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Assaying Virulence in 14 Clinical Isolates of Candida parapsilosis

Giles William Chickering
Worcester Polytechnic Institute

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Assaying Virulence in 14 Clinical Isolates of Candida parapsilosis

A Major Qualifying Project Submitted to the Faculty of Worcester Polytechnic Institute in Partial Fulfillment of the Requirements for the Degree of Bachelor of Science in Biology and Biotechnology

Giles Chickering

April 25, 2013

Report submitted to:
Professor Reeta Prusty Rao
**Abstract**

Fungi of the *Candida* genus have caused an increasing number of infections over the past 30 years, with *Candida albicans* being the most common species. *Candida parapsilosis*, however, is an emerging threat as the second most prevalent species. This study focused on characterizing virulence in 14 *C. parapsilosis* clinical isolates using a variety of *in vitro* and *in vivo* assays. The results reveal a correlation between relatively low levels of adhesion to acrylic surfaces, smooth colony morphology, and virulence.
Acknowledgments

I would like to thank Professor Reeta Prusty Rao for her direction and insight on this project and for hosting me in her laboratory at WPI’s Life Sciences and Bioengineering Center at Gateway Park. I would also like to sincerely thank Luca Issi for his continued guidance and assistance in all aspects of the project and the development of my approach towards research throughout the year.
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Introduction

While scientists have been working for the better part of a century to develop agents that seek out and destroy bacterial pathogens, most fungal infections are still difficult or impossible to treat clinically. A detailed survey showed that an average American hospital in the 1980s contained at least 15 different isolates of fungal pathogens in treatment centers. These same organisms were responsible for about 40% of all mortalities caused by nosocomial infections in that same time period (Jarvis 1995). Fungi, like their human hosts, are eukaryotic and challenging to treat. The fundamental similarities of their cellular mechanisms make it difficult to develop drugs that selectively target the fungal pathogen, while leaving the host unaffected.

These fungal infections are becoming an increasing public health concern as instances of infection are still relatively high in both countries with advanced medical technology and underdeveloped countries. Hospital patients and immunocompromised individuals are more susceptible to fungal infections of the bloodstream, which can turn fatal if not treated promptly. While medical science recognizes the presence of these diseases, the development of new treatments is rather slow, hence the relatively limited number of antifungal agents. Thus there is an unmet need for more avenues for antifungal therapy.

*Candida* spp are some of the most common causes of mycosis; particularly the species *Candida albicans*. Although this organism exists naturally on human skin and inside the GI tract, it can occasionally cause deep tissue or blood stream infections. As of 2010 an estimated 63,000 cases of *Candida*-based infections occurred in the United States with mortality rates up to 25% (Evans 2010). *C. albicans* is by far the most prevalent of these pathogens, causing nearly 66% of all Candida infections as of 2008 (Trofa, Gacser and Nosanchuk 2008). While *C. albicans* remains the predominant species, *Candida parapsilosis* has also been responsible for significant number of mortalities and has been increasing in prevalence over the past few years. This study characterizes the virulence of 14 clinical isolates of *C. parapsilosis* using a series of *in vivo* and *in vitro* assays.

Recent clinical studies in portions of Europe and Asia indicate that the presence of *C. parapsilosis* in blood samples is outnumbering the cases of *C. albicans* (Trofa, Gacser and Nosanchuk 2008). This pathogen is responsible for causing fungemia, especially in
immunocompromised patients, as a result of injury or preexisting conditions (Laffey and Butler 2005). The yeast can also cause endocarditis, peritonitis, ocular infections, and other ailments that all stem from the yeast successfully entering the blood stream (Trofa, Gacser and Nosanchuk 2008). One of the most significant facts about *C. parapsilosis* is that it is not limited to growth in the human body like *C. albicans* and *C. tropicalis*. With the ability to harbor itself in a variety of other living hosts and environments (see Table 1 for reference), it is important to profile the virulence of various isolates of this organism in case the increasing trend of infection does not cease.

**Phylogenetic Analysis of *C. parapsilosis***

*C. parapsilosis* has been classified and reclassified since the mid-1990s as the number of groups researching the pathogenic yeast has increased. The fungal species was originally classified in 1995 based on isoenzyme profiles and comparisons of specific DNA fragment sequences (Lin, et al. 1995). Group I is significantly larger than the other two groups classified in Lin et al.’s study, and contains all of the isolates used in the research detailed in this report (see Table 1). Nearly one decade later, Tavanti et al. reclassified the *C. parapsilosis* phylogeny into three separate species that closely resembled the groups used by Lin et al. These separate species were defined by analysis of 4 specific genes (*COX3, L1A1, SADH, SYA1*). The new species are illustrated below in Figure 1 (Tavanti, et al. 2005).
Figure 1: Candida phylogeny post-2005 classification (Tavanti, et al. 2005). *C. parapsilosis*, formerly three groups, is now classified as *C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis*.

The three new species (*C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis*) are outlined in the blue box with the former *C. parapsilosis* classifications in parentheses (Lin, et al. 1995). The 14 clinical isolates (Table 1) used in the experiments detailed in this report are officially classified as *Candida parapsilosis* (formerly Group 1) that were obtained from Geraldine Butler’s laboratory.

Table 1: *C. parapsilosis* Isolates used in this study were obtained from Geraldine Butler and were originally sourced by Tavanti, et al. 2005.

<table>
<thead>
<tr>
<th>Strain I.D.</th>
<th>Geographic Origin</th>
<th>Isolate Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>J931058</td>
<td>Belgium</td>
<td>Nail</td>
</tr>
<tr>
<td>103</td>
<td>London, UK</td>
<td>Anus</td>
</tr>
<tr>
<td>73/107</td>
<td>London, UK</td>
<td>Mouth</td>
</tr>
<tr>
<td>81/040</td>
<td>London, UK</td>
<td>Toe</td>
</tr>
<tr>
<td>J93063/1</td>
<td>Africa</td>
<td>Cat Hair</td>
</tr>
<tr>
<td>90-137</td>
<td>San Jose, USA</td>
<td>Orbital Tissue</td>
</tr>
<tr>
<td>74/046</td>
<td>Leeds, UK</td>
<td>Aortic Valve</td>
</tr>
<tr>
<td>J961250</td>
<td>Lisbon, Portugal</td>
<td>Nail</td>
</tr>
<tr>
<td>J931845</td>
<td>Japan</td>
<td>Unknown</td>
</tr>
<tr>
<td>J950218</td>
<td>USA</td>
<td>Unknown</td>
</tr>
<tr>
<td>81/041</td>
<td>Mayo Clinic, USA</td>
<td>Vagina</td>
</tr>
<tr>
<td>73/037</td>
<td>Leeds, UK</td>
<td>Vagina</td>
</tr>
<tr>
<td>CLIB214</td>
<td>Puerto Rico</td>
<td>Feces</td>
</tr>
<tr>
<td>CDC317</td>
<td>Clinical Isolate</td>
<td>Clinical Isolate</td>
</tr>
</tbody>
</table>
The variety of tissues these isolates were obtained from shows that this species is capable of living and causing infection in a variety of tissue types, ranging from cat hair to heart valves. Furthermore, the CDC317 isolate has been known to cause outbreaks of infection, and was received considerable attention as results were obtained (Kuhn, et al. 2004). We used a variety of in vitro and in vivo assays to characterize the virulence of these clinical isolates. We used a Candida albicans strain (SN250) as a control in many experiments, as it is known to be virulent and elicit a positive pathogenic response.

In vivo Virulence Assays

In this study we characterized isolates of C. parapsilosis by infecting the model host Caenorhabditis elegans. Nematodes have been used extensively to study virulence and immunity as they have been well studied, including a fully sequenced genome (Chiasson, et al. 2010). C. elegans evoke a similar immune response to pathogens that is genetically conserved in a wide variety of species including humans. Furthermore, their transparent body type allows visualization of metabolic activity in vivo, as well as a short life cycle, and relative ease of culturing and sustaining strains (Jain, Yun, et al. 2009). In addition, many microbial pathogens (including fungi) contain genetic elements that are required for pathogenicity in both mammals and C. elegans, leading to the conclusion that relative virulence levels among different C. parapsilosis isolates observed in nematode infections should be comparable to those seen in humans and other warm-blooded organisms (Sofro, Begun and Ausubel 2005).

We used a quantitative survival assay for measuring virulence of particular C. parapsilosis isolates by determining the life span of infected worms relative to other strains of the fungi as well as Candida albicans strains with established levels of very high virulence. This simple test produces results that are clear to interpret. It is considerably more difficult, however, to measure how significant an effect a disease-inducing agent has on a host before either organism dies. A specialized assay was required in this experiment to observe the virulence of the various isolates of C. parapsilosis apart from the lethality of the strains.

We used a qualitative marker for infection called the Deformed Anal Region (Dar) assay to measure relative virulence among isolates. Infected C. elegans show a swollen post-anal tail region phenotype when infected by fungal pathogens such as S. cerevisiae and C. parapsilosis.
(Jain, Yun, et al. 2009). This phenotype is visible under a light dissection microscope and does not require any type of probe, dye, or labeling procedure to observe. Another major benefit of this assay is the speed and convenience of the protocol, which takes about 1/3 the time of the survival assay. The Dar assay is more qualitative in nature, as multiple observers may differ on the condition of mildly-affected nematodes; however, the use of the qualitative survival covers discrepancies in virulence that may otherwise be debated.

**In vitro Virulence Assays**

Studying *C. parapsilosis* outside the host provided more depth in terms of visualizing growth and allowed further comparison between the isolates. Similar studies in *C. albicans* have shown that colony morphology can be correlated to virulence (Laffey and Butler 2005). Wrinkled colonies are typically more virulent, while mutants that render a smooth colony morphology are expected to show decreased virulence (Trofa, Gacser and Nosanchuk 2008). This morphology is easily visualized by culturing the organism on yeast production media and incubating for several days (Laffey and Butler 2005).

Studying the abilities of these organisms to adhere to a polystyrene surface also leads to inferences about the virulent capabilities of the fungi. Adhesion is the first step in infection, as the pathogen must attach itself to the tissue and cells undergoing infection. In most microbes, especially in *Candida albicans*, increased adhesion on abiotic substrates indicates a better ability to form biofilms and ultimately cause infection in a host (Trofa, Gacser and Nosanchuk 2008). This correlation also extends to experiments that test the ability of pathogens to adhere to epithelial cells, though such protocols are not included in this study.
Materials and Methods

Two *in vivo* assays for virulence were performed in this virulence study of 14 *Candida parapsilosis* isolates (Table 1). A Dar assay and survival assay were used to measure relative infection and mortality rates, respectively. A study of colony morphology and a polystyrene adhesion assay were also used to assess the virulence characteristics of the 14 isolates.

**Deformed Anal Region (Dar) Assay**

Three adult *C. elegans* worms were plated on nematode growth media (NGM) plates and incubated for 3-5 days at 20°C until enough young eggs were present on the plate to perform and egg preparation. Eggs were prepped by washing, suspending, and pelleting the *C. elegans* worms and eggs in M9 buffer and a mixture of Bleach diluted to 5.25% with DI H2O and .25 M NaOH. Once the washed eggs are suspended in M9 buffer, 20-30 eggs are plated in triplicate on fresh NGM plates spotted with a mixture of OP50 *E. coli* cocultured with an isolate of *C. parapsilosis* plus streptomycin (50mg/mL). These plates were incubated for 5 days at 20°C with the number of Dar phenotype worms counted on day 3, 4, and 5. A plate with an isolate of *C. albicans* (Isolate SN250) was used as a control, as it is known to produce Dar in 100% of infected worms. This procedure is modeled off recent developments of this assay (Jain, Yun, et al. 2009).

**Survival Assay**

An egg preparation was performed in using the same protocol described in the Dar assay. After two days at 20°C, 20 worms were transferred to fresh NGM plates (triplicate sets) spotted with a mixture of OP50 *E. coli* cocultured with an isolate of *C. parapsilosis* plus streptomycin (50mg/mL) that had incubated for 24 hours after spotting at 20°C (Chiasson, et al. 2010). The surviving worms were counted and transferred to fresh spotted and incubated plates every 24 hours until no worms remained. Worms that died as a result of crawling off the edges of the plates were censored in the post experimental analysis (Chiasson, et al. 2010).

**Colony Morphology**

Cultures of each isolate were grown overnight in 1X YPD broth and standardized to an equal concentration using a spectrophotometer. Isolates were spotted on freshly-poured YPD media
plates left to incubated at 30°C overnight before being parafilmed and sitting at 4°C for at least 14 days before morphology images were taken at 7.5X magnification.

**Adhesion to Abiotic Surfaces**

Cultures of *C. parapsilosis* were grown in SC media + 0.15% glucose + His + Leu + Trp + Uracil for 24 hours and plated in 96 well polystyrene plates (12 samples per isolate). The plates were incubated at 37°C for 4 hours before aspirating and dyed with crystal violet for 45 minutes, rinsed with DiH₂O and developed in 75% methanol for 30 minutes before reading at 590nm. The resulting OD readings varied significantly between isolates but there was strong consistency between trials; hence the use of a relative OD chart to view the data (Chiasson, et al. 2010).
Results

Both in vivo and in vitro techniques were used to characterize the relative virulence of 14 isolates of *Candida parapsilosis* (see Table 1 for full listing of isolate names). *Candida albicans* strains were used as experimental controls rather than benchmarks for virulence.

**In vivo Characterization**

The Deformed Anal Region (Dar) assay was used to show the relative severity of particular *Candida parapsilosis* isolates by infecting *Caenorhabditis elegans* with the fungi and observing the nematodes for a 5-day period. The final day of observation frequently showed the highest levels of Dar phenotype, and the average rates of expression are illustrated in Figure 2.

![Figure 2: Dar Phenotypes on Day 5. Isolate CDC 317 shows significantly higher average amounts of the infected phenotype, while most other isolates show no infection. The SN250 C. albicans isolate was used as a positive control.](image)

These average Dar percentages are taken from triplicate sets of plates, and show that CDC317 is producing high amounts of the Dar phenotype relative to the other isolates, which were mostly unable to produce the infected phenotype. The J950218 isolate also stands out with relatively high levels of Dar in comparison with the other isolates and the SN250 *C. albicans* control, which is known to be highly virulent. The isolates showing Dar phenotype were subjected to a survival assay that studied mortality rates of *C. elegans* with *C. parapsilosis* present.
Figure 3: The survival assay did not provide statistically conclusive results according to an analysis of variance and the short lifespan of the SN250 control plates in comparison with past studies. The figure does, however, raise questions to be answered in further studies.

While Figure 3 appears to show that isolate CDC 317 causes the fewest mortalities, an analysis of variance (α-level = 0.05) performed with the data indicated that the results are statistically inconclusive. The results of the experiment also show that the SN250 control strain of Candida albicans was less able to cause mortality than isolate J950218, even though the former is known to be highly virulent. Fitting liner trend lines indicated correlation coefficients (R^2 values) of less than 0.73 for each isolate; however, fitting logarithmic trendlines in Excel produced R^2 values between 0.87 (J950218) and 0.92 (CDC 317).

**In vitro Characterization**

In addition to studying the effects of Candida parapsilosis in a live host- Caenorhabditis elegans, several phenotypic assays were used to investigate the 14 isolates’ behavior in vitro. The various Candida parapsilosis clinical isolates were incubated (Figure 4) on spider media to simulate a carbon starvation conditions.
Figure 4: Colony morphology images of all 14 isolates of *Candida parapsilosis*. The isolates are separated into groups of totally smooth morphology (left column), uneven and non-wrinkling in the center 2 columns, and wrinkled morphology (right column).

The isolates of *C. parapsilosis* showed a wide range of morphology structure, ranging from totally smooth patches with minimal or no irregular indentation on the outer edge of the colonies (Figure 4, left column) to very wrinkled colonies (Figure 4, right column). While this species does not truly filament, the strong wrinkling morphology allows for simple qualitative grouping of the isolates (Laffey and Butler 2005). The isolates shown in the two center columns of Figure 4 show a less-smooth morphology with irregular edges but no wrinkling structure. The morphology of each isolate alone indicates little about virulence, but comparison with adhesion later revealed significant correlations with virulence.
Figure 5: Relative adhesion of all 14 isolates of *Candida parapsilosis* on polystyrene plates. The results are shown in percentages relative to the highest average adhesion value, which was found in isolate 103.

Pathogens must prove able to adhere to both biotic and abiotic surfaces in order to cause infection. A polystyrene adhesion assay (Figure 5) was used to conveniently determine the relative ability of the 14 isolates to adhere to a solid surface. The values in Figure 5 show that there was a wide range of adhesion abilities observed among the isolates. Few conclusions could arise from these data alone; however, correlations between adhesion, colony morphology, and infection rates became apparent after review.

**Discussion**

The overall goal of this project was to characterize relative virulence in 14 clinical isolates of the pathogenic fungi *Candida parapsilosis*. The assays used to assess the pathogens included both *in vivo* types (the Dar and survival assays) and *in vitro* (acrylic adhesion and colony morphology) studies. By comparing the data from these assays we were able to create a graphic representation (Figure 6) that allows easier visualization of the combined results and shows a correlation between low levels of adhesion, smooth colony morphology, and increased amounts of infection.
Figure 6: Comparison of Relative Adhesion with Colony Morphology and Dar Phenotype shows multiple correlations
Figure 6 illustrates that there is a positive correlation between smooth colony morphology and decreased adhesion to polystyrene surfaces. Furthermore these in vitro infection phenotypes show a negative correlation with the Dar phenotype, a marker for infection in a live host. The green bars indicate smooth colony morphology, showing uniform fields that are glossy, even, and have edges that do not wrinkle. Notice that the green bars show a strong presence towards the lower end of relative adhesion values. The wrinkled isolates, shown in red in Figure 6, also showed very high levels of adhesion (the lowest at 77%) and showed no Dar phenotype. The blue bars represent colonies with intermediate morphology phenotypes (see Figure 4). Note that this figure does not include the results obtained through the survival assay, which was statistically inconclusive and requires a more in-depth repeating to produce feasible results.

Isolate CDC 317 consistently showed a completely smooth morphology with no visible wrinkling. This isolate also produced an average Dar phenotype of 83% in C. elegans, indicating infection over than 275% more often than the next most effective isolate (J950218). What is most striking about this finding is not the abnormally high infection rate that is indicate by the Dar assay; CDC 317 was once responsible for one of the largest Candida parapsilosis outbreaks in the United States over a decade ago (Kuhn, et al. 2004). The striking discovery is the correlation of lower levels of adhesion with higher levels of infection in C. elegans as shown in Figure 6. This begs the question of why an organism that is less able to adhere to surfaces is more able to cause infected phenotypes in our model?

The answer to this question is not completely clear. One possible explanation for the low adhesion/high virulence correlation may rest in the inherent morphology of C. parapsilosis, which is incapable of forming “true filaments” (Laffey and Butler 2005). The less adhesive isolates of C. parapsilosis may simply be less able to hold onto the agarose plate surface, allowing the nematodes to ingest more of the low adhesion isolates than the wrinkled phenotypes, causing a stronger infection response in the hosts. The translation of this virulence into a mammalian cells would be less direct if this were the case, but past studies have shown inconclusive results regarding the abilities of different strains of C. parapsilosis to adhere to epithelial cells (Trofa, Gacser and Nosanchuk 2008). A beneficial future study would be to group cellular adhesion data by morphology as is shown in Figure 6 and to check for the same correlation between decreased adhesion values and increased infection rates.
As mentioned previously, the survival data did not show any statistically significant difference between the isolates in terms of mortality rates. Repeating this experiments in greater depth, repeating the trials with more individuals per trial with all 14 isolates would ideally provide results that correlate higher infection rates with lower rates of survival. If this were the case, it would provide more evidence to confirm the low adhesion/high infection rate observation, which would be a trait that *Candida parapsilosis* shares with few other pathogenic fungi. Conversely, if survival assays showed strong evidence that increased mortality rates correlated directly with higher relative adhesion levels, we could infer that infection rates in the Dar assay were higher for isolates with lower adhesion levels because the fungi was less simply able to hold onto plates and entered the hosts in higher volumes.

The implications of a survival assay with statistically significant differences in mortality rates is a critical next step in the study of *Candida parapsilosis* virulence. Additionally, resourcing the isolates used in future experiments may be beneficial, as the generally low infection rates in the Dar assay may suggest a decrease in virulence as a result of long-term cryogenic storage.

The ultimate result of this experimentation is the formation of a new hypothesis based on the observation that relatively low adhesion values appear to correlate with increased infection rates. Future studies that assess the survival rates of all 14 isolates can help determine if this increased infection leads to decreased levels of survival and can aid in confirming this new hypothesis and will be a critical step in continuing this research. A wide array of other assays will also help further assess the virulence of these 14 isolates, including superoxide resistance tests and macrophage phagocytosis assays, both of which will aid in assessing the resilience of *Candida parapsilosis*. 
Works Cited


