

# Characterization of the virulence of *Cryptococcus neoformans* strains in an insect model

Tejas Bouklas<sup>1,†</sup>, Elizabeth Diago-Navarro<sup>1,†</sup>, Xiaobo Wang<sup>2</sup>, Marc Fenster<sup>2</sup>, and Bettina C Fries<sup>1,2,3,4,\*</sup>

<sup>1</sup>Department of Medicine (Division of Infectious Diseases); Stony Brook University; Stony Brook, NY USA; <sup>2</sup>Department of Medicine (Division of Infectious Diseases); Albert Einstein College of Medicine of Yeshiva University; Bronx, NY USA; <sup>3</sup>Department of Microbiology and Molecular Genetics; Stony Brook University; Stony Brook, NY USA; <sup>4</sup>Department of Microbiology and Immunology; Albert Einstein College of Medicine of Yeshiva University; Bronx, NY USA; <sup>†</sup>These authors equally contributed to this work.

**Keywords:** *Cryptococcus neoformans*, fungal pathogenesis, *Galleria mellonella*, host pathogen, mice, virulence, waxworm

*Cryptococcus neoformans* is a fungal pathogen that causes disease in immunocompromised individuals, especially in HIV+/AIDS patients, and organ transplant recipients. Patients who present with cryptococcosis primarily die from chronic meningoencephalitis (CME), and disease may follow acute infection or reactivation of latent infection.<sup>1</sup> Despite the introduction of Combination Antiretroviral Therapy (cART) and anti-fungal therapy, over 600,000 deaths per year have been reported worldwide.<sup>1</sup> This is in large part due to the ability of the fungus to successfully infect a diverse range of hosts with different body temperatures.<sup>2</sup> In addition, current treatment regimens are expensive and difficult to provide in resource limited settings because they are very toxic and require careful monitoring. Consequently, efforts are constantly undertaken to identify new anti-fungal drug regimens with fewer side effects. To date, laboratories have relied on cell, tissue, and animal based models to investigate new drug targets. However, it remains unclear to what extent, the current infection models fully represent this complex host-pathogen interaction.

Several models currently exist to study *C. neoformans* infection. *In vitro* models include the use of cell lines; primarily, monocytes,<sup>3</sup> and macrophages,<sup>4</sup> and also dendritic cells, neutrophils, eosinophils, and lymphocytes.<sup>5</sup> In addition, *in vivo* models have been established and include vertebrate models of mouse, rat, and rabbit.<sup>6,7</sup> Because cryptococcal virulence has been suggested to evolve in more primitive organisms as *C. neoformans* was first an environmental fungus that became an accidental pathogen,<sup>8</sup> other host models

have been used to investigate its interaction. These models include invertebrates, such as *Caenorhabditis elegans*,<sup>9</sup> *Acanthamoeba castellanii*,<sup>10</sup> *Dictyostelium discoideum*,<sup>11</sup> and most recently the lepidoptera, *Galleria mellonella*.<sup>12</sup> *Galleria* is the greater wax moth and is used during its larval stage to screen for virulence with a diverse array of pathogens. This model affords several advantages, most important of which is that it can withstand mammalian body temperature, but also that its larvae are easy to inoculate, inexpensive, and have host hemocytes that are able to phagocytose *C. neoformans*. Therefore, this model has been used to test cryptococcal virulence, and antifungal efficacy.<sup>12</sup> Since 2005, when this model was introduced for *C. neoformans*, there have been 25 papers published using the *Galleria* model with *C. neoformans*. Because we observed some inconsistencies in select experiments with this model when compared with murine infection models, we undertook a systematic large scale study to compare the virulence of 20 different clinical *C. neoformans* strains with cellular, murine, and *Galleria* infection models. Our results demonstrate that while the model is beneficial to study some virulence parameters with standard passaged laboratory strains, virulence does not consistently correlate with murine infection models. We propose that *Galleria* infection does not conclusively model the host-pathogen interaction of *C. neoformans*, and most importantly cannot substitute for mammalian models of *C. neoformans* infections.

First, clinical *C. neoformans* strains (n = 20) were characterized with respect to

known virulence traits. Strains were randomly selected from our collection, and included both serotype A (n = 15) and serotype D (n = 5) strains from different parts in the world (United States of America and India). Also included was the standard strain, H99, which was first isolated from a patient at Duke University in 1978,<sup>13</sup> and is the predominant strain used in published *C. neoformans* literature. All strains were found to exhibit variable doubling times, cell, and capsule sizes in rich glucose media, and laccase activity in L-DOPA media (Table 1). Doubling times ranged from 2.7 h (strain H99) to 5.2 h (strain M8A), and laccase activity ranged from 0.886 U (strain I65) to 1.038 U (strain JEC21) normalized to 1.000 U of strain H99. Next, phagocytosis indices, killing rates, and intracellular replication rates (IPR) by co-cultivation in the murine macrophage cell line J774.16 were determined for all strains. Phagocytosis ranged from 8 - 55%, and accordingly intracellular killing and IPR were also found to be variable (Table 1). In summary, these randomly selected clinical *C. neoformans* strains exhibited marked variability with respect to virulence traits that were consistent with the previously reported variability of replicative life span (RLS) in these strains, which ranged from 8 generations (strain M8A) to 99 generations (strain M12A).<sup>14</sup>

Next, virulence of these strains was compared in the insect model, *Galleria mellonella*, as well as a murine intravenous (i.v.) infection model. *Galleria* (n = 20) were infected with 3 different doses [2 × 10<sup>4</sup>, 10<sup>5</sup>, or 10<sup>6</sup> colony forming units (CFU)] of individual strains in 3

\*Correspondence to: Bettina C Fries; Email: bettina.fries@stonybrookmedicine.edu

Submitted: 07/29/2015; Revised: 08/17/2015; Accepted: 08/19/2015

<http://dx.doi.org/10.1080/21505594.2015.1086868>

**Table 1.** *In vitro* characterization of clinical *C. neoformans* strains

Strain #	Strain serotype	Strain name	Doubling times (h)	Cell size ( $\mu\text{m}$ )	Capsule size ( $\mu\text{m}$ )	Laccase activity (U)	Phagocytosis indices (%)	Killing post phagocytosis (%)
1	A	H99	2.7 + 0.19	6.867 + 1.00	0.8566 + 0.35	1.000	48.38	35.92
2	D	I114	3.3 + 0.25	6.961 + 0.98	0.6491 + 0.43	0.922	32.96	-
3	A	I47	3.4 + 0.29	7.920 + 1.59	0.8348 + 0.57	0.968	33.46	20.55
4	A	I55	2.8 + 0.48	6.797 + 0.61	0.7312 + 0.24	0.955	36.75	25.62
5	A	I58	3.1 + 0.17	8.072 + 1.03	0.8870 + 0.28	1.020	44.00	19.51
6	A	I65	3.6 + 0.24	7.540 + 1.09	0.7031 + 0.51	0.886	17.79	27.63
7	A	ISG12	3.7 + 0.41	9.041 + 1.24	1.2440 + 0.43	0.894	18.06	12.11
8	D	J22	2.9 + 0.50	7.341 + 1.12	1.0770 + 0.47	0.889	24.35	23.01
9	D	J9	3.1 + 0.37	7.956 + 1.16	0.6764 + 0.42	0.914	8.31	46.37
10	D	JEC21	3.7 + 0.41	7.629 + 1.06	0.9114 + 0.50	1.038	34.25	30.29
11	A	M12A	2.9 + 0.17	6.286 + 0.81	0.6905 + 0.43	0.906	39.33	30.51
12	A	M12B	3.9 + 0.17	6.751 + 0.90	0.6162 + 0.48	0.937	55.71	17.43
13	A	M511B	3.2 + 0.19	8.157 + 0.94	1.1220 + 0.28	0.965	38.83	21.90
14	A	M7A	3.6 + 0.32	6.528 + 0.83	0.6809 + 0.43	0.896	20.42	43.06
15	A	M7E	4.3 + 0.10	7.467 + 1.30	1.0130 + 0.43	0.924	18.75	57.56
16	A	M8A	5.2 + 0.25	6.285 + 0.92	0.5851 + 0.37	0.943	16.10	56.79
17	A	M9A	4.6 + 0.27	6.938 + 1.14	0.6149 + 0.55	0.909	13.04	42.01
18	D	RC-2	3.3 + 0.46	11.100 + 1.51	2.8650 + 0.35	0.900	17.94	25.34
19	A	SB4	2.8 + 0.46	8.572 + 0.89	0.9422 + 0.51	0.917	24.36	34.96
20	A	W911A	3.6 + 0.28	8.884 + 1.57	1.3070 + 0.55	0.960	29.17	44.86

independent experiments. BALB/c mice ( $n = 8$ ) were infected i.v. with  $10^6$  CFU. Survival was observed daily and mortality was recorded (Fig. 1A). Virulence varied for clinical strains in both models. In the i.v. model, survival ranged from 8 days to 45 days (mice sacrificed at 45 days). Premorbid mice exhibited hunched backs, and decreased movement consistent with signs of secondary cryptococcal meningoencephalitis. Survival of *Galleria* correlated in the majority of the strains (70%) between the 3 doses ( $p < 0.05$ ) (Fig. 1B). However, most importantly, a relationship in survival between both animal models, *Galleria* and mice, was also not found (Fig. 1C).

Given the lack of correlation between the survival of *Galleria* and the survival of mice, we explored correlation with other commonly assessed virulence traits. These experiments established that intracellular phagocytosis or killing by murine macrophages, as well as laccase activity does not correlate with virulence in *Galleria*. Instead, killing by murine macrophages, and laccase activity were found to moderately correlate with murine survival ( $p < 0.05$ ) (Fig. 1D, E). Lastly, correlation with RLS, a variable, but also reproducible trait of yeast strains was examined. Previous studies have shown that older cryptococcal cells are more virulent;<sup>14</sup> however,

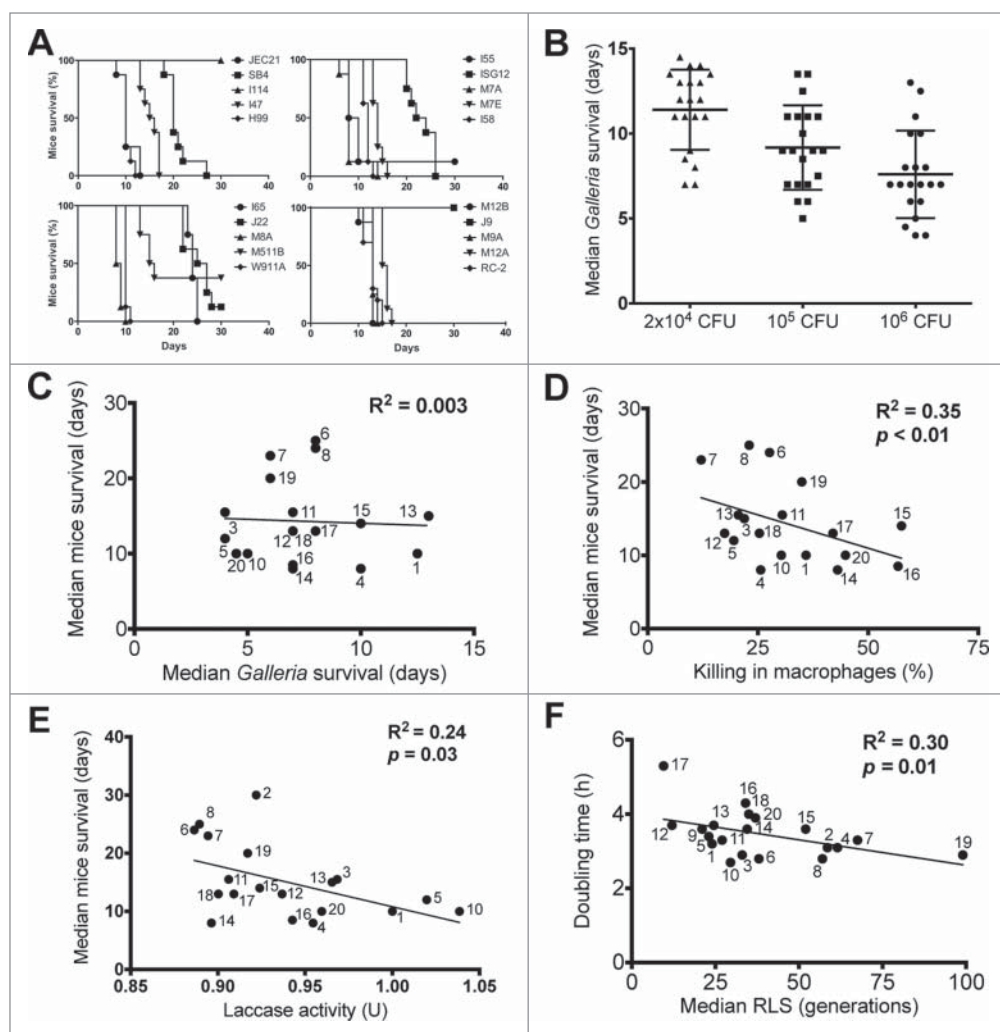
the relevance of the RLS of a strain to its virulence had not been examined. These data now demonstrate that although a strong correlation between doubling time and RLS in clinical strains was established (Fig. 1F), no correlation between the RLS of a strain and its virulence, either in *Galleria*, or in BALB/c mice was found.

The observed variability in the outcome of cryptococcal meningoencephalitis is attributed to differences in host immunity, as well as differences in potency of the virulence associated with individual *Cryptococcus* strains.<sup>1,15</sup> Consequently, many studies on mutants and potential drug targets seek to screen and compare the virulence of individual *Cryptococcus* strains. Phenotypic characterization of human infection with *C. neoformans* can be mimicked in different infection models.<sup>5-7,9,12,16</sup> Although murine infection models have been well established and have the advantage of close correlation with human pathology, strain virulence can also be assessed in more primitive infection models, such as *Galleria mellonella*, which are considerably cheaper.<sup>12</sup>

We compared *Galleria* and murine infection models, and also correlated outcomes with *in vitro* laccase activity, IPR, phagocytosis, killing, and growth times. Specifically, easily phagocytosed strains, namely “high-uptake” strains have been described as hypocapsular, have enhanced

laccase activity, and importantly have been associated with central nervous system fungal burden and patient death.<sup>15</sup> In our study, we found such high-uptake strains to have significantly enhanced laccase activity, and high virulence in mice, but not in *Galleria*. These data reaffirm that the virulence of clinical *C. neoformans* strains varies greatly, highlight some important differences between the various infection models, and ultimately suggest that the *Galleria* infection model cannot reliably predict virulence in the murine model. Notably, our high-uptake strains did not have a significantly smaller capsule size, nor a significantly smaller cell size. Capsular enlargement has been shown to be statistically similar among strains and not predictive of virulence.<sup>17</sup> One study has also concluded that the virulence for *C. neoformans* may be an emergent property, rather than deterministic in the *Galleria* model of infection.<sup>18</sup>

High-throughput screening of mutants and of antifungal agents seeks to work with the most cost efficient model. Virulence of *C. neoformans* is mediated by many different virulence traits, which interact with the host in a complex manner. The most important virulence traits are the presence of a polysaccharide capsule, as well the ability to grow at  $37^\circ\text{C}$ ;<sup>1,8</sup> however, multiple other genes contribute



**Figure 1.** Relationships between different strain-specific characteristics of *C. neoformans*. (A) Kaplan-Meier survival curves of mice infected with clinical *C. neoformans* strains divided into 4 arbitrary groups (top left: JEC21, SB4, I114, I47, H99; top right: I55, ISG12, M7A, M7E, I58; bottom left: I65, J22, M8A, M511B, W911A; bottom right: M12B, J9, M9A, M12A, RC-2). (B) Scatter plot of *Galleria* infected with variable doses of clinical *C. neoformans* strains (symbols represent the infection dose: triangle =  $2 \times 10^4$  CFU, square =  $10^5$  CFU, circle =  $10^6$ ). (C) No significant relationship was found between mice and *Galleria* survival. Symbol legends represent the assigned strain numbers listed in **Table 1**. Note that strains #2 and #9 were omitted from this comparison because infected mice were sacrificed at day 45 to terminate the study. Strains #12 and #18 are represented by the same symbol because they induced similar survival in mice and *Galleria*. (D) An inverse correlative relationship was found between mice survival and intracellular killing of *C. neoformans* strains in macrophages. Strains #2 and #9 were omitted from this comparison. (E) An inverse correlative relationship was found between mice survival and laccase activity of *C. neoformans* strains. (F) An inverse correlative relationship was found between the doubling time and the median replicative life span of *C. neoformans* strains. Strains #2 and #9 were omitted from this comparison. Correlation considered when Pearson's correlation coefficient had  $p < 0.05$ .

to virulence, and either their up- or down-regulation can alter virulence as was recently described even with *C. gattii* strains used to infect *Galleria*.<sup>17</sup> Several important findings were documented in this study. First, we established that internalization of the fungus did not depend on the doubling time of different *C.*

*neoformans* strains; however, strains with a long doubling time were killed intracellularly faster than strains with a short doubling time. Virulence in *Galleria* was dependent on the different doses of *C. neoformans* inoculated for most of the strains. Despite this finding, we were not able to find a significant correlation

between the virulence in *Galleria*, and in the most widely used animal model, the mouse. In the latter host, killing by macrophages and host survival were found to strongly correlate, but the same could not be established in *Galleria*. This suggests that variables usually reported to measure the virulence of a strain, i.e. phagocytosis and intracellular killing, are not being replicated in all the strains infecting the insect host, *Galleria*. These findings suggest that *C. neoformans* virulence data obtained in the *Galleria* model should be cautiously applied to the virulence of a pathogen in mammalian hosts. It should be acknowledged that for the 2 highly passaged laboratory strains, H99 and RC2, some correlation between *Galleria* and murine virulence was observed. It has been described that laboratory passage selects for fast growing, high passaged strains with phenotypes different from low passaged strains.<sup>19</sup> These findings also highlight the importance of relying on multiple models of infection, particularly with respect to high-throughput screening. One group<sup>20</sup> has found a way to reliably screen deletion mutants in a single strain using a multi-host approach that involves first, a *C. elegans* host, and second, a *Galleria* host, and this approach was successfully validated in a murine model of systemic cryptococcosis. This multi-host approach should greatly aid high-throughput screening of *C. neoformans* mutants and antifungal

agents when limited to a single strain and not to compare virulence among different strains.

Lastly, we analyzed the replicative life span of the different clinical isolates. This was done since the short-living mucoid variant of strain RC2,<sup>14</sup> is known to be hypervirulent in mice.<sup>21</sup> Also, for a pathogen, the

ability to live long and also divide fast could potentially aid in colonization; or alternatively, the ability to live short, but divide slower could aid in persistence. We found no correlation between RLS and virulence; however, interestingly an inverse correlation between doubling time and RLS was observed. This finding is not consistent with previously published data in *S. cerevisiae* that enhanced longevity often comes with a fitness cost.<sup>22</sup> However, more recent data in this yeast<sup>23</sup> highlights that genetic variability also contributes to variability in longevity. For facultative intracellular pathogens, such as *C. neoformans*, genetic variants could potentially be selected through the host response.

All animal experiments were carried out with the approval of the Albert Einstein College of Medicine Institute for Animal Studies under protocol number 20091015 as approved by the Institutional Animal Care and Use Committee at Einstein. *C. neoformans* strains used in this study are listed in Supplemental Methods (Table S2). Standard yeast culture media

as outlined (Table S3) were employed. *C. neoformans* cells (n = 100) were imaged at 100X magnification on an Olympus AX70 microscope. Pictures were taken with a Qimaging Retiga 1300 digital camera using the Qcapture Suite V2.46 software (Qimaging, British Columbia, Canada) and size was measured with Adobe Photoshop CS5 for Macintosh. Log phase *C. neoformans* cells in 2% YPD were diluted to an OD of 0.01 and grown at 37 °C with agitation for *in vitro* studies. Growth curves were determined in a Bioscreen-C Automated Growth Curve Analysis System (Growth Curves USA). Standard *in vitro* phagocytosis indices, and macrophage killing assays were done in J774.16 cells as previously described.<sup>24</sup> IPR and laccase activity was determined as previously described.<sup>15</sup> For infection with *Galleria mellonella*, a 10 µl suspension of 2 × 10<sup>4</sup>, 10<sup>5</sup>, or 10<sup>6</sup> *C. neoformans* cells was used to infect larvae (n = 20) (Vanderhorst Wholesale, Inc., OH) in the last proleg as described previously.<sup>16</sup> For infection with mice, 10<sup>6</sup> *C. neoformans*

cells were used to infect 6-8 week old female BALB/c mice (n = 8) (National Cancer Institute, Bethesda, MD) intravenously as previously described.<sup>25,26</sup> Standard statistical analysis and non-parametric tests, such as Student's t-test, Log-rank, Pearson correlation, and Wilcoxon rank sum tests were performed using Prism version 5 (Graphpad) or Microsoft Excel 2011 for Macintosh. Differences were considered significant if *p* < 0.05.

## Funding

B.C.F. is supported by NIH awards R01 AI059681 and R21 AI114259. For a portion of this work, T.B. was supported by the Institutional AIDS Training Grant, "HIV, AIDS and Opportunistic Infections" (T32 AI007501).

## Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

## References

- Perfect JR, Casadevall A. Cryptococcosis. *Infect Dis Clin North Am* 2002; 16:837-74, v-vi; PMID:12512184; [http://dx.doi.org/10.1016/S0891-5520\(02\)00036-3](http://dx.doi.org/10.1016/S0891-5520(02)00036-3)
- Mandal P, Banerjee U, Casadevall A, Nosanchuk JD. Dual infections with pigmented and albino strains of *Cryptococcus neoformans* in patients with or without human immunodeficiency virus infection in India. *J Clin Microbiol* 2005; 43:4766-72; PMID:16145139; <http://dx.doi.org/10.1128/JCM.43.9.4766-4772.2005>
- Alvarez M, Burn T, Luo Y, Pirofski LA, Casadevall A. The outcome of *Cryptococcus neoformans* intracellular pathogenesis in human monocytes. *BMC Microbiol* 2009; 9:51; PMID:19265539; <http://dx.doi.org/10.1186/1471-2180-9-51>
- Gilbert AS, Wheeler RT, May RC. Fungal Pathogens: Survival and Replication within Macrophages. *Cold Spring Harb Perspect Med* 2014; 5:a019661; PMID:25384769; <http://dx.doi.org/10.1101/cshperspect.a019661>
- Sabiiti W, May RC, Pursall ER. Experimental models of cryptococcosis. *International J Microbiol* 2012; 2012:626745; <http://dx.doi.org/10.1155/2012/626745>
- Carroll SF, Guillot L, Qureshi ST. Mammalian model hosts of cryptococcal infection. *Comparative Med* 2007; 57:9-17
- Goldman DL, Casadevall A, Cho Y, Lee SC. *Cryptococcus neoformans* meningitis in the rat. *Lab Invest* 1996; 75:759-70; PMID:8973471
- Casadevall A, Steenbergen JN, Nosanchuk JD. 'Ready made' virulence and 'dual use' virulence factors in pathogenic environmental fungi—the *Cryptococcus neoformans* paradigm. *Curr Opin Microbiol* 2003; 6:332-7; [http://dx.doi.org/10.1016/S1369-5274\(03\)00082-1](http://dx.doi.org/10.1016/S1369-5274(03)00082-1)
- Mylonakis E, Ausubel FM, Perfect JR, Heitman J, Calderwood SB. Killing of *Caenorhabditis elegans* by *Cryptococcus neoformans* as a model of yeast pathogenesis. *Proc Natl Acad Sci USA* 2002; 99:15675-80; PMID:12438649; <http://dx.doi.org/10.1073/pnas.232568599>
- Steenbergen JN, Shuman HA, Casadevall A. *Cryptococcus neoformans* interactions with amoebae suggest an explanation for its virulence and intracellular pathogenic strategy in macrophages. *Proc Natl Acad Sci USA* 2001; 98:15245-50; PMID:11742090; <http://dx.doi.org/10.1073/pnas.261418798>
- Steenbergen JN, Nosanchuk JD, Malliaris SD, Casadevall A. *Cryptococcus neoformans* virulence is enhanced after growth in the genetically malleable host *Dicystostelium discoideum*. *Infect Immun* 2003; 71:4862-72; PMID:12933827; <http://dx.doi.org/10.1128/IAI.71.9.4862-4872.2003>
- Mylonakis E, Moreno R, El Khoury JB, Idnurm A, Heitman J, Calderwood SB, Ausubel FM, Diener A. *Galleria mellonella* as a model system to study *Cryptococcus neoformans* pathogenesis. *Infect Immun* 2005; 73:3842-50; PMID:15972469; <http://dx.doi.org/10.1128/IAI.73.7.3842-3850.2005>
- Perfect JR, Casadevall A. The history of *Cryptococcus* and *Cryptococcosis*. *Cryptococcus: from human pathogen to model yeast* Washington, DC: ASM Press pp 2011:17-26
- Bouklas T, Pechuan X, Goldman DL, Edelman B, Bergman A, Fries BC. Old *Cryptococcus neoformans* cells contribute to virulence in chronic cryptococcosis. *MBio* 2013; 4:e00455-13; PMID:23943761; <http://dx.doi.org/10.1128/mBio.00455-13>
- Sabiiti W, Robertson E, Beale MA, Johnston SA, Brouwer AE, Loyse A, Jarvis JN, Gilbert AS, Fisher MC, Harrison TS, et al. Efficient phagocytosis and laccase activity affect the outcome of HIV-associated cryptococcosis. *J Clin Invest* 2014; 124:2000-8; PMID:24743149; <http://dx.doi.org/10.1172/JCI72950>
- Cotter G, Doyle S, Kavanagh K. Development of an insect model for the *in vivo* pathogenicity testing of yeasts. *FEMS Immunol Med Microbiol* 2000; 27:163-9; PMID:10640612; <http://dx.doi.org/10.1111/j.1574-695X.2000.tb01427.x>
- Firacative C, Duan S, Meyer W. *Galleria mellonella* model identifies highly virulent strains among all major molecular types of *Cryptococcus gattii*. *PLoS One* 2014; 9:e105076; PMID:25133687; <http://dx.doi.org/10.1371/journal.pone.0105076>
- Garcia-Solache MA, Izquierdo-Garcia D, Smith C, Bergman A, Casadevall A. Fungal virulence in a lepidopteran model is an emergent property with deterministic features. *MBio* 2013; 4:e00100-13; PMID:23631914; <http://dx.doi.org/10.1128/mBio.00100-13>
- Ronald J, Tang H, Brem RB. Genomewide evolutionary rates in laboratory and wild yeast. *Genetics* 2006; 174:541-4; PMID:16816417; <http://dx.doi.org/10.1534/genetics.106.060863>
- Desalermos A, Tan X, Rajamuthiah R, Arvanitis M, Wang Y, Li D, Kourkoumpetis TK, Fuchs BB, Mylonakis E. A multi-host approach for the systematic analysis of virulence factors in *Cryptococcus neoformans*. *J Infect Dis* 2015; 211:298-305; PMID:25114160; <http://dx.doi.org/10.1093/infdis/jiu441>
- Jain N, Li L, Hsueh YP, Guerrero A, Heitman J, Goldman DL, Fries BC. Loss of allergen 1 confers a hypervirulent phenotype that resembles mucoid switch variants of *Cryptococcus neoformans*. *Infect Immun* 2009; 77:128-40; PMID:18955480; <http://dx.doi.org/10.1128/IAI.01079-08>
- Delaney JR, Murakami CJ, Olsen B, Kennedy BK, Kaerberlein M. Quantitative evidence for early life fitness defects from 32 longevity-associated alleles in yeast. *Cell Cycle* 2011; 10:156-65; PMID:21191185; <http://dx.doi.org/10.4161/cc.10.1.14457>
- Stumperferl SW, Brand SE, Jiang JC, Korona B, Tiwari A, Dai J, Seo JG, Jazwinski SM. Natural genetic variation in yeast longevity. *Genome Res* 2012; 22:1963-73; PMID:22955140; <http://dx.doi.org/10.1101/gr.136549.111>
- Jain N, Cook E, Xess I, Hasan F, Fries D, Fries BC. Isolation and characterization of senescent *Cryptococcus neoformans* and implications for phenotypic switching and pathogenesis in chronic cryptococcosis.

- Eukaryotic cell 2009; 8:858-66; PMID:19411622; <http://dx.doi.org/10.1128/EC.00017-09>
25. Huffnagle GB, Yates JL, Lipscomb MF. Immunity to a pulmonary *Cryptococcus neoformans* infection requires both CD4+ and CD8+ T cells. *J Exp Med* 1991; 173:793-800; PMID:1672543; <http://dx.doi.org/10.1084/jem.173.4.793>
26. Mukherjee S, Lee S, Mukherjee J, Scharff MD, Casadevall A. Monoclonal antibodies to *Cryptococcus neoformans* capsular polysaccharide modify the course of intravenous infection in mice. *Infect Immun* 1994; 62:1079-88; PMID:8112842
27. Jain N, Wickes BL, Keller SM, Fu J, Casadevall A, Jain P, Ragan MA, Banerjee U, Fries BC. Molecular epidemiology of clinical *Cryptococcus neoformans* strains from India. *J Clin Microbiol* 2005; 43:5733-42; PMID:16272511; <http://dx.doi.org/10.1128/JCM.43.11.5733-5742.2005>
28. Casadevall A, Spitzer ED, Webb D, Rinaldi MG. Susceptibilities of serial *Cryptococcus neoformans* isolates from patients with recurrent cryptococcal meningitis to amphotericin B and fluconazole. *Antimicrob Agents Chemother* 1993; 37:1383-6; PMID:8328793; <http://dx.doi.org/10.1128/AAC.37.6.1383>
29. Kwon-Chung KJ, Edman JC, Wickes BL. Genetic association of mating types and virulence in *Cryptococcus neoformans*. *Infect Immun* 1992; 60:602-5; PMID:1730495