Investigating the Invertebrate Response to *Cryptococcus neoformans,* a Fungal Pathogen

By

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**Abstract**

*Cyrptococcus neoformans* (*Cn*)is a pathogen that kills more than 250,000 people each year. Several organisms are used as models to investigate pathogenesis of *Cryptococcus* such as larvae of the wax moth, *Galleria mellonella.* These larvae have phagocytic haemocytes which engulf and kill microbes similarly to mammalian neutrophils. To investigate aspects of the pathogen, a strain of *Cn* (H99W) was serially passaged in *Galleria* larvae 15 times to create an evolved strain, P15. Based on results of genomic analysis and mammalian infections of P15 and H99W, a peroxide induction study with *Galleria* was conducted to compare the immune response of the parent and evolved strain. To further analyze the characteristics of the evolved strain, a growth assay was conducted to determine changes in growth between P15 and H99W. The results showed a subdued immune response and faster growth in hemolymph by P15 compared to H99W.

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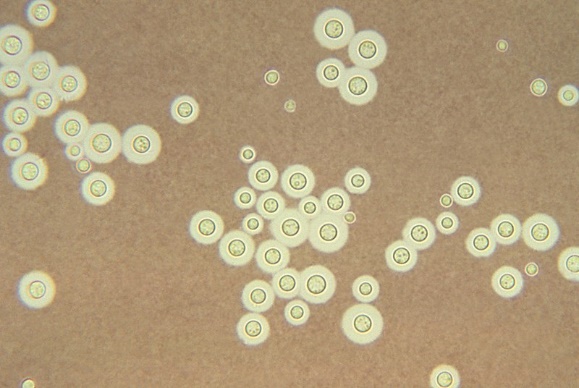
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**Introduction**

*Cryptococcus neoformans* (*Cn*)is a fungal pathogen that kills more than 250,000 people each year (8). Infections commonly occur through inhalation of spores. Paired with a compromised immune system, these infections result in life-threatening illnesses such as meningitis and encephalitis.

Once entering the human body, *Cn* can survive being engulfed by phagocytes and has been shown to be virulent formation of a polysaccharide capsule, production of melanin, and secretion of several degradative enzymes such as urease (10). The polysaccharide capsule protects *Cn* being engulfed as the slippery capsule does not allow phagocytes from being able to attach properly to the yeast (Figure 1).



**Figure 1: Image of *Cryptococcus neoformans.*** *Cn* with capsule depicted with light India ink at 100X (5)

Melanin production has been shown to make the yeast more resistant to antibody mediated phagocytosis (13). The production of degradative enzymes such as urease are necessary for the dissemination of the yeast into the central nervous system (7). Although the polysaccharide capsule and melanin serve as defense from phagocytosis, *Cn* can also survive phagocytosis. Residence within macrophages brings protection from other innate immune components such as antibodies, alveolar immune factors (3). The mechanism through which *Cn* survive within phagocytic vesicles of macrophages is not yet understood.

Several organisms are used as models to investigate pathogenesis of *Cn*, including mice, cultured mammalian cells, and larvae of the wax moth, *Galleria mellonella* (Figure 2). The larvae of the wax moth is used as a model of mammalian pathogenesis for a range of organisms such as *Klebsiella pneumonia, Pseudomonas aureginosa, Listeria monocytogenes* and *Cryptococcus neoformans* (9).



**Figure 2: Image of Galleria mellonella.** Galleria mellonella larva (2)

These larvae have phagocytic hemocytes which engulf and kill microbes similarly to mammalian neutrophils. Neutrophils kill microbes using reactive oxygen species (ROS). ROS are chemicals produced by the cells including peroxides and superoxides that kill engulfed microbes by destroying their membrane and specifically use hydrogen peroxide (H2O2) to kill engulfed microbes (11). *Galleria* larvae have been shown to produce H2O2 when infected with *Candida* (1).

In an effort to investigate aspects of intracellular replication, a strain of *Cn* (H99W) was serially passaged in *Galleria* larvae fifteen times, which equates to 100 generations and new strain, P15, was created (6). A sibling strain to P15, YPD15 is a strain that originated from H99W and was then passaged on Yeast Extract Peptone Dextrose (YPD) plates and adapted for the rich media environment. Strains P15 and YPD15 originate from H99W, which is a less virulent and less lethal strain compared to H99S. Previous research has shown neither H99W nor P15, were resistant to peroxide exposure *in vitro*. Both strains were equally susceptible to killing by macrophages *in vitro*, but P15 killed mice more than the original strain (6). Furthermore, genome sequencing analyses revealed genetic differences in between H99W and P15 and gene expression analyses revealed differences in growth-related gene. Genome sequencing identified a promoter for NADH dehydrogenase in P15 which could affect the ATP production and lead to a growth defect due to lack of energy resources. The difference in immune response by macrophages that utilize H2O2, and genome sequencing studies suggest the P15 strain is able to subdue normal H2O2 production in hemocytes. This is done perhaps by growing slowly, producing a large capsule, and adaptation of P15 in hemolymph will be increased above H99W.

**Thesis Statement**

The objective of this study was to: 1) compare hydrogen peroxide production of *G. mellonella* hemocytesinfected with different strains of *Cn:* H99W (wildtype), P15 (the evolved strain), and *Candida albicans* (which is known to induce H2O2-based killing in hemocytes), and 2) compare growth rates H99S, YPD15, H99W, and P15 strains in hemolymph, rich medium, minimal media, and a 1:1 ratio of minimal media and hemolymph (1). We hypothesized that the P15 strain should induce less ROS, more quickly in a hemolymph containing, and be more virulent than the parent strain H99W.

**Material and Methods**

***Galleria mellonella***

To conduct an immune response study of *Cn*,a role model of mammalian pathogenesis *Galleria mellonella* was used. These larvae were obtained from Armstrong Cricket Farm and were infectedless than one week after being shipped to the lab. Only healthy larvae were used for infection and were defined as ones that have a plump figure, which are moving around frequently, and have not pupated (Figure 3).

**Peroxide Induction and Infection**

H99W, P15 and *C. albicans* cultures were all started from frozen stock and grown in Yeast Extract Peptone Dextrose (YPD) at 37 °C for 24 hours prior to infection. These cultures were then washed with Insect Physiological Saline (IPS) to remove the media from cells and counted on a hemocytometer. Once the cells were counted and dilutions were made for each strain so that cell concentrations were controlled for, thirty larvae were infected with different strains of *Cn* and/or *C. albicans* with 10 larvae for each strain. Approximately 400,000 cells per mL of P15 and H99W of inoculate was made while 2,000,000 cells per mL of *Candida.* From these inoculate 5 uL of each was used to infect into the last pro-leg of 10 larvae of each strain. Thus, 2,000 cells of P15 and H99W and 10,000 cells of *Candida* were infected by injection into the pro-leg of the *Galleria*. The pro-leg is the last pair of legs on the larvae which gives a standard place for all infections (Figure 3). Standard safety protocols (i.e. sanitized area and gloves) were taken with all infections to minimize risk to the researcher.



**Figure 3: Image of *Galleria mellonella* Pro-Leg.** The pro-leg of *Galleria mellonella* being injected (4)

After 24 hours of incubation, hemocytes were isolated from hemolymph by clipping a terminal leg and the hemolymph was removed by squeezing it. Approximately 3 milligrams of 1-Phenly-2-thiourea crystals (PTU) were added to 1 mL of IPS to create a 1:200 dilution of PTU solution. From the PTU solution 100 μl was added to the extracted hemolymph of each strain to prevent melanization. Hemocytes were collected as pellets by centrifugation for 5 minutes at 0.7 rotations per minute, counted with a 1:2 dilution of Trypan Blue to differentiate between dead and living hemocytes and re-suspended in IPS. Cells were used immediately in the ThermoFisher Scientfic Hydrogen Peroxide Assay Kit to determine the relative levels of H2O2 in hemocytes (12). The assay was conducted by creating a 50uM Amplex Red, 1U/mL of Horse Radish Peroxidase (HRP) solution. This was done by creating of a solution consisting of 8.4 µL of Amplex Red solution with 8.4 µL HRP stock and 1.383 mL of IPS. Assays were performed in triplicate and each well used in a 96 well black plate. Each assay used 1000 cells per well in a total volume of 120 μl for each well. Thus, 20 μl of cells were added into each well and 100 μl of the 50 µM Amplex Red, 1U/mL HRP solution. An U5e plate reader was used to monitor florescence every 2 minutes for 12 minutes with excitation 560 nm and detection at 590 nm. All assays were normalized to cell counts, and all replicates were averaged.

**Growth Assay**

Cultures of H99S, H99W, and P15 were started from frozen stock and grown in YPD for 24 hours. These were then washed with Phosphate-buffered saline (PBS) three times to remove media, and counted using a hemocytometer. Dilutions were made to have 5000 cells per mL for each strain. These cells were then added to hemolymph extracted from healthy *Galleria*, YPD, minimal media, solution of 1:1 ratio of minimal media, and hemolymph to total a volume of 360 µL. To prevent melanization, all media containing hemolymph also contained 3 milligrams of PTU crystals. Next, since PTU crystals may affect growth, the rich media and minimal media were grown with and without crystals. For example, in one experiment there would be YPD media with all strains, and an YPD plus crystals with all strains to compare the changes. Thus, each strain was grown in YPD, YPD + PTU, Minimal Media, Minimal Media + PTU, Hemolymph + PTU and 1:1 mixture of minimal media and hemolymph + PTU. These cultures were then placed in multi well plates and growth was measured for 24 hours using the BioScreen C growth curve machine that read absorbance at 600 nm in each culture to determine growth and doubling times (the time it takes for the organisms to double in population). The growth curves were repeated three times with different concentrations of H99W, H99S and P15. Due to issues with settling of fungal cells over 24 hours, cells were re-suspended using sterile pipetting technique after the first 8 hours and then every 10 hours for the duration of the 24 hour study. The formula was used to calculate the doubling time is:

Time ( )

Doubling times are measured by identifying a growth point of OD past 0.1 and using this point as the Initial OD. Then, an OD reading 6 hours after the Initial OD was used as the final OD in the doubling time formula.

**Results**

**Peroxide Induction**

To understand the differences in the immune response in mammalian infections between P15 and H99W, a peroxide induction study was conducted. P15, H99W,and *C. albicans* were used to infect larvae through injection, hemocytes isolated and a peroxide assay was conducted to evaluate the production of H2O2, which is a marker for the immune response. Increased production of hydrogen peroxide correlates with a higher immune response by the *Galleria*. Results of this study in Figure 4 show the mock infected *Galleria* hemocytes had no production of H2O2 while *C. albicans* showed a positive response to the infection by producing a detectable amount of H2O2. This was expected for both controls and shows the experimental system behaved as expected. Furthermore, there are high levels of H2O2 produced from hemocytes from H99W infected larvae compared to mock-infected larvae.The experimental assay consistently showed lower production of hydrogen peroxide from hemocytes infected with P15 than with H99W (Figure 4). This data is consistent in all replicates so one representative experiment is shown in Figure 4.

**Figure 4: Peroxide Induction Assay of Cn Infected Hemocytes.**  The peroxide induction assay conducted by infecting Galleria larvae with H99W, P15, Candida, and Insect Physiological Saline. Absorbance was read at 600 nm immediately after the addition of cells into a solution of Amplex Red and HRP. All strains were averaged and normalized. The experiment was stopped at 11:31 as H99W saturated the spectrophotometer.

**Growth Curve Assay** Knowing hemocytes from larvae infected with the evolved strain P15 induced less peroxide than the parent strain, it was of interest to determine whether the evolved strain grows better than the parent strain in the evolved environment, which is *Galleria* hemolymph. Growth curve assays are typically done in large volume cultures over several days, but growth in small volumes would allow more media types to be tested feasibly. To test this, a new instrument, the BioScreen C growth curve machine, was utilized. To be able to use the instrument for growth curves, the settings needed to be optimized for growth of *Cn* cultures. Several trials were conducted in which amplitude and speed of shaking were changed. The settings chosen for this study were: continuous shaking and medium setting. Temperature was kept at a constant 37 °C with absorbance readings taken at 600 nm every hour for 24-36 hours.

For growth in rich medium, H99S, and P15 strain showed faster doubling times in two out of 3 experiments (1, 3) to H99W and YPD15 strain (Figure 5). For growth in rich medium with PTU, which inhibits melanization the P15 strain grew more slowly than in rich medium in two trials (2, 3), but H99W strain grew more quickly in 2 of 3 trials (1, 3). For growth in hemolymph with PTU, the evolved strain P15 consistently grew best (short doubling time compared to the other strains in 2 of 3 trial (1, 2) while h99S grew least well in 2 of 3 trails (2, 3) (Figure 7). For growth in minimal medium with hemolymph and PTU, H99W, and H99S strains grew better than YP15 and P15 strains, but all grew reasonably well (Figure 8). For growth in minimal media, growth was inconsistent for YPD15 and P15 strains. H9S and H99W strains grew well with approximately equal doubling time. However, all strains showed doubling times less than 4 hours for all replicates.

**Figure 5: YPD Trials Doubling Time.** Doubling times of strains in Yeast Extract Peptone Dextrose. Doubling times are measured by identifying a growth point and using this point as the initial OD. Then going to a standard amount of time of 6 hours past initial OD and using this final point as the final OD in the doubling time formula.

**Figure 6: YPD with Crystals Trials Doubling Time.** Doubling times of H99S, YPD15, P15 and H99W strains in Yeast Extract Peptone Dextrose with 1-Phenly-2-thiourea crystals. Doubling times are measured by identifying a growth point and using this point as the initial OD. Then going to a standard amount of time of 6 hours past initial OD and using this final point as the final OD in the doubling time formula.

**Figure 7: Hemolymph Trials Doubling Time.** Doubling times of H99S, YPD15, P15 and H99W strains in hemolymph media with 1-Phenly-2-thiourea crystals. Doubling times are measured by identifying a growth point and using this point as the initial OD. Then going to a standard amount of time of 6 hours past initial OD and using this final point as the final OD in the doubling time formula.

**Figure 8: 1:1 Minimal Media and Hemolymph Trials Doubling Time.** Doubling times of H99S, YPD15, P15 and H99W strains in 1:1 Minimal Media and Hemolymph with 1-Phenly-2-thiourea crystals. Doubling times are measured by identifying a growth point and using this point as the initial OD. Then going to a standard amount of time of 6 hours past initial OD and using this final point as the final OD in the doubling time formula. H99S, H99W grew quicker in all three trials compared to P15 and YPD15 in these trials.

**Figure 9: Minimal Media Trials Doubling Time.** Doubling times of H99S, YPD15, P15 and H99W strains in minimal media. Doubling times are measured by identifying a growth point and using this point as the initial OD. Then going to a standard amount of time of 6 hours past initial OD and using this final point as the final OD in the doubling time formula.

Since doubling times for these experiment were inconsistent, it was of interest to examine further the growth curves used to generate the doubling time. One trial is shown for each experimental medium (Figure 10-14). These curves show growth patterns that reflect expected stages of culture growth: lag, log, stationary phases (Figures 10-14). First, turbidity for H99S and P15 reached an absorbance of 1, which indicates saturation, before H99W and YPD15 in YPD (Figure 10). This is consistent with doubling times (Figure 5). In two out of the three growth assays in rich medium with PTU, H99W grew quicker and P15 slower (Figure 11), which is consistent with doubling times (Figure 6). Growth in hemolymph with PTU (Figure 12), P15 grew significantly more quickly than all other strains in two out of three trials and is consistent with doubling times (Figure 7). In Figure 13, P15 grew more slowly than other strains in two out the three trials in a 1:1 mixture of minimal media and hemolymph and is consistent with doubling times (Figure 8). The growth assays conducted with minimal media show no consistent pattern (Figure 14).

**Figure 10: YPD Growth Assay** *Cn* strains H99S, YPD15, P15 and H99Wgrown in Yeast Extract Peptone Dextrose. Data was collected over a 24-48 hour period on BioScreen C machine at 600 nm absorbance. H99S and P15 grew the quickest in two out of the three trials based on the doubling times and this growth assay.

**Figure 11: YPD with Crystals Growth Assay.** *Cn* strains H99S, YPD15, P15 and H99Wgrown in Yeast Extract Peptone Dextrose with 1-Phenly-2-thiourea crystals. Data was collected over a 24-48 hour period on BioScreen C growth curve machine at 600 nm absorbance.

**Figure 12: Hemolymph Trials Growth Assay.** *Cn* strains H99S, YPD15, P15 and H99Wgrown in hemolymph with 1-Phenly-2-thiourea crystals. Data was collected over a 24-48 hour period on BioScreen C machine at 600 nm absorbance.

**Figure 13: 1:1 Minimal Media and Hemolymph Growth Assay.** *Cn* strains H99S, YPD15, P15 and H99Wgrown in 1:1 mixture of minimal media and hemolymph with 1-Phenly-2-thiourea crystals. Data was collected over a 24-48 hour period on BioScreen C machine at 600 nm absorbance.

**Figure 14: Minimal Media Growth Assay.** *Cn* strains H99S, YPD15, P15 and H99Wgrown in minimal media. Data was collected over a 24-48 hour period on BioScreen C machine at 600 nm absorbance. Minimal media trials show no consistent pattern in the growth assays or doubling time.

**Discussion**

The peroxide induction study was conducted to determine the differences in the immune response between an evolved and parent strain of *Cn*. The results of this study show the evolved strain, P15, induced lower production of H2O2 from hemocytes than H99W (the parent strain) which suggest a lower immune response by *Galleria* hemocytes when infected with P15 rather than with H99W. The positive and negative controls showed expected results indicating the assay was functional. These results suggests of an unknown mechanism which P15 uses to subdue the ROS response of the hemocytes. There are two possible mechanisms through which P15 might reduce the immune response of *Galleria.* One mechanism is to avoid detection by hemocytes and another mechanism is to inhibit production of H2O2 from hemocytes. Based on the results of this study, P15 must somehow inhibits production of H2O2 because no detection of P15 by the hemocytes would result in no production of H2O2.

Based on the findings of the peroxide induction study and genomic analysis, a growth assay study was conducted for four different strains of *Cn* to understand the differences in growth between P15 and H99W*.* In Figure 10 and 11, H99S and P15 strains have similar doubling times, while YPD15 and H99W have longer doubling times with H99W having the longest in two out of three trials. This result is expected because H99S and P15 strains are more virulent and likely have faster doubling times in nutrient rich media. In addition, YPD15 and H99W are less virulent thus likely have longer doubling times. Furthermore, PTU crystals, which were used in hemolymph to prevent melanization, were inserted into YPD media to determine whether the PTU would affect the growth of *Cn.* H99W was observed to grow more quickly in YPD with PTU and grew the slowest in YPD only. P15 grew quickly in YPD and was the slowest to grow in YPD with PTU. This is indicates the crystals are affecting cells in a strain dependent manner. The mechanism for how PTU affect growth of cells is not understood.

In hemolymph, P15 grew quicker than H99S as it had a shorter doubling time than H99S in two out of the three trials. This suggests P15 has adapted to the hemolymph environment. In 1:1 ratio of minimal media and hemolymph, H99S and H99W grew the fastest while YPD15 and P15 had longer doubling times. This result for P15 is not unexpected, because previous data shows P15 grew slower in minimal media (6). This suggests that something in minimal media may be affecting the availability of a nutrient in hemolymph so that P15 does not grow quickly. Lastly, the experiments with minimal media are inconsistent thus should be repeated.

Through the peroxide induction and growth assay experiments there is now further evidence of the virulent nature of *Cryptococcus neoformans.* Creating an evolved strain of *Cn* in a controlled environment which is more lethal, creates a reduced immune response and can grow more quickly than the original strain in a specified environment such as hemolymph shows the remarkable adaptability of the yeast to its environment.

To further investigate the evolved strain, a growth assay should be conducted with the traditional method to measure the growth curve of the strains in minimal media. This method requires measuring the absorbance of cells every 3 hours for 48 hours using a spectrophotometer which makes it less efficient. This will also be conducted to evaluate the accuracy of the data received from the BioScreen C growth curve machine.

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