida albicans* biofilm growth. Positive biofilm growth was defined as measureable sessile cell metabolic activity (Figure 7). Antimicrobial coated urinary catheter segments did not support growth and were therefore excluded. Intravenous needles were capable of supporting biofilm, but they proved difficult to prepare and were a potential safety hazard. Therefore, they were also eliminated. Silver ion antimicrobial coated urinary catheters did allow for growth. Red rubber urinary catheters supported growth to a similar extent as the silver coated catheters. As such, the cheaper and more readily available Bard® red rubber foley catheter was chosen as the best clinically relevant substrate to study the difference in *Candida albicans* biofilm production and susceptibility.

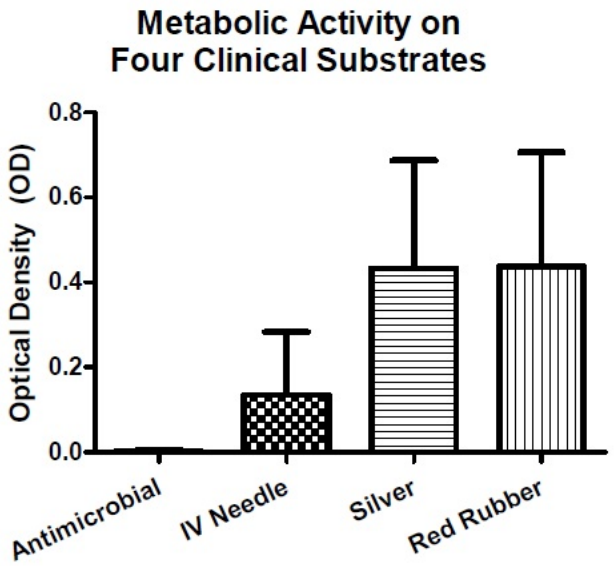

Figure 7: Substrate Testing. Four Candida albicans isolates were grown on four different clinical substrates, in duplicate. Mean optical density and standard deviation are shown here. Metabolic activity varied across substrates.

3.2 NARROW CATHETER

3.2.1 Quantification

Biofilm production of all 18 clinical *Candida albicans* isolates was quantified following 48 hours of growth on 0.5cm x 1cm catheter segments, as detailed above. Each test (crystal violet, dry weight, sessile metabolic activity, and planktonic metabolic activity) was performed in duplicate on each experiment day; Table 1 shows the average results for each isolate by each method, as well as select descriptive statistics.

Table 1: Biofilm Production on a Narrow Catheter by Four Methods. Values shown are the averaged result of two catheter segments for each isolate analyzed on the same day. Summary statistics are listed at the bottom.

Site of Collection	Catheter Association	Isolate	Crystal Violet (OD)	Dry Weight (mg)	Sessile XTT (OD)	Planktonic XTT (OD)
Bloodstream	Yes	732	0.027	0.250	0.070	0.262
		779	0.042	1.950	0.332	0.224
		816	0.043	1.950	0.312	0.257
		819	0.031	0.600	0.107	0.337
		829	0.051	1.650	0.141	0.452
	No	207	0.035	2.100	0.384	0.218
		623	0.030	1.600	0.269	0.132
		778	0.017	1.700	0.169	0.106
		857	0.005	1.250	0.866	0.058
		896	0.038	0.900	0.891	0.681
Urine	Yes	O-141	0.042	0.500	1.018	0.521
		O-363	0.012	3.150	0.892	0.698
		O-763	0.016	3.000	0.809	0.605
		O-772	0.136	1.550	0.698	0.760
	No	O-239	0.082	1.650	0.633	0.593
		O-766	0.104	2.200	0.618	0.686
		O-767	0.066	0.550	0.304	0.812
		O-835	0.082	0.400	0.612	0.749
Blood Median			0.070	1.625	0.506	0.602
Urine median			0.074	1.600	0.666	0.692
Site of Collection: P-value			0.897	0.859	0.360	0.203
Catheter Median			0.076	1.650	0.496	0.577
Non-Catheter Median			0.066	1.550	0.698	0.686
Catheter Association: P-value			0.863	0.965	0.162	0.162
Overall Minimum			0.005	0.250	0.253	0.058
Overall Median			0.071	1.625	0.626	0.637
Overall Maximum			0.136	3.150	1.018	0.889
Overall Standard Deviation			0.040	0.848	0.247	0.178

3.2.2 Correlation of Quantification Methods

Spearman correlations were used to assess the consistency of the commonly reported methods of biofilm production used in this study (Figure 8). There were no significant correlations between any of the tested methods on this substrate. The lack of correlation by planktonic XTT was expected – it is a measure of the activity of free cells in the environment, not those adherent to the catheter. All other methods measure identical populations of cells, and it is reasonable to expect these methods would indeed correlate. The two measures of biomass, crystal violet and dry weight, should correlate, but that is not seen here. The lack of correlation suggests that at least one of the methods is insufficient for accurately quantifying biofilm. The differing results in biofilm production by method for each isolate imply that at least one of these methods is inadequate at quantifying biofilm production.

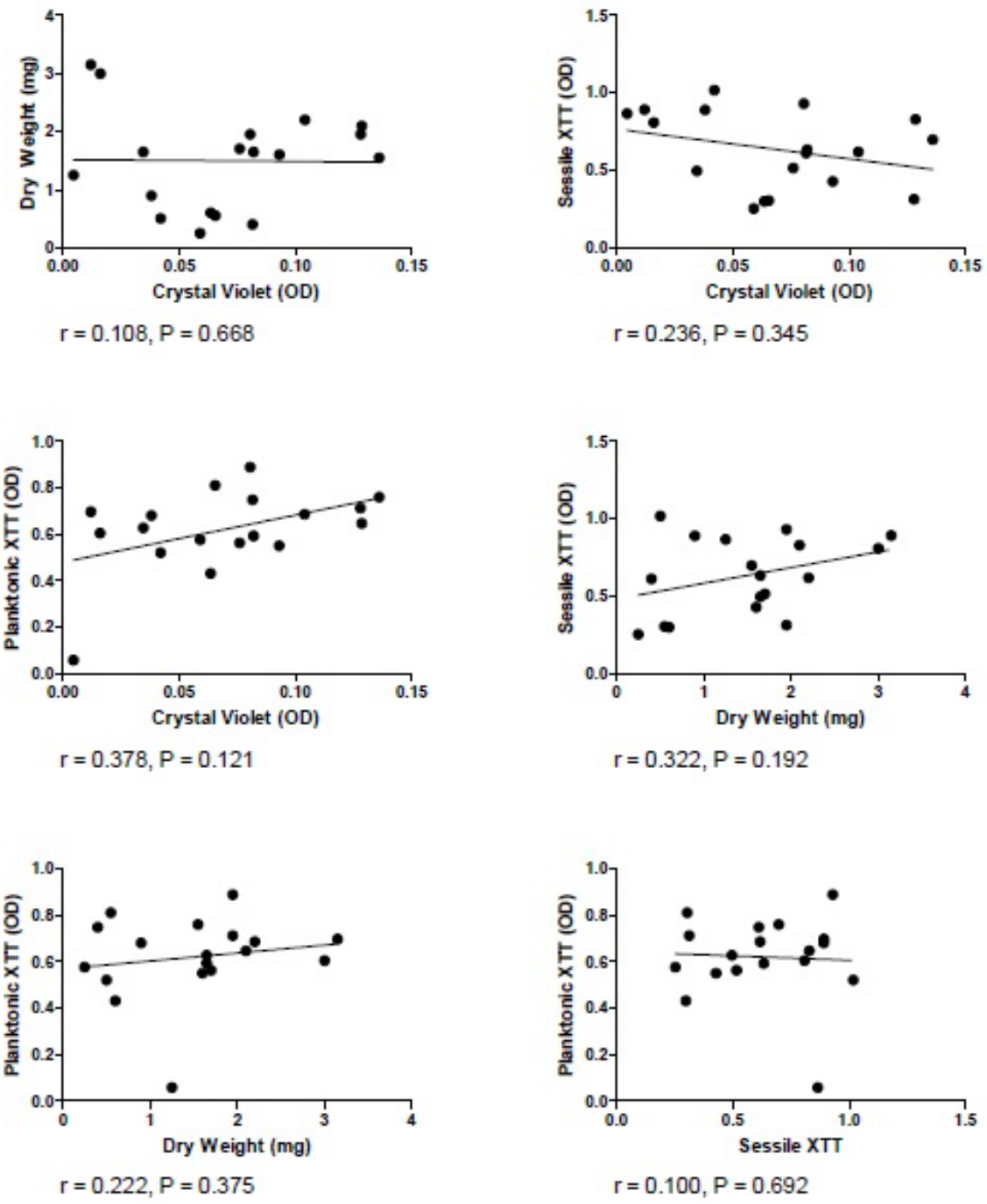

Figure 8: Correlation of Methods of Biofilm Production on Narrow Catheter. There were no significant correlations across tested methods.

3.2.3 Production by Catheter Association

Clinical isolates were chosen based on the presence of a catheter at the collection site for ≥3 days at time of collection, or no catheter association. Biofilm production was then examined based on this classification. Isolates exposed to a foreign device, such as a catheter, for an extended period of time may adapt for increased ability to grow adherent to these devices. Despite the expectation that these populations would differ in biofilm production, there was no difference in bioactivity or biomass production based on collection through a catheter (Figure 9).

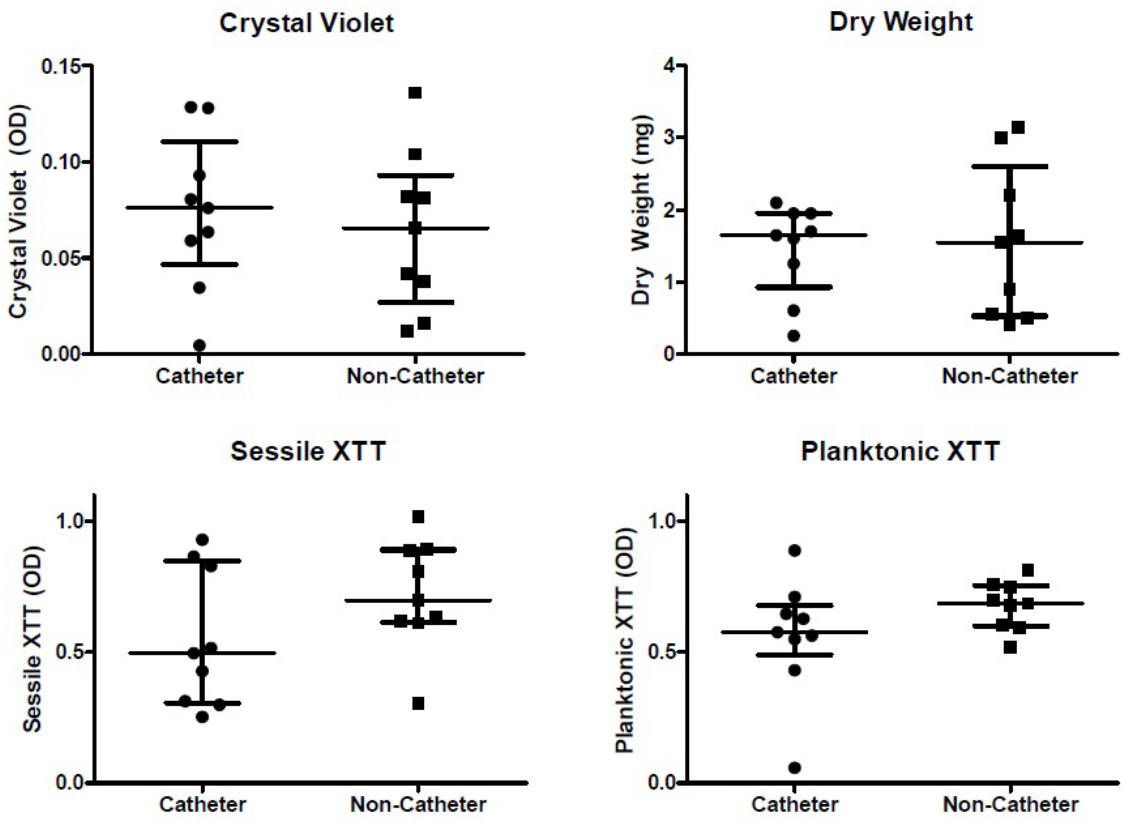

Figure 9: Biofilm Production on Narrow Catheter by Catheter Association. Biofilm production of isolates collected through a catheter that had been in place ≥3 days as compared to production of isolates that were not associated with a long-term catheter, by all four methods. No statistical differences were found between the groups for any method, as determined by the Mann-Whitney U test. Catheter associated and non-catheter associated medians for each method, respectively: crystal violet: 0.076 vs 0.066, P = 0.863; dry weight: 1.65 vs 1.55, P = 0.965; sessile XTT: 0.496 vs 0.698, P = 0.162; and planktonic XTT: 0.577 vs 0.686, P = 0.162. These values are also found in Table 1.

3.2.4 Production by Clinical Site of Collection

To ascertain if statistical differences existed between isolates collected from the bloodstream versus the urine, quantification results of 48 hour growth on narrow rubber catheter were compared. The Mann-Whitney U test was performed for each method individually (Figure 10, Table 1). Differences in the physical environment from which these isolates were collected, such as pH, availability of nutrients, and vessel flow, may cause behavioral changes in the isolates, including the ability to produce biofilm. However, biomass as measured by crystal violet or dry weight did not vary by clinical collection site. Similarly, XTT measures of metabolic activity did not vary for planktonic or catheter associated cells.

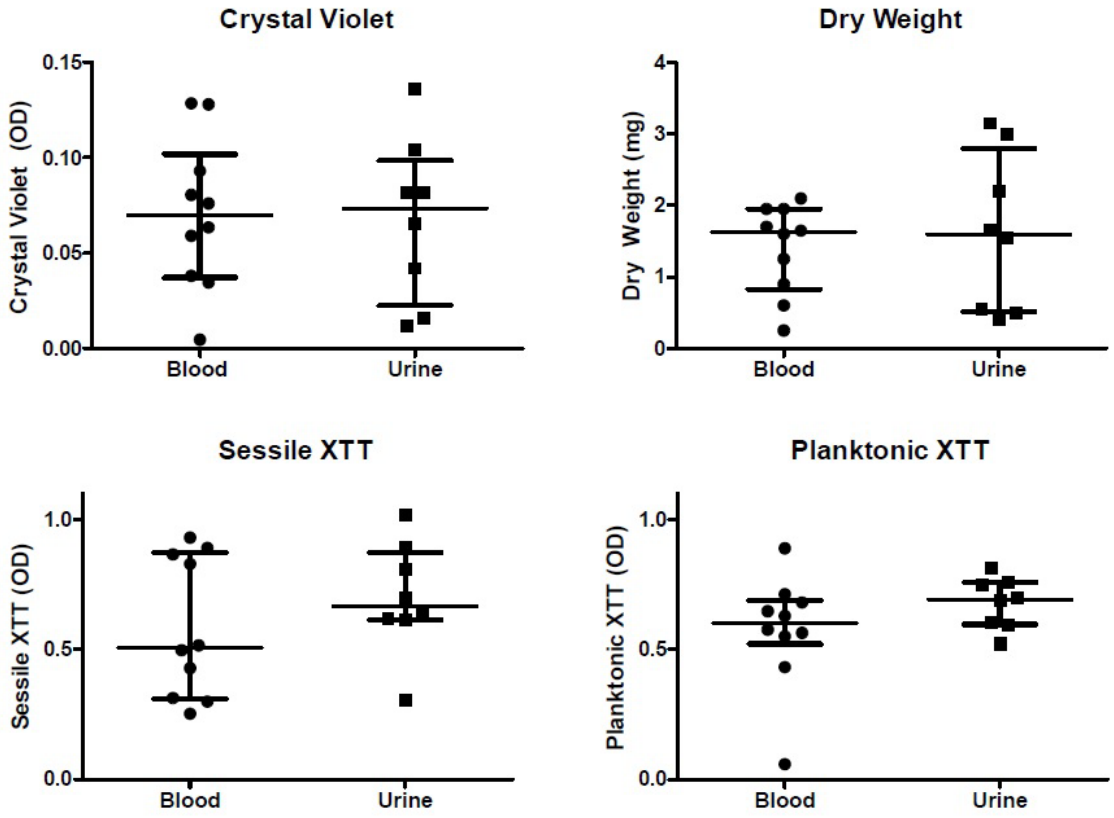

Figure 10: Biofilm Production on Narrow Catheter by Clinical Collection Site. Biofilm production of isolates collected from the bloodstream as compared to the production of isolates collected from the urine, by all four methods. No statistical differences were found between the groups for any method, as determined by the Mann-Whitney U test. Bloodstream and urinary medians for each method, respectively: crystal violet: 0.070 vs 0.074, P = 0.897; dry weight: 1.625 vs 1.6, P = 0.859; sessile XTT: 0.506 vs 0.666, P = 0.360; and planktonic XTT: 0.602 vs 0.692, P = 0.203. These values are also found in Table 1.

3.3 WIDE CATHETER

3.3.1 Quantification

Extent of biofilm production was quantified for all 18 clinical *Candida albicans* isolates following 48 hours of growth on 0.6cm x 1cm catheter segments, as detailed in the Experimental Design. Each test (crystal violet, dry weight, sessile metabolic activity, and planktonic metabolic activity) was performed in duplicate; average results for each isolate by each method, as well as select descriptive statistics can be found in Table 2.

Table 2: Biofilm Production on a Wide Catheter by Four Methods. Values shown are the averaged result of two catheter segments for each isolate analyzed on the same day. Summary statistics are listed at the bottom.

Site of Collection	Catheter Association	Isolate	Crystal Violet (OD)	Dry Weight (mg)	Sessile XTT (OD)	Planktonic XTT (OD)
Bloodstream	Yes	732	0.051	1.950	0.267	0.352
		779	0.054	2.350	0.327	0.483
		816	0.070	4.000	0.330	0.429
		819	0.030	1.400	0.149	0.368
		829	0.042	0.950	0.243	0.431
	No	207	0.073	1.900	0.629	0.527
		623	0.043	1.000	0.200	0.256
		778	0.047	1.100	0.098	0.273
		857	0.033	0.000	0.158	0.098
		896	0.042	0.000	0.507	0.474
Urine	Yes	O-141	0.110	0.550	0.382	0.525
		O-363	0.102	0.500	0.592	0.419
		O-763	0.054	4.317	0.399	0.360
		O-772	0.037	2.617	0.225	0.657
	No	O-239	0.058	3.717	0.302	0.506
		O-766	0.078	4.250	0.312	0.277
		O-767	0.040	0.500	0.148	0.453
		O-835	0.052	0.950	0.232	0.346
Blood Median			0.045	1.250	0.255	0.398
Urine median			0.056	1.784	0.307	0.436
Site of Collection: P-value			0.142	0.593	0.633	0.408
Catheter Median			0.047	1.400	0.243	0.368
Non-Catheter Median			0.054	0.950	0.312	0.453
Catheter Association: P-value			0.269	0.929	0.297	0.258
Overall Minimum			0.030	0.000	0.098	0.098
Overall Median			0.051	1.250	0.284	0.424
Overall Maximum			0.110	4.317	0.629	0.657
Overall Standard Deviation			0.022	1.455	0.151	0.128

3.3.2 Correlation of Quantification Methods

Once again, Spearman correlations were used to investigate the correlation between each of the methods, as the data presented is not normally distributed (Figure 11). Similar expectations existed for this slightly larger substrate as for the narrow catheter. Since planktonic XTT tests a distinct population of cells it was not expected to show correlation, and again this method did not correlate. Biomass as measured by dry weight and crystal violet did not correlate again, despite the expectation that these methods would align. Interestingly though, for this substrate, a strong correlation exists between crystal violet and sessile metabolic activity ($r = 0.721$, $P < 0.001$). No other methods correlated.

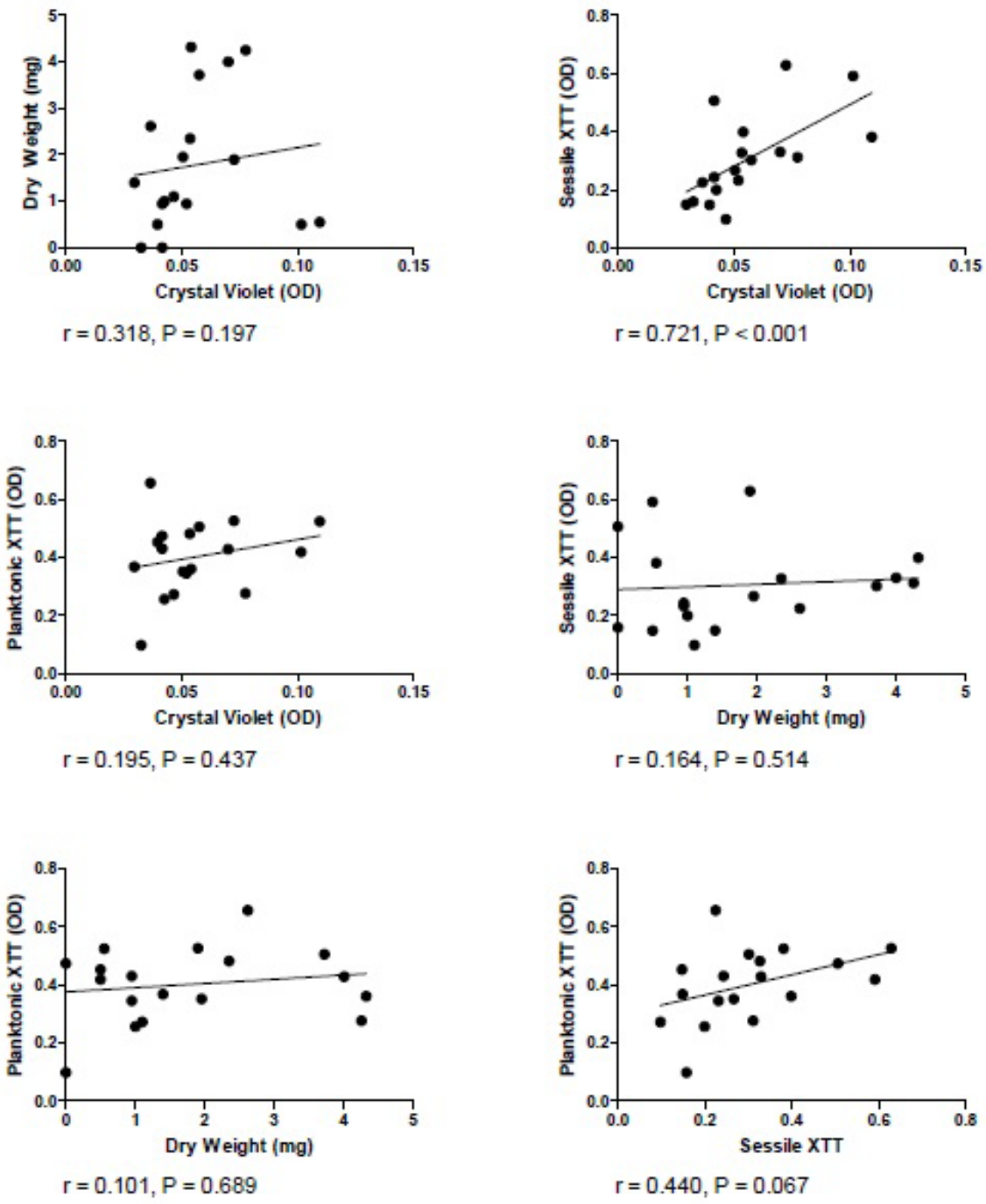

Figure 11: Correlation of Methods of Biofilm Production on Wide Catheter. Sessile metabolic activity correlated well with measures of crystal violet. No other methods correlated significantly.

3.3.3 Production by Catheter Association

Quantification results by each method were analyzed by the Mann-Whitney U test to determine if significant differences exist between biofilm production and association with (or absence of) a catheter at time of collection. Once again, measures of both bioactivity and biomass production showed no correlations as stratified by catheter association (Figure 12).

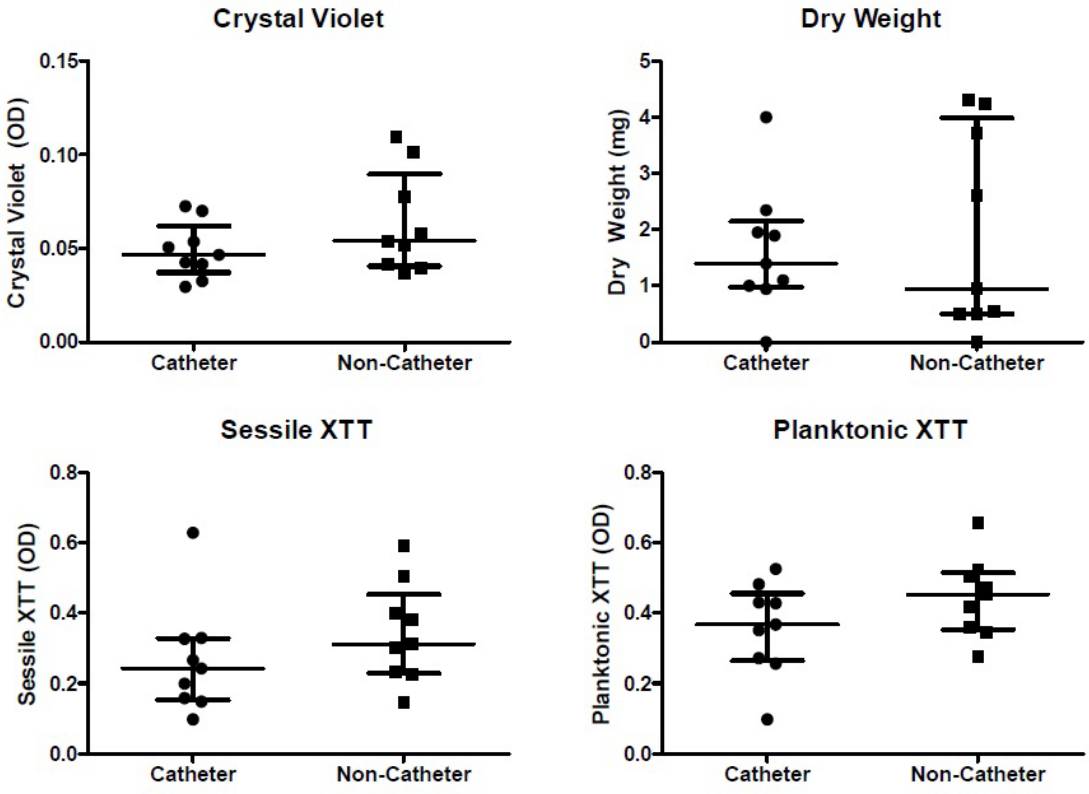

Figure 12: Biofilm Production on Wide Catheter by Catheter Association. Biofilm production of isolates collected through a catheter that had been in place ≥3 days as compared to production of isolates that were not associated with a long-term catheter, by all four methods. No statistical differences were found between the groups for any method, as determined by the Mann-Whitney U test. Catheter associated and non-catheter associated medians for each method, respectively: crystal violet: 0.047 vs 0.054, P = 0.269; dry weight: 1.4 vs 0.95, P = 0.929; sessile XTT: 0.243 vs 0.2312, P = 0.297; and planktonic XTT: 0.368 vs 0.453, P = 0.258. These values are also found in Table 2.Table 2

3.3.4 Production by Clinical Site of Collection

Biofilm production by each method was tested by Mann-Whitney U test to determine if significant differences existed between isolates collected from the bloodstream versus urine (Figure 13, Table 2). No significant differences were seen in this dataset.

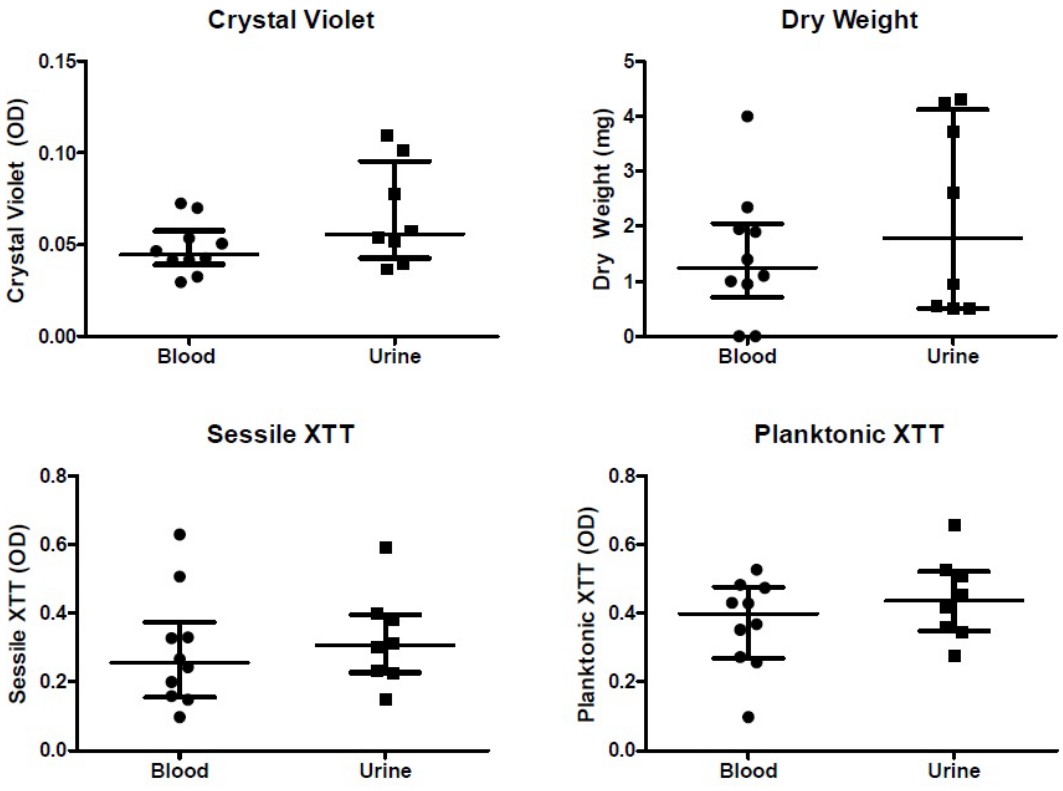

Figure 13: Biofilm Production on Wide Catheter by Clinical Collection Site. Biofilm production of isolates collected from the bloodstream as compared to the production of isolates collected from the urine, by all four methods. No statistical differences were found between the groups for any method, as determined by the Mann-Whitney U test. Bloodstream and urinary medians for each method, respectively: crystal violet: 0.045 vs 0.056, P = 0.142; dry weight: 1.25 vs 1.784, P = 0.593; sessile XTT: 0.255 vs 0.307, P = 0.633; and planktonic XTT: 0.398 vs 0.436, P = 0.408. These values are also found in Table 2.

3.4 METHODOLOGICAL VARIABILITY

On each day, biofilms were quantified on two separate catheter segments for each method of biomass quantification and two separate segments for bioactivity. Results from these same-day duplications were compared by approximating the Spearman rank correlation coefficient, r_s, for each method. Individually, these values, found in Table 3, show the reliability of each test for each isolate. Reliability increases as values approach one. This test does not assume normality, appropriate for the small number of observations presented here. Associations are unchanged when analyzed by the intraclass correlation coefficient (ICC), which assumes normality. ICC is a proportional measure of variance [43]. Using the ICC, correlations between the narrow and wide catheter were 0.759, 0.420, 0.353, and 0.424 for crystal violet, dry weight, sessile and planktonic metabolic activity, respectively.

Table 3: Experimental Variability as Determined by Spearman Rank Correlation Coefficient. Spearman rank correlation coefficient (r_s) for each tested method.

		Crystal Violet	Dry Weight	Sessile XTT	Planktonic XTT
NARROW CATHETER	**Approximation of Spearman r_s**	0.951	0.401	0.923	0.894
WIDE CATHETER	**Approximation of Spearman r_s**	0.67	0.793	0.767	0.791

3.5 ANTIFUNGAL SUSCEPTIBILITY

3.5.1 Standard Minimum Inhibitory Concentrations

Standard (planktonic) MICs were determined according to CLSI guidelines for AMB, CSP, and FLUC. Analysis showed that MICs did not vary by collection site or with the presence of a catheter at time of collection, as would be expected. Results are detailed in Table 4.

Table 4: Standard and Sessile Minimum Inhibitory Concentrations. MICs shown are visual consensus results from testing on two separate days. All MICs shown are in µg/ml.

Site of Collection	Catheter Association	Isolate	Standard MICs			Sessile MICs		
			AMB	CSP	FLUC	AMB	CSP	FLUC
Bloodstream	Yes	**732**	0.25	0.12	0.12	1	0.12	512
		779	0.25	0.12	>64	2	≤0.12	512
		816	0.25	0.12	0.12	2	>8	>512
		819	0.25	0.12	0.12	≤0.12	0.25	256
		829	0.25	0.12	>64	0.25	≤0.12	128
	No	**207**	0.25	0.12	>64	1	≤0.12	>512
		623	0.25	0.12	0.12	≤0.12	≤0.12	16
		778	0.25	0.12	>64	1	>8	512
		857	0.12	0.12	0.12	≤0.12	≤0.12	8
		896	0.12	0.12	>64	≤0.12	≤0.12	256
Urine	Yes	**O-141**	0.25	0.12	>64	4	0.25	512
		O-363	0.25	0.12	0.12	4	4	512
		O-763	0.25	0.12	0.12	0.5	≤0.12	>512
		O-772	0.25	0.06	1	0.25	≤0.12	512
	No	**O-239**	0.25	0.12	0.06	1	0.12	>512
		O-766	0.25	0.12	>64	2	0.12	>512
		O-767	0.25	0.12	0.12	0.25	≤0.12	≤8
		O-835	0.25	0.25	0.25	≤0.12	≤0.12	512

3.5.2 Sessile Minimum Inhibitory Concentrations

Each of three antifungals (AMB, CSP, and FLUC) were applied to 48 hour old biofilms grown on wide catheter segments for 24 hours. Sessile MICs were visually defined by a 50% reduction in metabolic activity for caspofungin and fluconazole and a 100% reduction for amphotericin B, as compared to control (no drug) (Table 4). MICs were analyzed by site of collection and catheter association; no statistical differences were found.

3.5.3 Correlations of Biofilm Production and Antifungal Susceptibility

Spearman correlations were used to evaluate the relationship between biofilm production and sessile MICs for AMB, CSP, and FLUC. Biofilms are known to be associated with decreased resistance, therefore high producing isolates would be expected to have higher MICs.

Correlations were strong between AMB MICs and both crystal violet ($r = 0.827$, $P < 0.001$) and sessile metabolic activity ($r = 0.520$, $P = 0.026$) (Figure 14). CSP did not associate with any quantification method of biofilm (Figure 15). FLUC MICs correlated best with biofilm production; three of four tested methods were found to have a significant relationship (Figure 16Figure 16). Both measures of biomass production, crystal violet and dry weight, significantly correlated with FLUC MICs ($r = 0.739$, $P < 0.001$ and $r = 0.772$, $P < 0.001$, respectively). In addition, sessile metabolic activity as measured by XTT correlated well with FLUC MICs ($r = 0.576$, $P = 0.012$).

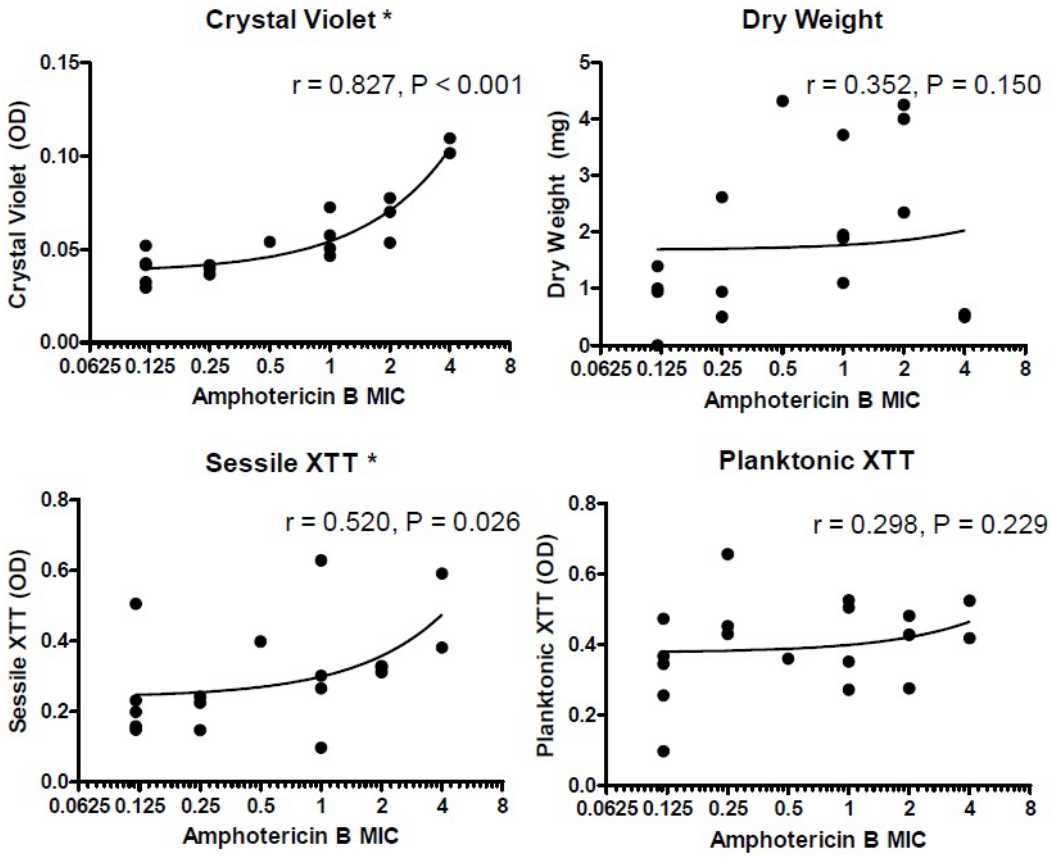

Figure 14: Correlation of Sessile Amphotericin B MICs and Quantification Methods. Crystal violet and sessile metabolic activity correlate well with AMB MICs. Spearman correlation data is shown with linear regression. Note: The x-axis is in log 2 scale for ease of viewing the doubling dilution scale of susceptibility testing, therefore the linear regression plot is curved. * denotes $P < 0.05$.

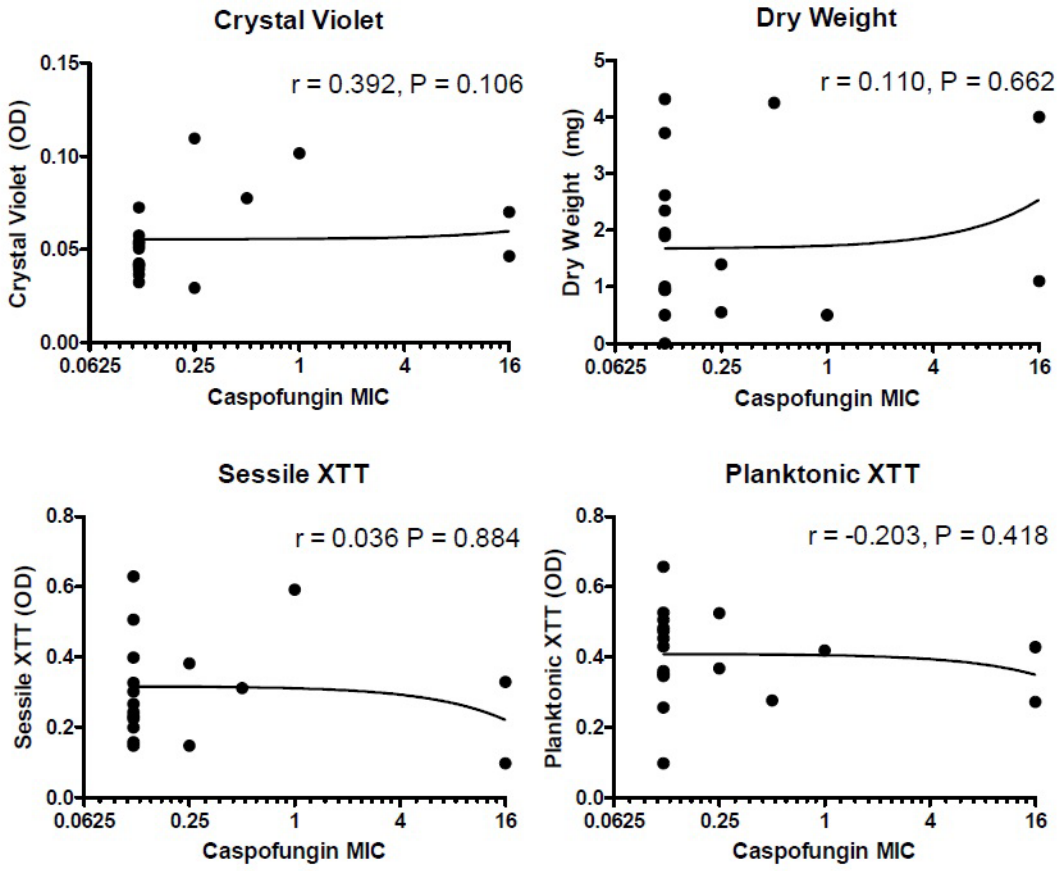

Figure 15: Correlation of Sessile Caspofungin MICs and Quantification Methods. CSP MICs did not correlate to any method quantifying biofilm production. Note: The x-axis is in log 2 scale for ease of viewing the doubling dilution scale of susceptibility testing. Spearman correlation data is shown with linear regression. Note: The x-axis is in log 2 scale for ease of viewing the doubling dilution scale of susceptibility testing, therefore the linear regression plot is curved.

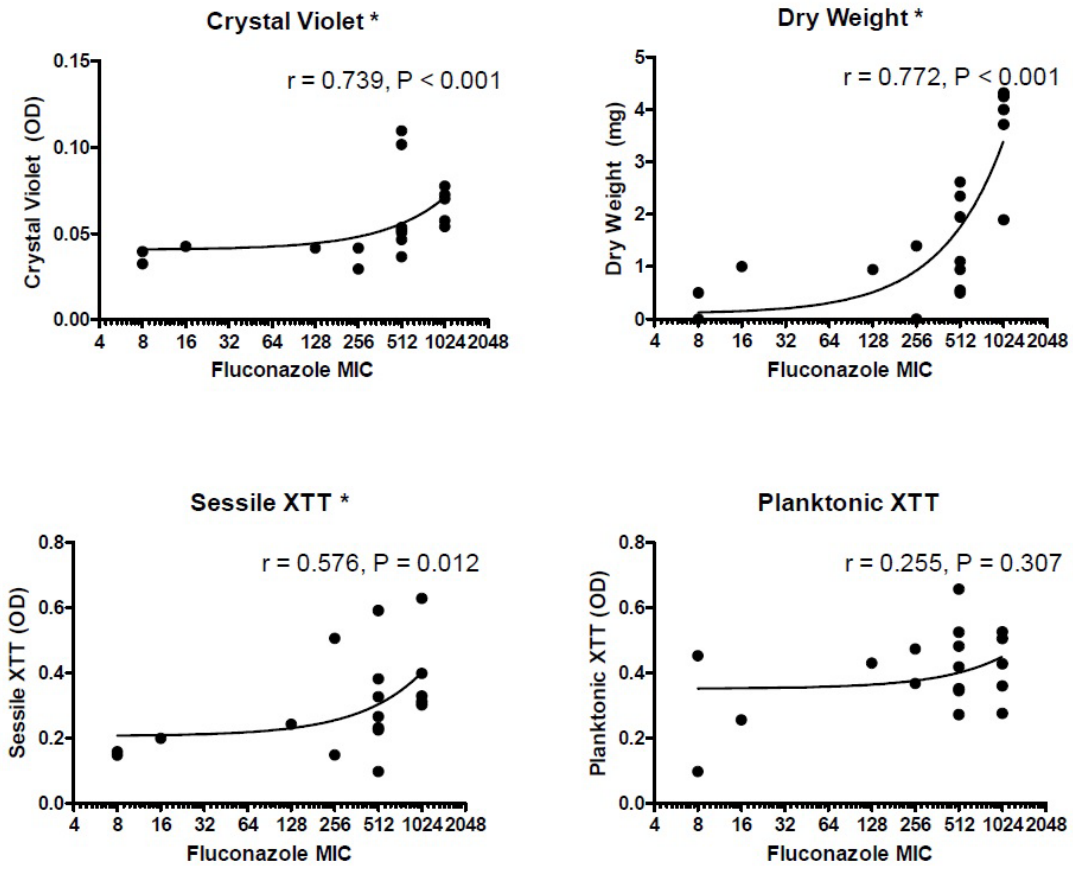

Figure 16: Correlation of Sessile Fluconazole MICs and Quantification Methods. Crystal violet, dry weight and sessile metabolic activity all correlate well with FLUC MICs. Spearman correlation data is shown with linear regression. Note: The x-axis is in log 2 scale for ease of viewing the doubling dilution scale of susceptibility testing, therefore the linear regression plot is curved. * denotes $P < 0.05$.

3.5.4 Paradoxical Effect

The presence of paradoxical growth of *Candida albicans*, defined as growth in consecutive wells greater than 2 dilutions above the MIC, was assessed for all antifungals [44]. Paradoxical growth in standard or sessile tests was seen only with CSP. Paradoxical growth is well described for Candida in the presence of CSP, though the clinical relevance is questionable given that the effect is eliminated in the presence of human serum [44]. When tested by standard methods, none of the isolates showed paradoxical growth at 24 hours; after 48 hours, the effect appeared in two of the 18 isolates.

Paradoxical growth of biofilm associated cells is previously described [27, 45]. Studies of Candida biofilm paradoxical growth in the presence of caspofungin often use much higher drug concentrations than were used here; therefore, it was unclear whether the effect would be observed. Indeed, paradoxical activity of biofilms was seen after 24 hours of caspofungin exposure in 11 of 18 isolates (Table 5). There were no significant differences in the presence or absence of paradoxical growth between isolates with or without catheter association (Fisher's exact test $P = 0.348$) or bloodstream and urine isolates (Fisher's exact test $P = 0.630$). Paradoxical growth did not correlate to sessile MIC for any drug (tested by Mann-Whitney U-test, AMB: $P = 0.449$, CSP: $P = 0.959$, and FLUC: $P = 0.178$).

Table 5: Paradoxical Growth in the Presence of Caspofungin. Based on visual MIC readings, two isolates exhibited paradoxical growth at 48 hours by standard methods. Visual reading of sessile MICs showed 11 of 18 with paradoxical growth, noted here by shading. All MICs shown are in µg/ml. For isolates with MIC > 8, one of the replications showed clearance and paradoxical growth.

Site of Collection	Device Association	Isolates	Standard	Sessile
			CSP	CSP
Bloodstream	Yes	732	0.12	≤0.12
		779	0.12	≤0.12
		816	0.12	>8
		819	0.12	0.25
		829	0.12	≤0.12
	No	207	0.12	≤0.12
		623	0.12	≤0.12
		778	0.12	>8
		857	0.12	≤0.12
		896	0.12	≤0.12
Urine	Yes	O-141	0.12	0.25
		O-363	0.12	4
		O-763	0.12	≤0.12
		O-772	0.06	≤0.12
	No	O-239	0.12	0.12
		O-766	0.12	0.12
		O-767	0.12	≤0.12
		O-835	0.25	≤0.12

4.0 DISCUSSION

The purpose of this study was to test biofilm production by *Candida albicans* across various reported methods of quantification and analyze the results based on catheter association, clinical collection site, and sessile antifungal susceptibility. Each of the four methods of biofilm quantification (crystal violet, dry weight, planktonic and sessile activity) exhibited strengths and weaknesses (Table 6).

Table 6: Summary of Strengths and Weaknesses for Each Quantification Method.

	Strengths	Weaknesses
Crystal Violet	- Simple to execute - Results are based on colorimetric change - Visual and OD interpretations are possible - All supplies and reagents are found in standard microbiology labs	- Does not distinguish between living and dead structures - Narrow range of results may be indicative of an insensitive test
Dry Weight	- Simple to perform and analyze - No special equipment is required	- Does not distinguish between living and dead structures - Pre-weighing segments can be time consuming - Reliability is questionable - Requires 24 hours of additional time to allow for drying
Metabolic Activity – Planktonic and Sessile	- Once prepared, simple to carry out - Results are based on colorimetric change - Visual and OD interpretations are possible - Most of assay run time is hands-off	- Preparation and storage of enzyme introduces more technically challenging concepts - Does not account for non-metabolically active structures, such as the ECM - Requires purchase of specific reagents

From a technical standpoint, biomass assessment by crystal violet is simple to accomplish and results are rapidly obtained. This method was highly reliable for the narrow catheter and the wide catheter, albeit to a lesser degree (Table 3). This shows that technical duplicates (within the same day) correlated very well. The modestly disparate results between catheter sizes indicate inter-day variability. The narrow range of all values, regardless of catheter size, may be an

indication of an insensitive test (Figure 9, Figure 10). Future studies designed to test the intra- and inter-day variability for biofilm quantification techniques are needed.

Biomass was also calculated by dry weight. This method is the simplest of tested methods to execute. Pre-weighing of catheter segments to determine net biofilm weight rather than sonication or scraping attempted to reduce variability due to loss of sample or incomplete removal. However, some flaking or loss of biofilm structure was observed. The lack of reliability in this method can be seen in the markedly lower Spearman r_s for the narrow catheter tests (Table 3). Wide catheter testing showed a much higher r_s. It is possible that by the second round of testing, there was unconscious technical adjustment due to experience. Technical expertise may improve reliability for this test. The inability of dry weight to correlate with other methods is supported by the variability shown in this study and is consistent with current literature [29]. The wide range in results, nearly 3mg for the narrow catheter, and over 4mg for the wider segments, may be an indication of the erratic nature of this test, or that the isolates themselves are capable of producing drastically different structures. Contingent upon additional testing, including vast microscopic imaging, this wide range holds true, if reproducibility can be achieved, this test may be highly effective at distinguishing differences in biofilm production across isolates.

Methods of biomass quantification are inherently unable to differentiate between living and non-living structures, therefore neither of these methods should be used alone to account for a clinical Candida albicans isolate's ability to produce biofilm.

Once considered a highly irreproducible method for biofilm quantification, the testing of metabolic activity of Candida biofilms has become more clearly defined in recent years [22, 30, 39]. Assay set up is fairly uncomplicated, and interpretation of results by optical density is

simple. There are still limitations. For example, inherent variability in enzyme activity, particularly by individual lot can cause problems in interpretation and comparison.

Measurements of planktonic activity are less commonly reported in the literature as an indication for biofilm growth as compared to measures of sessile metabolic activity. Still, planktonic cell measurement is important because it represents the activity of cells which are released in the final stage of biofilm maturation – "dispersal," or release of portions of sessile structure into the surrounding environment. Reports in the literature suggest that these cells are different from as compared to non-biofilm associated planktonic cells [46]. It appears that measures of planktonic activity are fairly reliable, regardless of catheter size (Table 3). Future studies focusing specifically on rate of dispersal may indicate a relationship between the extent of dispersal (or the rate of maturation) planktonic cellular activity.

Sessile XTT is a more direct method of biofilm quantification and it is simple to perform. If incubation time and concentration are kept consistent, sessile XTT provides a good indication for the extent of biofilm metabolic activity. Correlation between duplicates was very good for the both sizes of catheter (Table 3). It is important to note again that this method does not account for differences in production of ECM. As has been mentioned here already, the ECM is at least in part responsible for decreased drug susceptibility, and therefore its presence is important [10]. Biomass on the other hand, does account for ECM production. In quantifying biofilms, sessile metabolic activity should not be used alone to measure biofilm production.

Correlation results substantiate the claim that limited association exists between quantification methods. Indeed, biomass as determined by crystal violet and sessile metabolic activity of growth on wide catheter segments were the only methods to correlate. This correlation can most likely be attributed to an increased number of living and dividing cells rather than an

increase in metabolically inactive ECM. Similar correlations have been recently reported in the literature, leading to the introduction of proposed methodological cutoffs to classify the production of biofilm using crystal violet of sessile metabolic activity [47]. Further validation of these cutoffs is needed to determine both if they are reproducible and if they are clinically relevant.

Based on the strengths and weaknesses of each tested method, along with correlation data, it is reasonable to support the use of two tests when assessing biofilm production of clinical isolates. Ideally, a method for determining each biomass and bioactivity would be used, to ensure that over or underproduction of ECM was not mistaken for presence or absence of a strong biofilm phenotype. The ECM is indeed part of the overall biofilm structure, but it is not metabolically active and therefore cannot be measured by XTT reduction. As dry weight is not a particularly dependable method, use of crystal violet to determine biomass would be best. Crystal violet is easily run in parallel with sessile metabolic activity. Together, these methods provide a good indication as to the extent of biofilm production by clinical isolates.

It is reasonable to propose that the biofilm phenotype is altered upon exposure to a foreign device. Surrounding environments will elicit different cellular responses, and genetic differences have been shown exist between Candida in its planktonic lifestyle as compared to the biofilm lifestyle [48-51]. In an effort to accurately assess the impact of catheter association, nine of the isolates chosen for this study were collected through catheters that had been in place for at least three days. After this amount of time, it would be reasonable to assume that the organism had associated with the catheter. Remarkably, no difference was seen in biofilm production for *Candida albicans* collected from a catheter as compared to no catheter being present, despite the hypothesis that biofilm production would be greater for isolates exposed to a catheter *in vivo*.

The overall biomass and bioactivity were not differentially altered in these populations. There are a number of explanations for this lack of distinction between groups. Namely, the organisms may be capable of adapting to the *in vitro* environment quickly, eliminating any changes which occur *in vivo* by the time testing occurs *in vitro*. Additionally, the methods may be insufficiently sensitive to detect any differences which exist. It is also possible that no differences exist.

In an analogous manner, the human bloodstream and urine are physically distinct environments for organism growth. They offer similar challenges to microbial growth such as vessel flow and drug exposure. Nutrient sources and pH, for example, vary between the sites, likely causing adaptation in infecting organisms. Adaptations are often connected to better survivability. Yet, the increased ability to form biofilm does not obviously prove beneficial at either of these sites. Why these organisms change from the planktonic to biofilm lifestyle is unclear. Against the original hypothesis that differing *in vivo* conditions will alter biofilm production, production did not vary by clinical site of collection by any method in this study. Again, it may be that testing conditions allowed adaptation, effectively erasing any differences that may occur within the human body. This finding is consistent with similar reports in the literature – Shuford, et al. noted that there were no differences in dry weight and metabolic activity across invasive versus non-invasive sites [23]. Keeping in mind that the methods used between studies are not yet fully standardized, comparisons between that study and this report are tenuous.

Decreased susceptibility of clinical *Candida albicans* biofilms to AMB is not often reported. Five of eighteen (27.7%) isolates tested showed a greater than 2-fold increase in AMB MICs between standard and sessile populations. Sessile AMB MICs correlated well with biofilm production as measured by crystal violet and sessile XTT. This is consistent with the belief that

biofilm ECM is able to ensnare or otherwise prevent drugs from accessing their targets, in this case the cell wall component ergosterol. The clinical significance of these findings is unclear. The magnitude of MIC increase was markedly lower with AMB than with FLUC – the other drug to correlated increased biofilm production with increased MIC.

Decreased susceptibility to the azoles is a widely recognized feature of Candida biofilms [13, 25]. Azole efflux pumps are triggered in the earliest stages of biofilm formation, even without drug pressure, though the clinical relevance of this phenomenon is not well understood [52, 53]. A greater than 2-fold increase in fluconazole MIC was observed in 12 of 18 (67%) tested isolates. This included 8 of 9 urinary isolates, suggesting that there is a predisposition of these isolates to become resistant to fluconazole. It is plausible that these isolates have been exposed to this drug previously, thereby hastening adaptation, especially considering that fluconazole is excreted in the urine, 60-80% unchanged. Fluconazole MICs correlated with three of the four methods of biofilm production: crystal violet, dry weight, and sessile XTT. Together, the high percentage of isolates exhibiting increased MICs in the biofilm state along with the correlation to three methods of biofilm quantifications are highly suggestive that the biofilm lifestyle is inherently predisposed to decreased fluconazole susceptibility.

Caspofungin MICs did not correlate with any method of biofilm production. As shown in Table 4, there was little change between planktonic and sessile susceptibility. Drug activity was maintained, suggesting CSP still able to inhibit β-glucan synthase. Interestingly, a recent study suggests there is much less β-glucan present in Candida biofilms than previously thought [11]. This calls to question how CSP is so effective against these cell populations considering the target of drug activity (β-glucan) is not a large component of the cell wall structure. In all, CSP was the most effective at attenuating biofilm activity as compared to AMB and FLUC among the

isolates we tested. Only three isolates exhibited a greater than 2-fold increase in CSP MIC. While MICs remained low, i*n vitro* paradoxical growth of Candida biofilms in the presence of CSP was observed, consistent with current literature [27, 45]. Of the isolates tested here, 61% exhibited paradoxical growth in the presence of CSP. Testing did not go above 16µg/ml. There is evidence that paradoxical growth occurs at concentrations well above 16µg/ml for *Candida albicans* biofilms. Paradoxical growth may occur in a higher percentage of our tested isolates, though the range of tested drug here did not allow capture of that growth. Clinical relevance of paradoxical growth remains unknown; the increased prevalence of this effect shown in the biofilm lifestyle suggests that the two phenotypes may be linked. Further investigation is needed to determine if common pathways exist.

Consistent with current literature, higher MICs were seen in biofilm-associated cells as compared to planktonic cells [12, 22, 23]. Using standard MIC results obtained from a clinical microbiology laboratory may be insufficient when assessing appropriate dosing levels required to eradicate an infection. However, it is impractical to suggest methods such as those described here should be utilized in routine testing. First, not all infections are biofilm-associated. Secondly, even if biofilm growth is suspected, it may be difficult to discern whether an infection is associated with a device or if there is a mucosal biofilm seeding a device. Finally, even if methods could be perfected, and novel testing products, such as the Calgary biofilm device, became readily available, the time required to grow and subsequently test susceptibility produces unusable data [54]. Patient care requires faster turnaround than is currently feasible for susceptibility testing of Candida biofilms.

A major limitation of this study is the small sample size; however, this study was intended as a pilot study. Additionally, there was no confirmation of biofilm structure by

microscopy, as is often standard in similar studies. Despite these limitations, this report gives evidence for the potential use of sessile metabolic activity in combination with crystal violet measurement to determine the extent of biofilm production by clinical isolates. These methods could be employed in further testing to determine differences between clinical isolates that were not identified here.

5.0 CONCLUSION

In conclusion, this study shows that clinical *Candida albicans* isolates form distinct biofilms on clinical catheter segments, as confirmed by four methods of quantification. Data presented here address the three main objectives of my thesis project. First, reproducible methods of *Candida albicans* biofilm growth and quantification were established. The combination of sessile XTT and crystal violet are recommended as methods of quantification in future studies, rather than the use of only one method of quantification. Second, comparison of biofilm production did not vary by catheter association or clinical collection site, suggesting the biofilm phenotype is similar throughout in many *in vivo* environments. Third, strong relationships between biofilm production and sessile MICs for amphotericin B and fluconazole were shown. If these relationships hold true for biofilms produced *in vivo*, the presence of biofilms may need to be considered when interpreting clinical susceptibility data, as results may be falsely low.

BIBLIOGRAPHY

1. Press, E.G., R.K. Shields, and C.J. Clancy, *Candida Biofilm: Clinical Implications of Recent Advances in Research.* Current Fungal Infection Reports, 2014. **8**(1): p. 72-80.
2. Chandra, J., et al., *Biofilm formation by the fungal pathogen Candida albicans: development, architecture, and drug resistance.* J Bacteriol, 2001. **183**(18): p. 5385-94.
3. Zhu, Z., et al., *Time course analysis of Candida albicans metabolites during biofilm development.* J Proteome Res, 2013. **12**(6): p. 2375-85.
4. Baillie, G.S. and L.J. Douglas, *Role of dimorphism in the development of Candida albicans biofilms.* J Med Microbiol, 1999. **48**(7): p. 671-9.
5. Paramonova, E., et al., *Hyphal content determines the compression strength of Candida albicans biofilms.* Microbiology, 2009. **155**(Pt 6): p. 1997-2003.
6. Romanowski, K., et al., *Candida albicans isolates from the gut of critically ill patients respond to phosphate limitation by expressing filaments and a lethal phenotype.* PLoS One, 2012. **7**(1): p. e30119.
7. Mazaheritehrani, E., et al., *Human pathogenic viruses are retained in and released by Candida albicans biofilm in vitro.* Virus Res, 2014. **179**: p. 153-60.
8. Flemming, H.C. and J. Wingender, *The biofilm matrix.* Nat Rev Microbiol, 2010. **8**(9): p. 623-33.
9. Xie, Z., et al., *Candida albicans biofilms do not trigger reactive oxygen species and evade neutrophil killing.* J Infect Dis, 2012. **206**(12): p. 1936-45.
10. Nett, J.E., et al., *Interface of Candida albicans biofilm matrix-associated drug resistance and cell wall integrity regulation.* Eukaryot Cell, 2011. **10**(12): p. 1660-9.
11. Zarnowski, R., et al., *Novel entries in a fungal biofilm matrix encyclopedia.* MBio, 2014. **5**(4): p. e01333-14.
12. Mathé, L. and P. Van Dijck, *Recent insights into Candida albicans biofilm resistance mechanisms.* Curr Genet, 2013.
13. Tobudic, S., et al., *Antifungal susceptibility of Candida albicans in biofilms.* Mycoses, 2012. **55**(3): p. 199-204.
14. Kojic, E.M. and R.O. Darouiche, *Candida infections of medical devices.* Clin Microbiol Rev, 2004. **17**(2): p. 255-67.
15. Ramage, G., J.P. Martinez, and J.L. Lopez-Ribot, *Candida biofilms on implanted biomaterials: a clinically significant problem.* FEMS Yeast Res, 2006. **6**(7): p. 979-86.
16. Chavez-Dozal, A.A., et al., *In Vitro Analysis of Finasteride Activity against Candida albicans Urinary Biofilm Formation and Filamentation.* Antimicrob Agents Chemother, 2014. **58**(10): p. 5855-62.
17. Pfaller, M.A. and D.J. Diekema, *Epidemiology of invasive candidiasis: a persistent public health problem.* Clin Microbiol Rev, 2007. **20**(1): p. 133-63.

18. Kett, D.H., et al., *Candida bloodstream infections in intensive care units: analysis of the extended prevalence of infection in intensive care unit study.* Crit Care Med, 2011. **39**(4): p. 665-70.
19. Pappas, P.G., et al., *Clinical practice guidelines for the management of candidiasis: 2009 update by the Infectious Diseases Society of America.* Clin Infect Dis, 2009. **48**(5): p. 503-35.
20. Andes, D.R., et al., *Impact of treatment strategy on outcomes in patients with candidemia and other forms of invasive candidiasis: a patient-level quantitative review of randomized trials.* Clin Infect Dis, 2012. **54**(8): p. 1110-22.
21. Cuellar-Cruz, M., et al., *The effect of biomaterials and antifungals on biofilm formation by Candida species: a review.* Eur J Clin Microbiol Infect Dis, 2012. **31**(10): p. 2513-27.
22. Pierce, C.G., et al., *A simple and reproducible 96-well plate-based method for the formation of fungal biofilms and its application to antifungal susceptibility testing.* Nat Protoc, 2008. **3**(9): p. 1494-500.
23. Shuford, J.A., et al., *In vitro biofilm characterization and activity of antifungal agents alone and in combination against sessile and planktonic clinical Candida albicans isolates.* Diagn Microbiol Infect Dis, 2007. **57**(3): p. 277-81.
24. Calderone, R.A.C.C.J., *Candida and candidiasis*. 2012, Washington, DC: ASM Press.
25. Kalya, A.V. and D.G. Ahearn, *Increased resistance to antifungal antibiotics of Candida spp. adhered to silicone.* J Ind Microbiol, 1995. **14**(6): p. 451-5.
26. Shields, R.K., et al., *The presence of an FKS mutation rather than MIC is an independent risk factor for failure of echinocandin therapy among patients with invasive candidiasis due to Candida glabrata.* Antimicrob Agents Chemother, 2012. **56**(9): p. 4862-9.
27. Melo, A.S., A.L. Colombo, and B.A. Arthington-Skaggs, *Paradoxical growth effect of caspofungin observed on biofilms and planktonic cells of five different Candida species.* Antimicrob Agents Chemother, 2007. **51**(9): p. 3081-8.
28. Ramage, G., et al., *Standardized method for in vitro antifungal susceptibility testing of Candida albicans biofilms.* Antimicrob Agents Chemother, 2001. **45**(9): p. 2475-9.
29. Chandra, J., P.K. Mukherjee, and M.A. Ghannoum, *In vitro growth and analysis of Candida biofilms.* Nat Protoc, 2008. **3**(12): p. 1909-24.
30. Kuhn, D.M., et al., *Uses and limitations of the XTT assay in studies of Candida growth and metabolism.* J Clin Microbiol, 2003. **41**(1): p. 506-8.
31. Frade, J.P. and B.A. Arthington-Skaggs, *Effect of serum and surface characteristics on Candida albicans biofilm formation.* Mycoses, 2011. **54**(4): p. e154-62.
32. Costa, A.C., et al., *Methods for obtaining reliable and reproducible results in studies of Candida biofilms formed in vitro.* Mycoses, 2013.
33. Bernhardt, H., M. Knoke, and J. Bernhardt, *Efficacy of anidulafungin against biofilms of different Candida species in long-term trials of continuous flow cultivation.* Mycoses, 2011. **54**(6): p. e821-7.
34. Uppuluri, P., A.K. Chaturvedi, and J.L. Lopez-Ribot, *Design of a simple model of Candida albicans biofilms formed under conditions of flow: development, architecture, and drug resistance.* Mycopathologia, 2009. **168**(3): p. 101-9.
35. Uppuluri, P. and J.L. Lopez-Ribot, *An easy and economical in vitro method for the formation of Candida albicans biofilms under continuous conditions of flow.* Virulence, 2010. **1**(6): p. 483-7.

36. Kucharikova, S., et al., *Detailed comparison of Candida albicans and Candida glabrata biofilms under different conditions and their susceptibility to caspofungin and anidulafungin.* J Med Microbiol, 2011. **60**(Pt 9): p. 1261-9.
37. Li, X., Z. Yan, and J. Xu, *Quantitative variation of biofilms among strains in natural populations of Candida albicans.* Microbiology, 2003. **149**(Pt 2): p. 353-62.
38. Djordjevic, D., M. Wiedmann, and L.A. McLandsborough, *Microtiter plate assay for assessment of Listeria monocytogenes biofilm formation.* Appl Environ Microbiol, 2002. **68**(6): p. 2950-8.
39. Nett, J.E., et al., *Optimizing a Candida biofilm microtiter plate model for measurement of antifungal susceptibility by tetrazolium salt assay.* J Clin Microbiol, 2011. **49**(4): p. 1426-33.
40. Chandra, J., et al., *Antifungal resistance of candidal biofilms formed on denture acrylic in vitro.* J Dent Res, 2001. **80**(3): p. 903-8.
41. Institute, C.a.L.S., *Reference method for broth dilution antifungal susceptibility testing of yeasts.* Approved standard M27-A3, 2008. **3rd Edition**.
42. Hawser, S.P., et al., *Comparison of a 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-t etrazolium hydroxide (XTT) colorimetric method with the standardized National Committee for Clinical Laboratory Standards method of testing clinical yeast isolates for susceptibility to antifungal agents.* J Clin Microbiol, 1998. **36**(5): p. 1450-2.
43. McGraw, K.O. and S.P. Wong, *Forming inferences about some intraclass correlation coefficients.* Psychological Methods, 1996. **1**(1): p. 30-46.
44. Shields, R.K., et al., *Paradoxical effect of caspofungin against Candida bloodstream isolates is mediated by multiple pathways but eliminated in human serum.* Antimicrob Agents Chemother, 2011. **55**(6): p. 2641-7.
45. Ferreira, J.A., et al., *Biofilm formation and effect of caspofungin on biofilm structure of Candida species bloodstream isolates.* Antimicrob Agents Chemother, 2009. **53**(10): p. 4377-84.
46. Uppuluri, P., et al., *Dispersion as an important step in the Candida albicans biofilm developmental cycle.* PLoS Pathog, 2010. **6**(3): p. e1000828.
47. Marcos-Zambrano, L.J., et al., *Production of biofilm by Candida and non-Candida spp. isolates causing fungemia: Comparison of biomass production and metabolic activity and development of cut-off points.* Int J Med Microbiol, 2014.
48. Blankenship, J.R. and A.P. Mitchell, *How to build a biofilm: a fungal perspective.* Curr Opin Microbiol, 2006. **9**(6): p. 588-94.
49. Deveau, A. and D.A. Hogan, *Linking quorum sensing regulation and biofilm formation by Candida albicans.* Methods Mol Biol, 2011. **692**: p. 219-33.
50. Hornby, J.M., et al., *Quorum sensing in the dimorphic fungus Candida albicans is mediated by farnesol.* Appl Environ Microbiol, 2001. **67**(7): p. 2982-92.
51. Finkel, J.S. and A.P. Mitchell, *Genetic control of Candida albicans biofilm development.* Nat Rev Microbiol, 2011. **9**(2): p. 109-18.
52. Mateus, C., S.A. Crow, Jr., and D.G. Ahearn, *Adherence of Candida albicans to silicone induces immediate enhanced tolerance to fluconazole.* Antimicrob Agents Chemother, 2004. **48**(9): p. 3358-66.

53. Ramage, G., et al., *Investigation of multidrug efflux pumps in relation to fluconazole resistance in Candida albicans biofilms.* J Antimicrob Chemother, 2002. **49**(6): p. 973-80.
54. Ceri, H., et al., *The Calgary Biofilm Device: New Technology for Rapid Determination of Antibiotic Susceptibilities of Bacterial Biofilms.* J Clin Microbiol, 1999. **37**(6): p. 1771-6.