A comparative virulence study clinical isolates of *Candida*

A Major Qualifying Project Report submitted to the Faculty of Worcester Polytechnic Institute in partial fulfillment of the requirements for the Degree of Bachelor of Science in Biology & Biotechnology.

Submitted By:

Natalie Fabrizio

Brittney Lambert

Nina Murphy-Cook

Submitted to: Dr. Reeta Rao

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Abstract

A 2013 report from the CDC published a "watch list" of antimicrobial resistant "super-bugs". Among these, fluconazole-resistant *Candida* was the only fungal species identified as a serious public health threat. More recently, *Candida auris* is emerging as a multidrug-resistant fungal pathogen with high mortality rates. *C. auris* is often resistant to all three classes of antifungal drugs and tends to occur in hospitalized patients with the same risk factors as infection with any *Candida* spp. This has prompted the Centers for Disease Control and Prevention (CDC) to issue a call-to-action to *"better understand, contain and stop the spread of this drug-resistant fungus"*. Here we use *Caenorhabditis elegans* as a model host to measure the virulence of clinical isolates of *C. auris, C. tropicalis,* C. *dubliniensis, C. lusitaniae*, and *C. krusei* as compared to a reference strain *Candida albicans*.

Introduction

The CDC released a report titled "Antibiotic Resistance Threats in the United States, 2013" where they raised concerns of antibiotic resistance in U.S. hospitals. These were categorized as urgent, serious, or concerning. The report lists the most threatening "superbugs". The only fungal species listed in threat level "serious" is fluconazole-resistant *Candida,* the rest being bacteria (Antibiotic Resistance Threats in the United States, 2013). This article alerted health care professionals to the importance of finding a solution and began the search to understanding the mechanisms of *Candida*.

Antimicrobial resistance and antifungal therapies

"Superbugs" are organisms like bacteria and fungi that have somehow acquired resistance to drugs that were once used to treat patients who were infected with them. These organisms are a major concern for health professionals all over the world. With limited numbers of drugs, doctors are running out of treatment options for their patients. Thousands of patients all over the world are already dying as a result of these drug-resistant organisms, and it will only get worse without action (Antibiotic Resistance Threats in the United States, 2013). This is especially concerning for *Candida*, due to the already-limited number of drugs that are available to treat fungal infections.

The three major classes of antifungal drugs are azoles, polyenes, and echinocandins (Kauffman, 2017). Different species of *Candida* are resistant to different classes of drugs. *C. auris* (*C. auris*)*,* for example, is more concerning than other species of *Candida* because it has shown resistance to all three classes of antifungals in different isolates (Kauffman, 2017).

While it is important to investigate virulence and pathogenicity of this fungal species, it is most important to remember that resistance to antifungals is a serious public health concern (CDC, 2013). There are only a few antifungal agents available due to similarities that exist between fungal cells and animal cells. There are few differences that can be exploited to target only fungal cells which limits expansion. Two commonly used classes of drugs - polyenes and azoles - target ergosterol which is a component in the fungal cell membrane (Ford *et al*, 2015). Echinocandins are another class of antifungal which targets a different cell wall component, 1,3-β-D-glucan (Singh-Babak, 2012). Figure 1 below shows the mechanisms of action of the different classes.

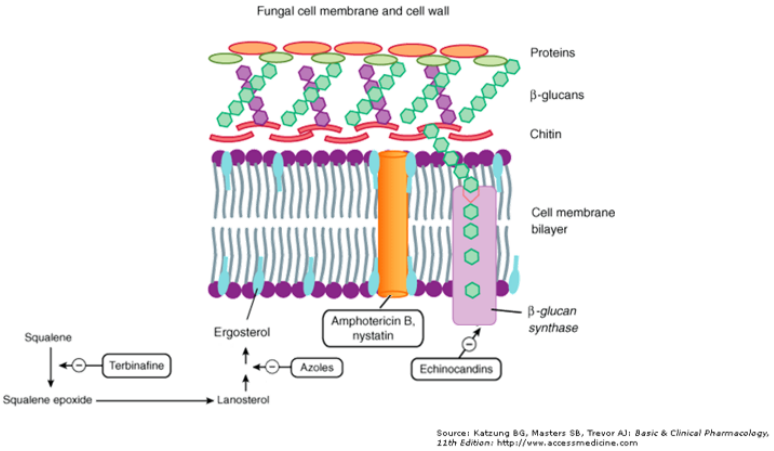


Figure 1: Antifungals target the cell surface

There are multiple mechanisms through which a pathogen acquires resistance to an antifungal. The first is that the yeast acquire a higher expression of their drug efflux pumps (Coste *et al*, 2006, Srinivasan *et al*, 2014). Drug efflux pumps work as transport proteins that remove toxic materials from within the yeast cell. This helps because when a yeast is exposed to the drug and it enters into the cell, the pumps are able to remove the toxic drug from the yeast. Overexpression of the genes that produce the drug efflux pumps is what commonly helps *C. albicans* acquire its drug resistance (Coste *et al*, 2006, Prasad *et al*, 2014).

The second mechanism is the occurrence of phenotypic alterations at the target site for the drug (Srinivasan *et al*, 2014). This can occur through mutations and changes of the target enzyme which prevent the drug from recognizing any familiar source and preventing treatment. Although it is uncommon, several studies have shown that non-*albicans* species of *Candida* including *krusei* and *lusitaniae* are very likely to develop polyene-resistance through phenotypic alterations (Srinivasan *et al*, 2014).

The third mechanism of resistance is the recombination in the genome that make the drug less effective (Srinivasan *et al*, 2014). There have been resistance genes that have been discovered in fungi (Marie *et al*, 2009). Mutations in the genome of the fungi that make them resistant to drugs are advantageous to the fungi in terms of evolution. Because of evolution, the only fungi that survive when an antifungal is introduced are also the ones that reproduce, leading to a new population of fungi that are more drug resistant.

The final mechanism is that organisms produce biofilms that protect the drugs from entering (Srinivasan *et al*, 2014). Biofilm formation is very common. Biofilm is the formation of a film by the fungus to prevent drugs from entering into the cells. This protects the fungi from the drugs and provides them with a safe area to reproduce and thrive (Chandra *et al*, 2001). Biofilm

formation is the natural way for many different bacteria and fungi to grow because of the protection that it provides (Nobile *et al,* 2015).

Candida auris **background**

Candida auris is a specific fungal species that causes serious infections. These infections are most common in long term hospital and nursing home patients who are immunosuppressed, or have a central venous catheter (CDC, 2017). The *C. auris* outbreaks seemed to arise simultaneously worldwide with outbreaks occurring in over fifteen countries. The first outbreak of *C. auris* was documented in Japan in 2009 (CDC, 2017). As of October 31, 2017, ten outbreaks have been identified in the United States with testing that traced the sources of outbreak to different countries. The states that have documented outbreaks are New York, Massachusetts, Connecticut, New Jersey, Maryland, Florida, Oklahoma, Illinois, Indiana, and California (CDC, 2017). The CDC mapped these outbreaks, which can be seen in Appendix A. New York State currently has the highest number of *C. auris* cases in the U.S. with 97 cases in its hospitals (Lutterloh, 2017). The majority of cases occurred in New York City with other cases occurring at hospitals outside the city range. Out of the 97 cases, there were 20 deaths. All documented mortalities came with comorbid conditions (Lutterloh, 2017).

There have been cases within the U.S. in which the patient had recently stayed in healthcare facilities in another country and contracted the infection there. This specific scenario occurred in India, Pakistan, South Africa, and Venezuela (CDC, 2017). Other countries that have reported multiple outbreaks are Canada, Colombia, India, Israel, Kenya, Oman, Pakistan, Panama, South Korea, South Africa, Spain, the United Kingdom, and Venezuela (CDC, 2017). Singular cases that arose have been reported from Germany, Japan, Kuwait, and Norway (CDC, 2017). A geographic map of these outbreaks can be seen in Appendix A. Globally there may be more undetected or undocumented outbreaks of *C. auris* in these countries, or in "uninfected" countries.

Although the sources of each of these outbreaks differs, it has been found that *C. auris* infections are spread through contact with the environment. The environment includes contact with infected people as well as contact with infected surfaces. The popular press (CNN in May of 2017) has reported that *C. auris* has been isolated from mattresses, bed rails, countertops and windowsills weeks after an infected person has left the hospital room (Scutti, 2017). This suggests that the fungus can survive on any surface for a relatively long period of time.

The CDC tested *C. auris* isolates that were collected from different countries for their resistance to different drugs. Multidrug resistance is an important concern because antifungals that will be effective against *C. auris* are not available, meaning fatalities will be unavoidable. The resistance of each of the 10 *C. auris* isolates studied in this experiment can be seen in Table 1. The isolates of *C. auris* are ordered from least overall resistant to most overall resistant.

Table 1: Resistance of C. auris isolates to antifungals

		Azoles				Echinocandins			nt Analog	Polyene
Lab ID#	Bank #	Fluconazole	Voriconazole	Posaconazole	Itraconazole	Caspofungin	Anidulafungin	Micafungin	Flucytosine	Amphotericin B
F317-cau1	381	4	0.03	0.06	0.125	0.125	0.25	0.125	2	0.38
F318-cau2	382	16	0.5	0.5	1	0.5	0.25	0.25	0.125	0.38
F315-cau3	383	128	4	0.5	0.5	16	1	1	0.5	0.38
F316-cau4	384	128	1	0.5	1	16	2	2	0.5	0.5
F319-cau5	385	>256	16	1	1	0.5	1	0.5	0.5	0.5
F320-cau6	386	>256	16	0.5	0.5	0.5	1	0.25	0.5	0.5
F321-cau7	387	8	0.6	0.25	0.5	0.25	0.5	0.5	8	0.75
F322-cau8	388	>256	2	0.25	0.5	1	0.5	0.125	0.125	1.5
F323-cau9	389	256	4	0.125	0.25	0.5	1	0.25	128	4
F324-cau10	390	>256	8	0.5	1	0.5	1	0.25	128	4

As seen in Table 1, nearly all isolates that have been tested by the CDC are highly resistant to fluconazole. About one third were resistant to amphotericin B (a polyene), and a small number were resistant to echinocandins (Passi, 2017). Infections with *C. auris* are often deadly when there is a combination of resistance to all three major classes of antifungals.

Other drug resistant *Candida*

Candida is a genus of yeast that is a common cause of infection worldwide. It is usually found in the intestinal tract and can also be found on mucous membranes and skin without any health problems (CDC, 2017). However, when there is an overgrowth of *Candida* in humans, it can lead to the progression of infection, illness, or death (CDC, 2017). *Candida* species have evolved in the last few decades, with new species emerging, typically in healthcare settings. A phylogenetic tree of *Candida* can be seen in Figure 2. *C. auris* has been cultured from various types of infections including deep tissues, bloodstream, superficial wounds, and ear (CDC, 2017). The symptoms vary based on the site of the infection, which makes it difficult to diagnose.

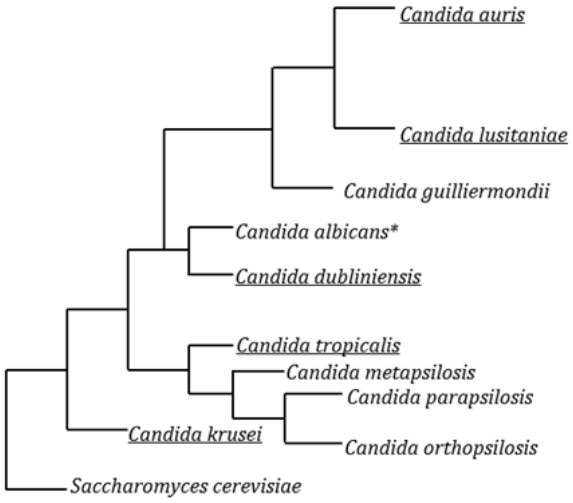


*Figure 2: Phylogenetic tree of Candida. Underlined species were used in this experiment. *reference strain.*

In order to test against the ten isolates of *C. auris*, six other closely-related species of *Candida* were examined and served as experimental references. The different species included *C. albicans, C. krusei, C. lusitaniae, C. tropicalis,* and *C. dubliniensis.* The control that was used throughout this experiment was *C. albicans*. The control is found in the gastrointestinal and genitourinary tracts, and has shown very little drug resistance (Nobile *et al* 2015). The other species of *Candida* are secondary reference strains and have been heavily-researched prior to this study. The *Candida* reference strains are also resistant to antifungals. Referencing different species throughout the experiment allows for greater overall understanding of *C. auris* and its mechanism.

Virulence assays

In order to complete an experiment and appropriately address the problem, a model host must first be chosen. A similar study was completed in the past on *Galleria melonella*, which is a species of moth. There are various other *in vivo* animal models that can be used to study *Candida* (Harwood, Rao, 2014). These include mice, zebrafish, fruit flies, and nematodes (Harwood, Rao, 2014). Each model has its own advantages and limitations. Because of the nature of this experiment, the model hosts were nematodes. Benefits from this choice include that they are easily controlled, they can be frozen indefinitely, and they allow for a large sample size (Harwood, Rao, 2014). The only disadvantage is that nematodes do not have phagocytes, so the virulence demonstrated in the nematodes may not translate well to a human host (Harwood, Rao, 2014).

According to the study done on the invertebrates *Galleria melonella*, *C. albicans* is the most pathogenic species in the *Candida* genus. There are some strains of *auris* that were comparable in virulence to *C. albicans*, but *C. albicans* was found to be the most pathogenic (Borman *et al*, 2016). Each *Candida* strain in the *Galleria* study was tested on "at least 10" *Galleria melonella* larvae. This study informed the decision to use *Candida albicans* as the reference strain for all other strains.

The host organism for this experiment is wild-type *Caenorhabditis elegan* N2 (*C. elegan*). These organisms are nematodes that share many genes and molecular pathways with humans. They also have a similar number of genes to humans as well as functional counterparts within the genes (Riddle, 1997). *C. elegans* reproduce quickly which makes them a good model host for this assay and allows for higher statistical significance with a greater number of hosts. Taking advantage of their quick reproduction cycle allowed for this study to use 90 *C. elegans* per fungal strain and increase statistical significance.

There are policies in place at most healthcare facilities already, such as hand washing and linen disposal. However, another factor that should be taken into considered is antifungal stewardship. This means that clinicians need to be aware of the risks and benefits of treating patients with

different antifungal agents and weighing the options before treatment to lower the risk of future resistance (Chowdhary *et al* 2016). It is important to understand the biology of *C. auris* including its virulence mechanisms to develop effective therapies. This will ultimately be used in healthcare facilities to limit the spread of nosocomial infections.

Project Goal

The goal of this project is to compare the virulence of 16 isolates of *Candida* to a reference strain of *C. albicans* (SC5314). The reference strain has been studied in depth and has been identified as the most pathogenic species of *Candida* (Borman *et al*, 2016). We will use *C. elegans* as an in vivo model to examine the virulence of each *Candida* isolate and compare to the reported antifungal resistance of each species.

Materials and Methods

Strains and Media

Ten *Candida auris* isolates (Table 1) were obtained from the Center for Disease Control (CDC). All *C. auris* strains were frozen upon arrival to avoid contamination during future experiments. Morphological analysis on differential growth media (CHROMagar) and relative resistance to fluconazole will be measured to further verify strains prior to use in this experiment.
Prior to each assay the strains were streaked onto Yeast-Peptone-Agar (YPD) plates and stored at 20°C. Liquid cultures were prepared by adding *C. auris* from those stock plates using a sterile inoculating loop to 5 mL liquid YPD and left to incubate overnight (14-18 hours) at 30°C in a roller drum. The 10 *C. auris* experimental isolates were compared to seven other strains of *Candida* species including *C. albicans* (1), *C. lusitaniae* (2), *C. tropicalis* (1), *C. dubliniensis* (1), *C. krusei* (2). Each *Candida* strain was grown following the same protocol as described for *C. auris*. The *Escherichia coli* strain OP50 stock solution was grown in Luria Broth (LB) and stored at 4°C. *C. albicans* was used in each survival assay as a control.

NGM Media

NGM is nematode growth media and is made for the *C. elegans* to live on for the duration of the assay. The NGM was prepared in a 6,000 mL erlenmeyer flask. The dry ingredients were added to 1,950 mL of water. Dry ingredients included 6g NaCl, 5g peptone, and 40g agar. The erlenmeyer flask with ingredients and the tube used for pouring plates were autoclaved in cycle 2 for 15 minutes. The wet ingredients were added to the NGM after sterilization. Wet ingredients included 2 mL 1M $MgSO_4$ x $7H_2O$, 2 mL 1M $CaCl_2$, 50mL 1M KPO_4, 1mL 0.5% cholesterol in EtOH. The prepared NGM was poured into plates and left overnight to solidify.

N2 Worm Maintenance

The *C. elegans* strain used for this experiment is the N2 line derived in 1964 by Sydney Brenner. This strain has been maintained in laboratories ever since and is now referred to as N2 (Riddle, 1997). In order to maintain the model host, the adult N2s were moved every two days onto new

plates. These new plates were prepped with 20 μL of the E Coli strain OP50, which was the food for the *C. elegans.*

Egg Preparation

A stock plate of *C. elegans* was prepared two days prior to egg prep. A slice of the stock plate was cut out and placed onto Nematode Growth Agar Media (NGM) that was seeded with OP50. These *C. elegans* were left to grow for 2 days at room temperature. On day 0 of experimentation the plates were flooded with 10 mL of M9 buffer to dislodge *C. elegans* from the media and this solution was centrifuged for 2 minutes at 3500 rpm. The supernatant was removed and the pellet of *C. elegans* was resuspended in a solution containing 1 mL NaOH, 2 mL H_2O and 1 mL bleach. The 15 mL conical tube was inverted for 2-3 minutes until the adult *C. elegans* were dissolved completely. The solution was then centrifuged for 2 minutes at 2500 rpm. The supernatant was removed once again and 200 μL of M9 was added. 20 μL of each M9 and egg solution was pipetted onto each subsequent plate. These plates were NGM plates that were also seeded with 20 μL OP50. Each 20 μL should yield approximately 30 eggs.

Dar Assay

The Dar assay is a sign of infection in the *C. elegans*. Dar stands for deformed anal region and can be identified as a small bump on the post anal region (Figure 9). This assay was completed on days 4 and 5 of each survival assay. This means that of the total *C. elegans* on each plate, the percentage that exhibited Dar was calculated. Typically when *C. elegans* are infected with *Candida*, the percentage Dar is about 80%.

Survival Assay

To prepare plates for *C. elegans* transfer after initial egg preparation plates were seeded with a specific fungus (Figure 4). 500 μL of each liquid culture of *Candida* were pipetted into microcentrifuge tubes and centrifuged at 13.2 rcf for 10 minutes. Supernatant was removed and the pellet was resuspended in 250 μL of deionized H_2O. Mixture was centrifuged at 13.2 rcf for 5 minutes. The supernatant was removed once more and the pellet was resuspended in 500 μL of deionized H_2O. The optic density (O.D.) was measured using a solution of 100 μL liquid culture and 900 μL deionized H_2O. A calculation was carried out to find the volume of liquid culture to be plated using the O.D. measurements. 20 μL of a liquid culture and deionized H_2O solution were plated onto corresponding NGM (Figure 3). The NGM plates were incubated at 20°C for approximately 24 hours.

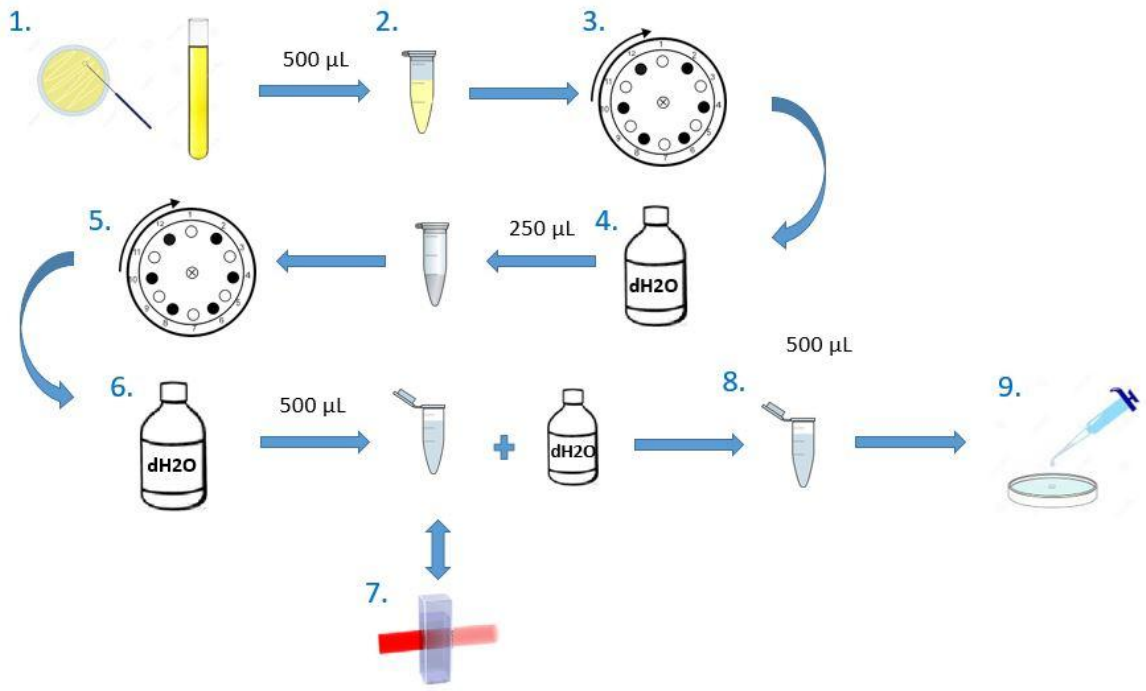


Figure 3: Steps to preparing plates for assay. Step 1: Prepare liquid cultures 14-16 hours before. Step 2: Add 500 µL of liquid culture to microcentrifuge tube. Step 3: spin down using microcentrifuge. Step 4: Resuspend in autoclaved water. Repeat steps 2-4 for 5-6. Step 7. Measure the optic density of the sample to calculate the correct ratio of culture/water. Step 8: Combine calculated amounts of culture and water into a new microcentrifuge tube. Step 9: Pipette 20µL solution onto labeled NGM plates.

C. elegans were counted every day. The number of alive, dead, and missing *C. elegans* was observed and documented every day. Every other day *C. elegans* were transferred to fresh seeded plates to prevent new eggs from hatching. The continuous transfer allowed for less confusion between original *C. elegans* and newly hatched *C. elegans*. A platinum metal pick was used to transfer each live *C. elegans* to the new NGM plate. The plates were incubated at 20°C again for about 24 hours.

Statistical Analysis

Results were quantified using GraphPad Prism, which is a program designed to analyze biological data. This program calculated both log rank and Gehan-Breslow Wilcoxon p-values, as well as the mean time to death (MTD) from the inputted survival information for each experimental isolate compared to *C. albicans*. Statistical significance is defined as a p-value < 0.05. Two analysis tools were used in this experimentation in order to provide significant evidence that a p-value is accurate. With the different factors that both analysis take into consideration, the results that are measured as p-value < 0.05 are significant. The Gehan-Breslow Wilcoxon analysis counted the *C. elegans* that left the plate in the first couple of days as "censored" which means that they were removed from the counts in following days. This test

specifically assumes that data from the early times are more accurate than later times and weights it all accordingly. The number of censored *C. elegans* was taken as the total number of nematodes on that day minus total number of nematodes from the previous day. Mean time to death (MTD) is reported as the number of days where the total number of *C. elegan* population was 50 percent. This was also compared among all of the isolates.

Results

In order to streamline the survival assay and mitigate confounding variables, a specific assay protocol was followed to prepare the *C. elegans* and the fungal species. Before the official start date of the experiment, *C. elegans* were grown on NGM plates with OP50 for two days until they became full adults. To prepare for the assay these plates were exposed to bleach in order to dissolve adult worms and leave the eggs to hatch on plates seeded with the corresponding fungal strain (Figure 4).

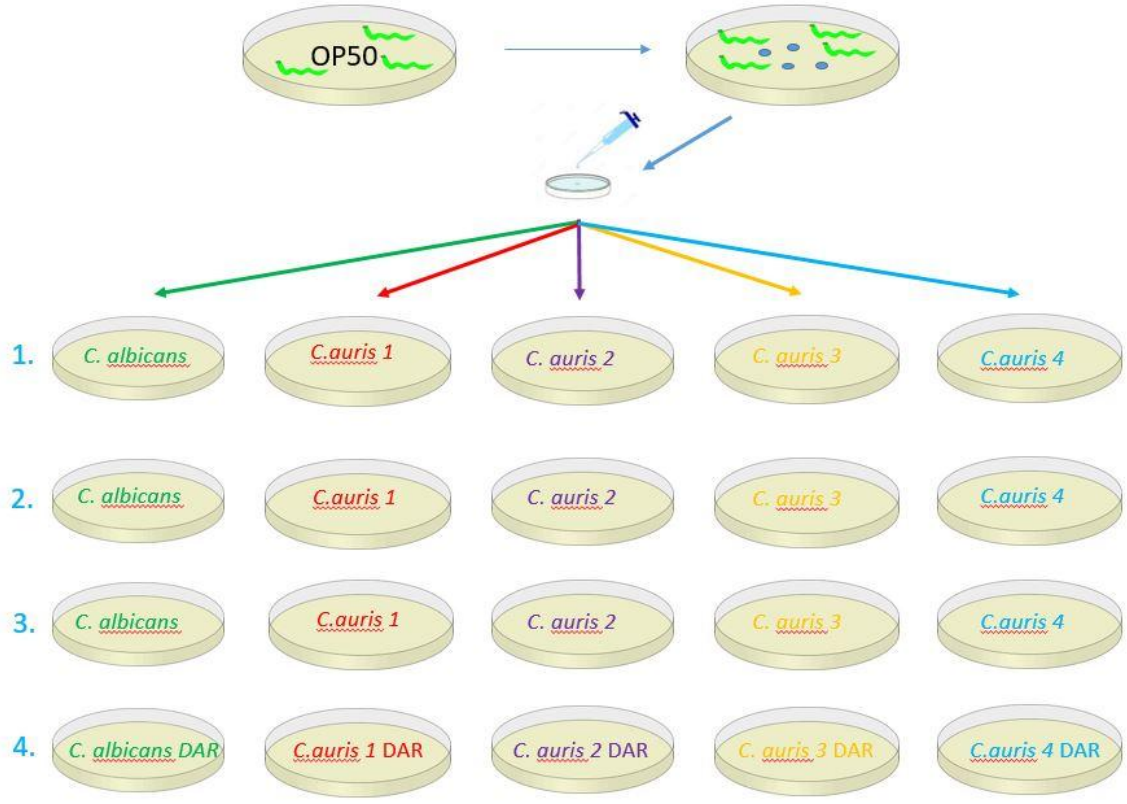
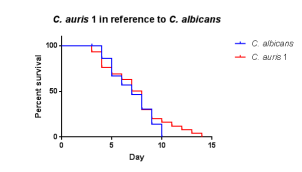
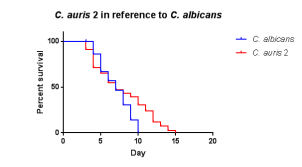
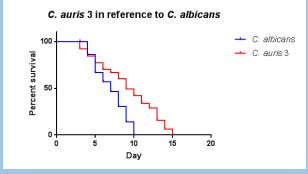
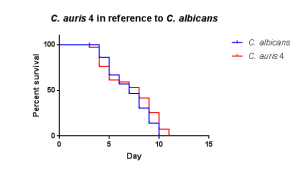
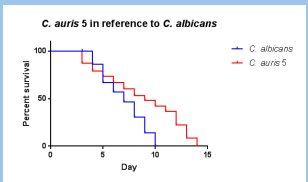
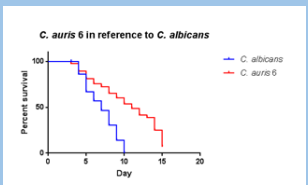


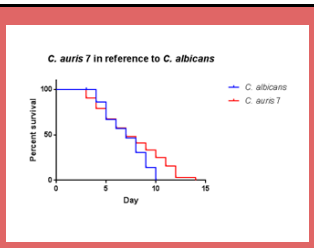
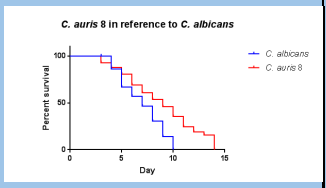
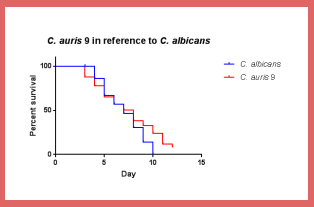
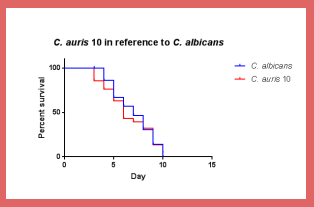
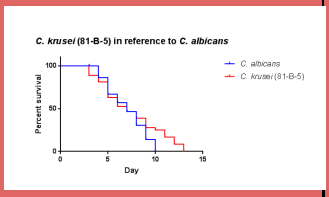
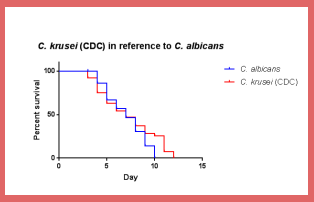
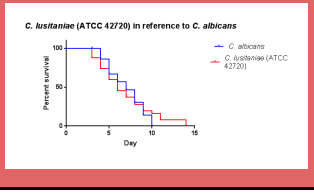
Figure 4: A diagram example of the setup of an assay. Worms were grown on plates with OP50 and left to sit and lay eggs. After 2-3 days the plates were exposed to a bleach solution described in the methods section of this paper. 20 µL of egg solution were pipetted onto plates previously seeded with the Candida isolate being tested.

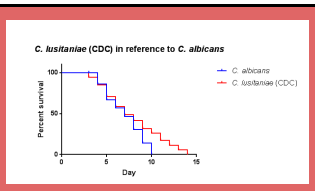
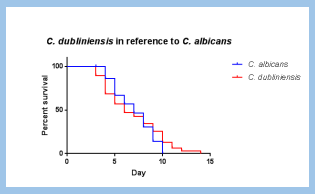
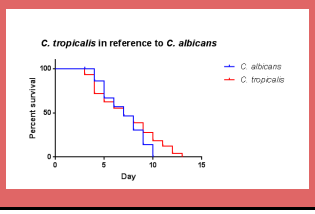
The data gathered from this assay were quantified using GraphPad Prism. The program output the mean time to death and p-values for both log rank analysis and Gehan-Breslow Wilcoxon analysis. Significance is defined as p-value < 0.05.The results of these three statistical tests can

be seen in Table 2. Rows highlighted in blue are significant and rows highlighted in red are insignificant.

Table 2: Results from Survival Assays and Statistical Analysis for all Candida Isolates.

***Candida* Species**	**Survival Curve**	**MTD**	**Log Rank**	**Gehan-Breslow Wilcoxon**
C. auris 1		8	0.3999	0.6847
C. auris 2		7	0.0196	0.5050
C. auris 3		9	0.0002	0.0959
C. auris 4		8	0.4618	0.6349
C. auris 5		9	0.0041	0.7750
C. auris 6		11	< 0.0001	0.0002

C. auris 7		7	0.1418	0.7401
C. auris 8		9	0.0003	0.0351
C. auris 9		8	0.2443	0.3829
C. auris 10		6	0.4275	0.0893
C. krusei (81-B-5)		7	0.2409	0.5594
C. krusei (CDC)		7	0.2827	0.4164
C. lusitaniae (ATCC 42720)		6	0.6868	0.0610

C. lusitaniae (CDC)		7	0.0686	0.6693
C. dubliniensis		6	0.8590	0.0484
C. tropicalis		7	0.5216	0.2609

While *C. auris* 1 is the least drug resistant and *C. auris* 10 is the most drug resistant, the measured virulence for *Candida* isolates do not reflect the same relationship.
When infected with the fungal species *C. albicans*, the *C. elegans* typically died within 10 days with a MTD of 7 days. Each *C. auris* isolate was tested in reference to *C. albicans*. The complete survival curve of all 10 *C. auris* isolates in reference to *C. albicans* can be seen in Figure 5.

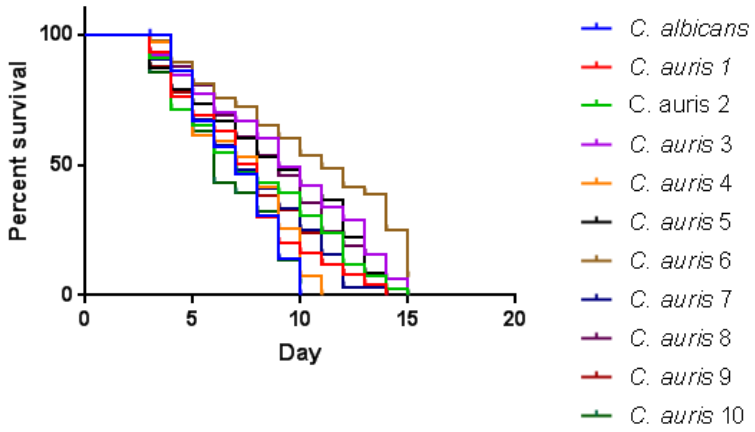


Figure 5: C. auris isolates in reference to C. albicans

The royal blue curve that ends at day 10 is the reference strain, *C. albicans*. The initial hypothesis predicted that there would be a correlation between the known drug resistance of the *C. auris* isolates and their measured virulence. These results suggest that the measured data does not support the hypothesis relating drug resistance and virulence. There was no direct correlation

identified that could explain the order of *C. auris* virulence that was measured. *C. auris* 6 was found to be the least virulent and *C. auris* 10 was found to be the most virulent. Selected survival curves were analyzed in a separate assay and the combined graph can be seen in Figure 6. In this assay, *C. auris* isolates 1, 3, 5, and 10 were examined in reference to *C. albicans*. These four isolates were specifically chosen for comparison because they represent the four clades of *C. auris* that the Rao Lab at WPI is currently focusing on.

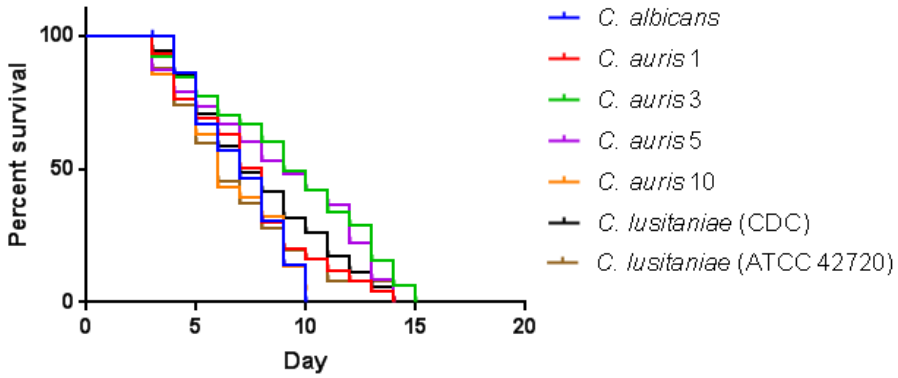


Figure 6: Comparison of C. auris isolates 1, 3, 5 and 10 in reference to C. albicans and C. lusitaniae.

This graph shows highly drug resistant *C. auris* 10 as almost identical to *C. albicans*. *C. auris* 10 was not found to be statistically different than *C. albicans* with a MTD of 6 and p-values much greater than 0.05. *C. auris* 3 and *C. auris* 5 were similar in virulence, both having a MTD of 9 days and p-values for log rank < 0.05. *C. auris* 1 followed the same trends as *C. auris* 10 and *C. albicans* until about day 9 where the final surviving worms had gradual death. *C. auris* 1 was not found to be statistically significant and had a MTD of 8 days. The individual survival curves for each isolate can be found in Table 2 and Appendix B.

C. krusei* is drug resistant yet less virulent as compared to *C. albicans

In order to further understand and examine the virulence of *Candida*, six other species of *Candida* were used in the assay and compared to *C. albicans*. These included *C. dubliniensis*, *C. krusei* (CDC), *C. krusei* (81-B-5), *C. lusitaniae* (CDC), *C. lusitaniae* (ATCC 42720), and *C. tropicalis*. The survival curve comparing these species in reference to *C. albicans* can be seen in Figure 7.

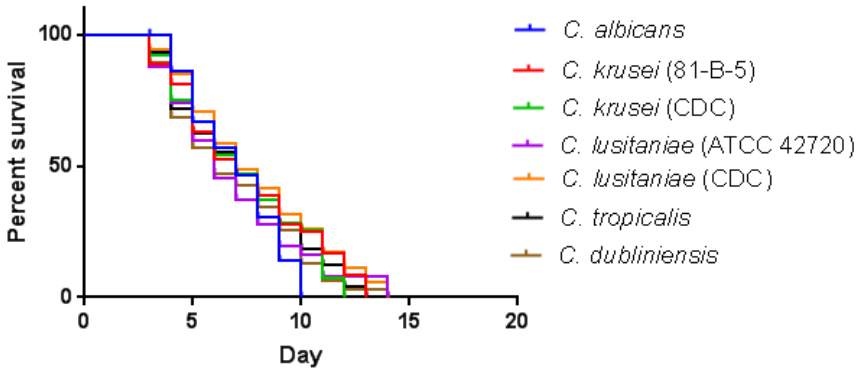


Figure 7: Candida species in reference to C. albicans

The results from this comparison suggest that all of the experimental species were less virulent than *C. albicans.* The least virulent species comparatively was found to be *C. dubliniensis*. *C. dubliniensis* was determined to have a MTD of 6 days and a p-value of 0.0484 on the Gehan-Breslow Wilcoxon test. This species had a log rank p-value of 0.8590. All other species had p-values > 0.05 when compared to *C. albicans* for both statistical analysis determining that the majority of the survival of the *C. elegans* was similar to *C. albicans*. Hosts infected with *C. krusei* (ATCC 42720), *C. krusei* (CDC), *C. lusitaniae* (CDC) and *C. tropicalis* had a MTD of 7 days. Although *C. lusitaniae* (ATCC 42720) was found to be statistically similar to *C. albicans* with a p-value > 0.05, it had an early MTD of 6 days. *C. lusitaniae* (CDC) compared to *C. lusitaniae* (ATCC 42720) was found to have a log rank p-value > 0.05 and a Gehan-Breslow Wilcoxon p-value of 0.0450. This difference in p-values is most likely due to the weighted differences between the two statistical analyses. The results suggest that there is a difference in the virulence of the *C. lusitaniae* isolates.

The two isolates of C. krusei were analyzed side by side and found to have a log rank p-value=0.7050 and a Gehan Breslow Wilcoxon p-value of 0.9643.Both isolates had a MTD of 7 days. These results suggest that the two isolates were very similar in virulence. Due to the known high drug resistance of *Candida krusei* (81-B-5), it was expected that there would be high virulence associated with it. *C. krusei* shows innate resistance to fluconazole (Cuomo *et al*, 2017). There have also been instances of resistance to echinocandins (Cuomo *et al*, 2017). Higher doses of polyenes are being required to treat *C. krusei* infections in recent years (Kauffman, 2017). *C. albicans*, on the other hand, shows significantly lower levels of resistance across all three classes of drugs. There was no statistically significant differences between *C. krusei* (81-B-5) and *C. albicans*. This suggests that the drug resistance is unrelated to the virulence.

***C. auris 1* and *C. auris 2* vary in drug resistance, but have similar virulence.**

The results suggest that there is no relationship between virulence and drug resistance in these *C. auris* isolates. *C. auris* 1 was the first strain isolated and was isolated from a patient's ear. *C. auris* 1 also showed very little drug resistance as seen in Table 1. *C. auris* 2 was the next to be

isolated and has been found to have a classic mutation for drug resistance. However, these clear differences in the drug resistance did not correlate to any difference in the measured virulence. The MTD for both isolates was found to be 7 days. While both isolates were statistically insignificant according to the Gehan-Breslow Wilcoxon analysis (*C. auris* 1 = 0.6437 and *C. auris* 2 = 0.5050), the log rank analysis measured *C. auris* 2 as statistically significant with a p-value = 0.0196 while this same test measured the p-value of *C. auris* 1 = 0.2777. The survival curve comparing *C. auris* 1 and *C. auris* 2 in reference to *C. albicans* can be seen in Figure 8.

A previous study (Lockhart et. al., 2017) examined mutations found to contribute to drug resistance in *C. auris*. In this study they compared Erg11 amino acid sequences in *C. albicans* and *C. auris*. Three "hot-spot" amino acid substitutions were found that significantly increased fluconazole resistance (Lockhart et. al, 2017). These mutations include F126T, Y132F, and K143R. They determined that these mutations were likely what gives the fungus its azole resistance. These mutations altered the target protein of the azoles, ergosterol, so that resistance could be achieved. *C. auris* 1, the first *auris* isolate, did not have this classic mutation.

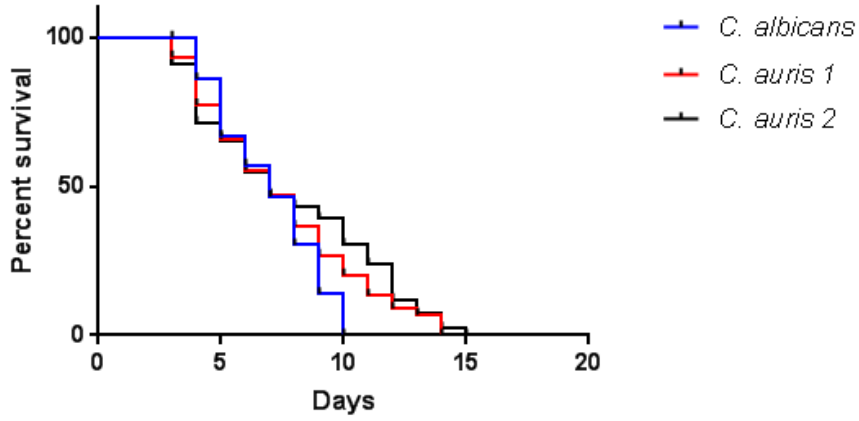


Figure 8: Comparison of C. auris isolates 1 and 2 in reference to C. albicans.

The results suggest that drug resistance and virulence do not correlate. The reasons for this lack of correlation is unknown. It is possible that when the fungus gains a mutation for drug resistance, it may result in a fitness cost. The fungus then might be more resistant to certain drugs but it is less likely to reproduce and succeed from an evolutionary standpoint.

Relative Dar phenotype varied in each *Candida* species

An additional analysis in this experiment was the observation of deformed anal regions (Dar) of the *C. elegans*. As a reminder, the presence of the Dar phenotype indicates that the worm has been infected by the fungus (Figure 9).

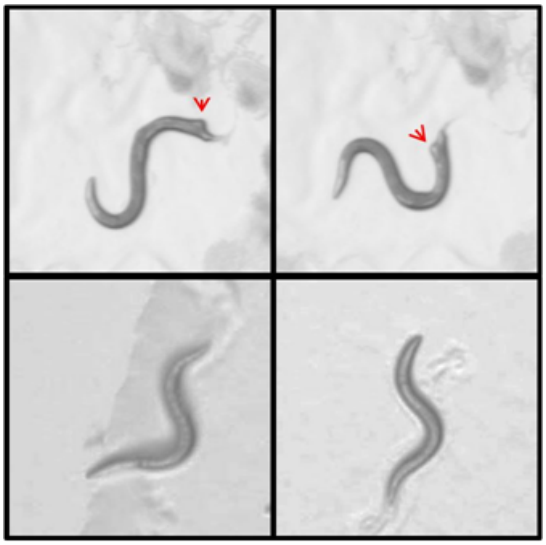


Figure 9: Image comparing observed Dar phenotype in C. elegans infected with C. lusitaniae (CDC) (a and b) to uninfected C. elegans fed the same fungus (c and d). The red arrows are pointing to the Dar phenotype.

In the bottom two figures the *C. elegans* are uninfected and do not show the Dar phenotype. In the top two boxes of the figure the Dar phenotype is shown by the bump on the right tail end of the worm (indicated by the red arrow). This bump is the physical representation of the phenotype and the indicator of infection.

For each *Candida* isolate, the percentage of Dar was determined based on the entire population. One plate was prepared specifically to observe Dar on the fourth or fifth day of the assay. On those days, the number of worms with Dar were counted and the percentage was determined out of 30 total adult worms (there were a total of 30 worms on each plate). Figure 10 shows the Dar percentages determined in this study for all 17 strains of *Candida*.

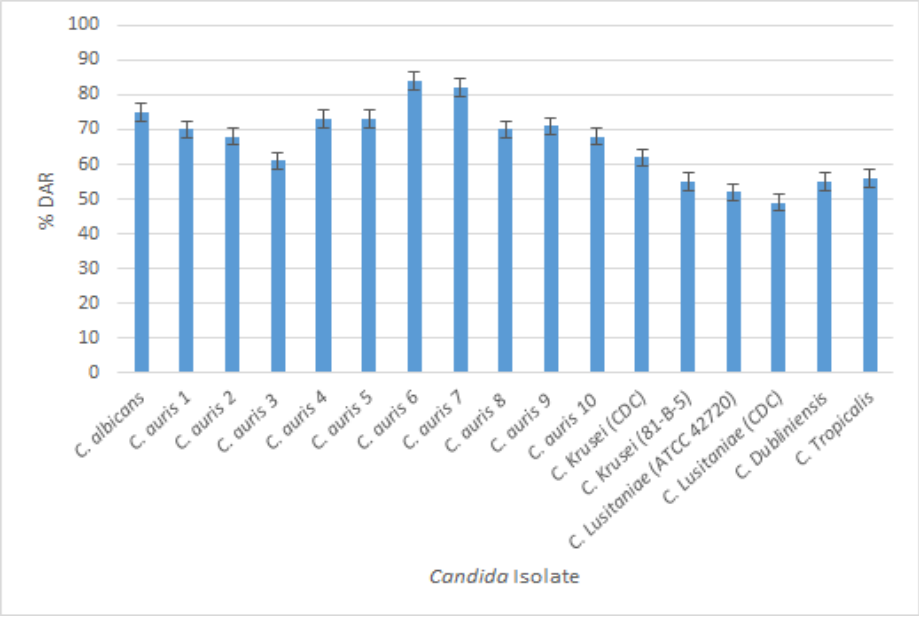


Figure 10: Relative Dar phenotype of Candida species

While this graph does not show specific trends in the percentage of the Dar phenotype, there are several notable differences. *C. auris* 6 and *C. auris* 7 both show significantly high percentages of Dar. The secondary reference strains including *C. krusei, C. lusitaniae, C. dubliniensis*, and *C. tropicalis* all had low percentages compared to *C. albicans* (Figure 11).

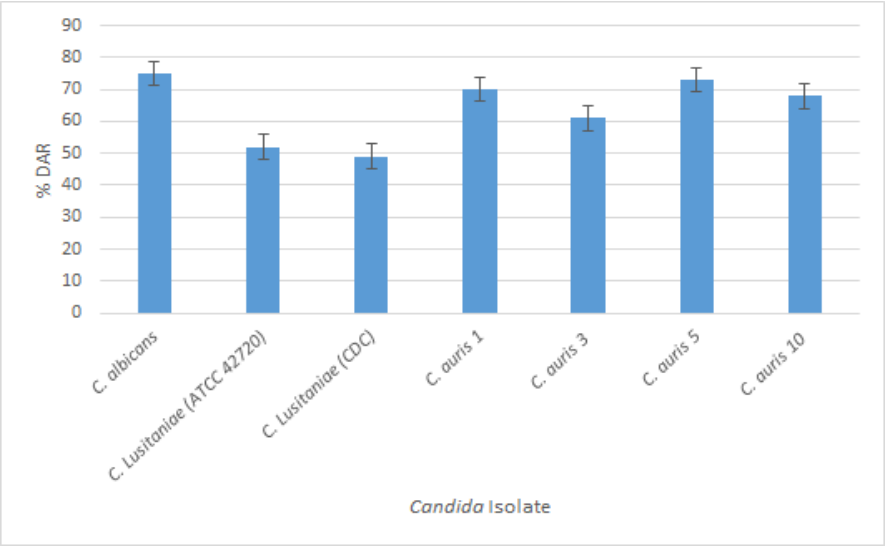


Figure 11: Relative Dar phenotype of Candida reference strains in reference to C. albicans

Figure 11 shows a direct comparison of *C. albicans* to the secondary reference strains. These results suggest that there was a low percentage of *C. elegans* that displayed the Dar phenotype on Day 5 of the assay. Additional specific bar graphs comparing the Dar phenotypes between *Candida* species can be found in Appendix C. The Dar phenotype percentages were further compared to virulence and can be seen in Table 3.

Candida Species	Mean Time to Death	Dar %
C. albicans	7	75
C. auris 1	8	70
C. auris 2	7	68
C. auris 3	9	61
C. auris 4	8	73
C. auris 5	9	73
C. auris 6	11	84
C. auris 7	7	82
C. auris 8	9	70
C. auris 9	8	71
C. auris 10	6	68
C. krusei (81-B-5)	7	55
C. krusei (CDC)	7	62
C. lusitaniae (ATCC 42720)	6	52
C. lusitaniae (CDC)	7	49
C. dubliniensis	6	55
C. tropicalis	7	56

high virulence with high Dar %
high virulence with low Dar %
low virulence with high Dar %
low virulence with low Dar %

Table 3: Relative Dar percentages compared to virulence (MTD) in Candida species

In this table a high virulence was classified as 6-8 days and a low virulence was classified as 9-11 days. A high Dar percentage was classified as any value greater than 65% and a low Dar percentage was classified as anything under 65%. With this comparison of data, the overall results of the Dar assay suggest that there is no direct correlation between the virulence measured and the percent of Dar measured.

Discussion and Conclusion

This experiment was a comparative study of the virulence of various *Candida* isolates and a map of correlation between virulence and drug resistance. The *C. albicans* strain SC5314 was used as a reference strain for these studies. The test strains used in this study included *C. krusei* (81-B-5), *C. krusei* (CDC), *C. lusitaniae* (ATCC 42720), *C. lusitaniae* (CDC), *C. tropicalis*, *C. dubliniensis,* and 10 different isolates of *C. auris*. Our results suggest while there are statistically significant differences between five of the ten strains of *C. auris* and the reference strain *C. albicans,* there are no direct correlations between the virulence of *C. auris* and their relative drug resistance. To test virulence we observed the survival rates of the model host *C. elegans* when infected with the different strains of *Candida*. The gathered data was analyzed using the software Graphpad Prism. Typically the *C. elegans* would survive between 10-15 days during each assay. The nematodes infected with the *C. auris* strains tend to survive longer than those infected with *C. albicans*. When working with *C. krusei* (81-B-5) and *C. krusei* (CDC), it was found that although this species is known to have high drug resistance, it was measured as one of the least virulent. These results pose a paradox that the virulence of the *Candida* isolates are unrelated to the drug resistance which have a direct correlation with mortality during nosocomial infections. Here we discuss the drug resistances of each of the experimental strains.

Prior to the start of this experiment, the drug resistance of each *C. auris* strain were known. With this prior knowledge it was predicted that there would be a potential correlation between the measured virulence and drug resistance of each strain. The grouping of *Candida* were numbered in order of their relative drug resistance. *C. auris* 1 was ranked as the overall least drug resistant and *C. auris* 10 was ranked as the overall most drug resistant. The other species of *Candida* also had known drug resistances. *C. krusei* is known to have significant drug resistance, with high doses of azoles and polyenes (Amphotericin B) required (Kauffman, 2017). Echinocandins are typically useful in treating a patient with *C. krusei*, but there have been cases of resistance to this class as well (Kauffman, 2017). *C. lusitaniae* is normally very susceptible to both echinocandins and azoles but can quickly acquire resistance to Amphotericin B (Kauffman, 2017). *C. tropicalis* is normally susceptible to all three classes of antifungals, but there has been increasing resistance to caspofungin and fluconazole (Kauffman, 2017). Lastly, *C. dubliniensis* is usually susceptible to all three classes of antifungals, though there was an outbreak of fluconazole-resistant strains in AIDS patients in the 1990's (Kauffman, 2017). The gathered data from this experiment determined that the relative virulences did not follow the same pattern and did not seem to follow a distinct pattern at all. Most of the *C. auris* strains killed all of the *C. elegans* about 1-3 days after the *C. albicans*.

The results of this experiment are able to confirm that the drug resistance and the virulence of these *Candida* isolates are not related. The measured p-values and MTD for all experimental strains of *Candida* in reference to *C. albicans* can be seen in Table 2. The least virulent isolate of *C. auris* was found to be *C. auris* 6, with a MTD of 11 days. This isolate differed from reference strain *C. albicans* according to the statistical analysis with a log rank p-value of < 0.0001 and a Gehan-Breslow Wilcoxon p-value of 0.0002. *C. auris* 2 was found to be the second most virulent isolate with a MTD of 7 days, a log rank p-value of 0.0196 and a Gehan-Breslow Wilcoxon p-value of 0.5050. *C. auris* 3, 5, and 8 were also considered statistically significant according to the analysis and each had a MTD of 9 days. *C. auris* 3 had a log rank p-value of 0.002 and a Gehan-Breslow Wilcoxon p-value of 0.0959. *C. auris* 5 had a log rank p-value of 0.0041 and a Gehan-Breslow Wilcoxon p-value of 0.7750. *C. auris* 8 had a log rank p-value of 0.0003 and a Gehan-Breslow Wilcoxon p-value of 0.0351. As previously mentioned the difference between Gehan-Breslow Wilcoxon statistical analysis and the log rank analysis is that Gehan-Breslow Wilcoxon assumes that data from earlier time points is more accurate than later time points and weights the entire assay accordingly. This difference in statistical analysis is why an isolate of *Candida* could be statistically significant on one test, but not on the other.

C. auris 1 was the first isolates of *C. auris* and was taken from a human ear. The next strain that was isolated was *C. auris* 2 which is known to have a mutation in its ERG11 that resulted in drug resistance. Although *C. auris* 2 was known to be more drug resistant, there was minimal difference in virulence between *C. auris* 1 and *C. auris* 2. Both isolates were measured to have a MTD of 7 days. *C. auris* 2 was found to be statistically significant according to the log rank test with a p-value of 0.0196 and a Gehan-Breslow p-value of 0.5050. *C. auris* 1 was not found statistically significant with p-values in both tests > 0.05. These results oppose the original hypothesis relating drug resistance and virulence and suggest that the drug resistance of *C. auris* 2 did not have an effect on its virulence.

Although there are a variety of ways that a microorganisms can acquire resistance to drugs. *C. auris* has emerged as a serious pathogen that is resistant to multiple antifungal agents. The mechanism of which *C. auris* acquires antifungal resistance is currently unknown. It could use a combination of mechanisms as well as only use a single mechanism. The treatment and prevention of *C. auris* is limited until the mechanism of antifungal resistance is determined. This unknown reason for antifungal resistance of *C. auris* is likely the reason that it has become an issue in hospitals. The complication is accentuated because *C. auris* is able to acquire drug resistance much faster than other treatable fungal infections such as *C. albicans.* Additionally, the diagnosis of *C. auris* is often misidentified as other species including *C. haemulonii*, *Rhodotorula glutinis*, or *Saccharomyces cerevisiae* (Sarma *et al* 2017). As of right now there is no fail proof treatment for a patient with a *C. auris* infection.

This difference in drug resistance is due to the fact that *C. auris* is haploid and *C. albicans* is diploid. An experiment was done on *Saccharomyces cerevisiae*, another type of yeast, in which both haploid and diploid populations were tested for survival in different stressful situations (Gerstein *et al*, 2010). The haploid yeast were able to adapt and mutate much more easily than the diploid yeast in all seven environments that were tested. There is little understood about the reasoning why this is the case.

The results of this experiment can lead to important future applications to further understanding of *C. auris* and aid in the continued search for treatment. Needed information can be gathered through a continuation of experiments using *C. elegans* to obtain data and further investigate virulence of *C. auris*. If the services allowed the research team would like to see the survival assay carried out on the 10 *C. auris* isolates and *C. albicans* while applying different antifungals. This combination of drug resistance observation and measure of virulence could provide researchers with more information. Another opportunity to investigate the data further could include conducting this experiment or further experimentation involving *C. auris* using more complex model hosts such as mice or rats. Mice and rats provide a more complex organism that has closer biological and genetic similarities to humans. Overall the search for answers about drug resistance and *C. auris* is a continued journey that will require collaborative investigation in order to stop the fungus in its mutating tracks.

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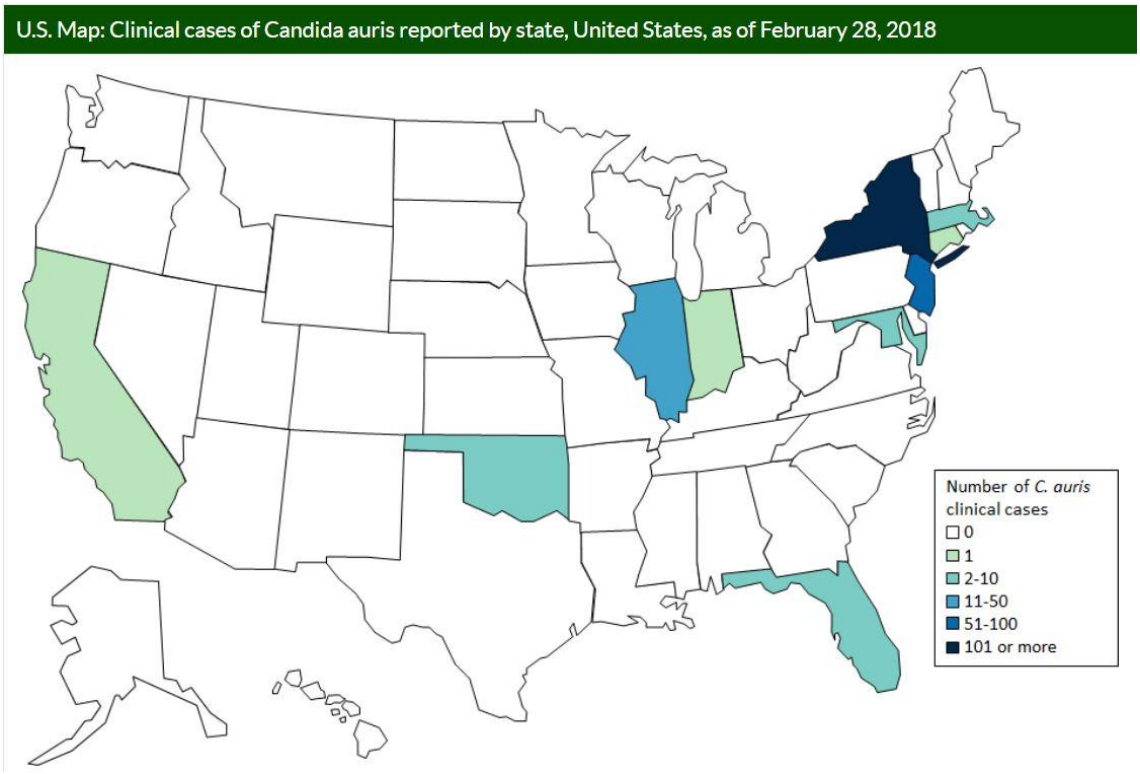
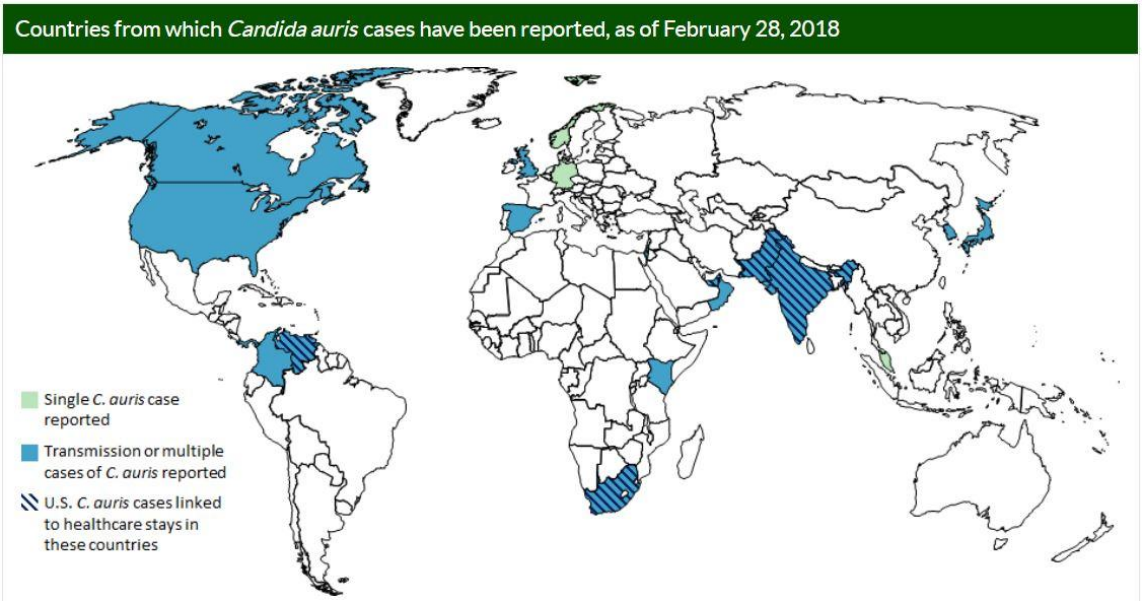
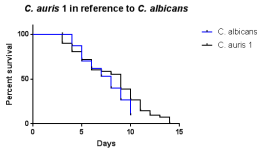
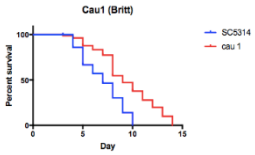
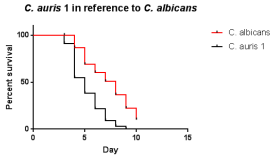
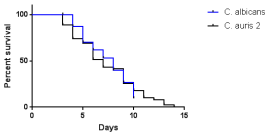
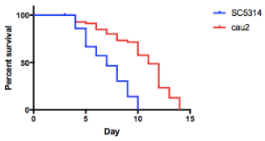
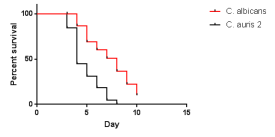
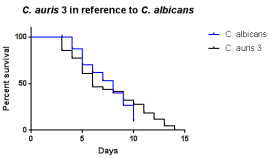
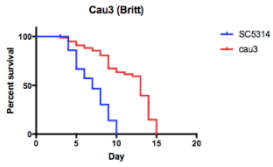
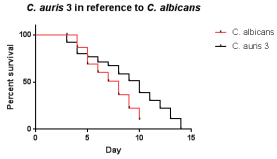
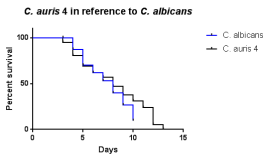
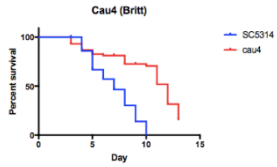
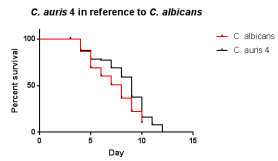
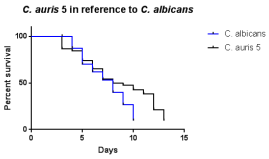
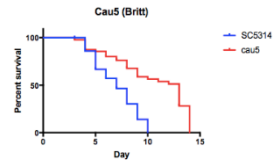
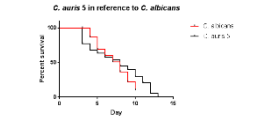
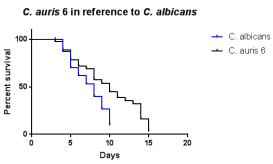
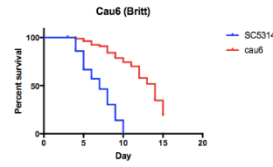
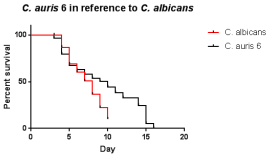
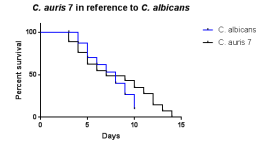
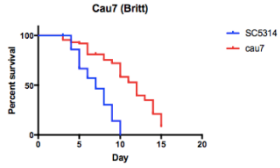
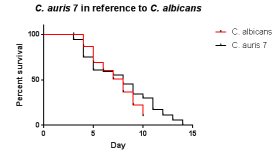
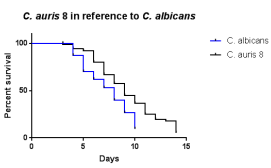
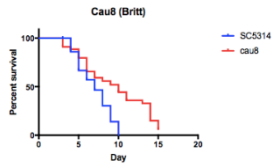
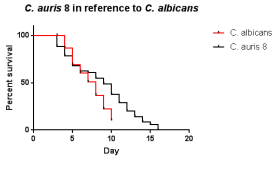
Appendix

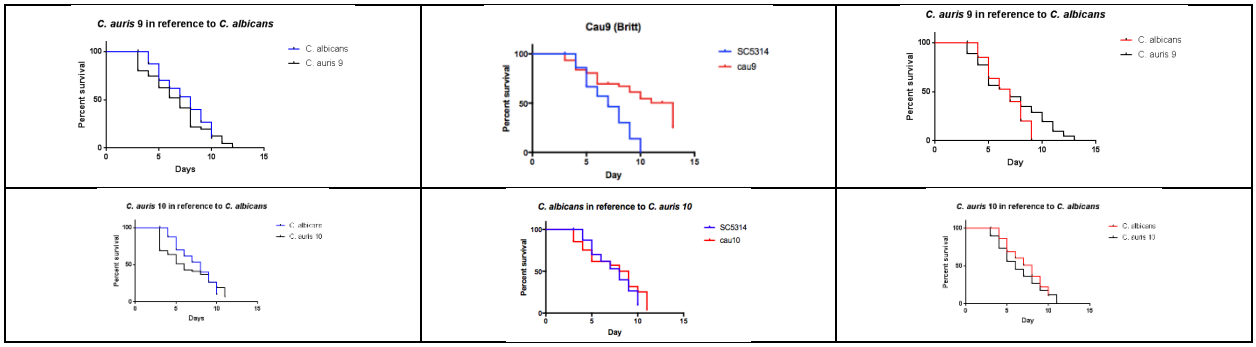
Appendix A. CDC Reported Maps Tracking *C. auris* Outbreaks



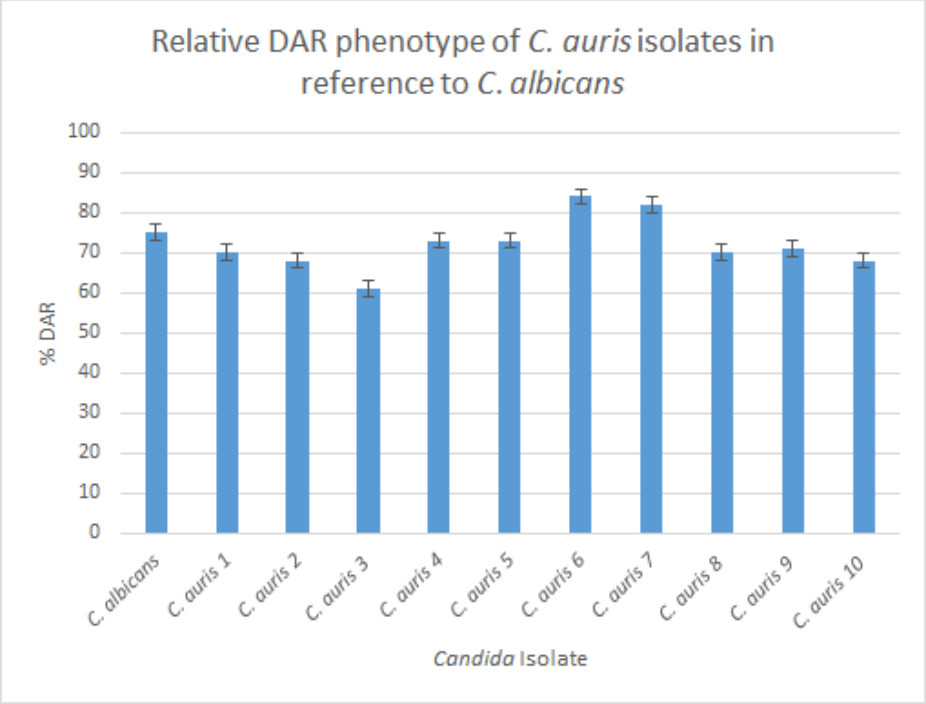


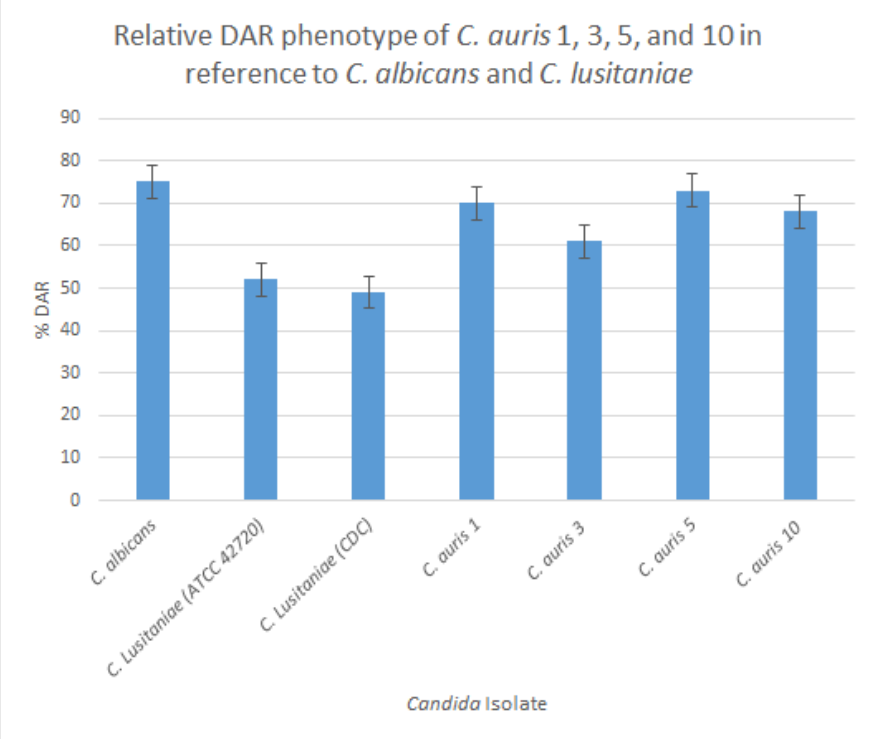
Appendix B: Individual Survival Curves in Reference to *C. albicans*

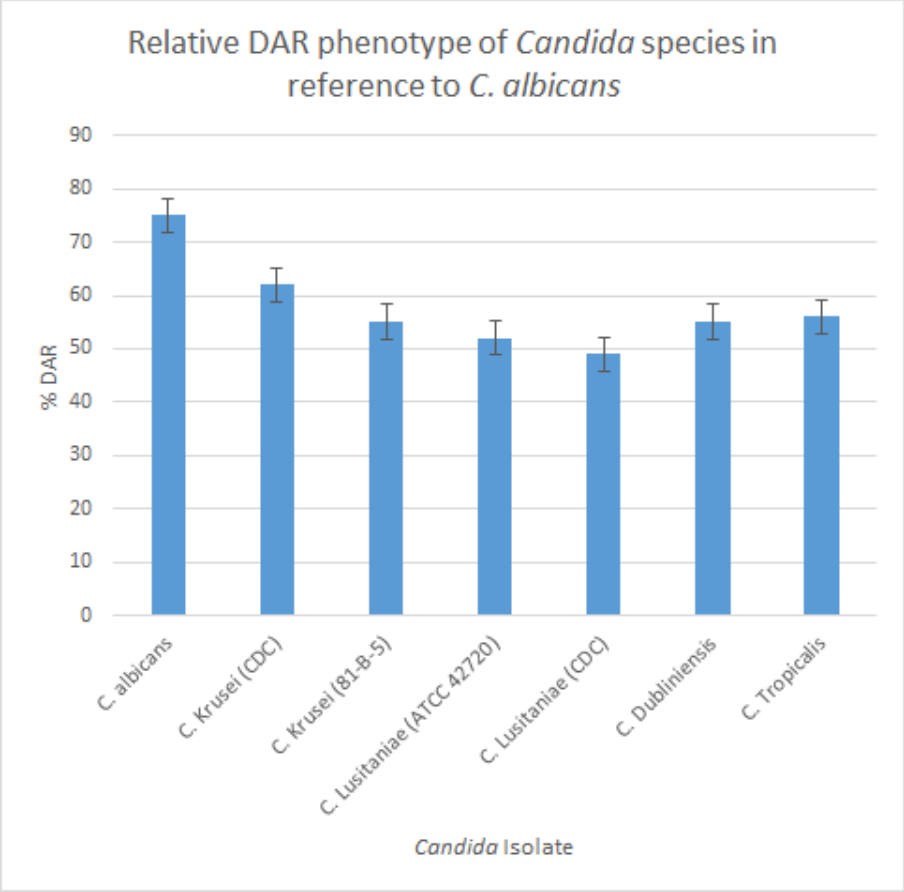
Natalie	Brittney	Nina
		
		
		
		
		
		
		
		


C. auris 9 in reference to C. albicans
Percent survival
Days
C. albicans
C. auris 9
Cau9 (Britt)
Day
SC5314
cau9
C. auris 9 in reference to C. albicans
C. albicans
C. auris 9
C. auris 10 in reference to C. albicans
C. albicans in reference to C. auris 10
SC5314
cau10
C. auris 10 in reference to C. albicans

Appendix C. Dar Assay Graphs






Relative DAR phenotype of *Candida* species in reference to *C. albicans*
% DAR
90
80
70
60
50
40
30
20
10
0
C. albicans
C. Krusei (CDC)
C. Krusei (81-B-5)
C. Lusitaniae (ATCC 42720)
C. Lusitaniae (CDC)
C. Dubliniensis
C. Tropicalis
Candida Isolate