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Phenotypic Differences of *Cryptococcus* Molecular Types and Their Implications for Virulence in a *Drosophila* Model of Infection

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Compared to *Cryptococcus neoformans*, little is known about the virulence of the molecular types in *Cryptococcus gattii*. We compared *in vitro* virulence factor production and survival data using a *Drosophila* model of infection to further characterize the phenotypic features of different cryptococcal molecular types. Forty-nine different isolates were inoculated into wild-type flies and followed for survival. *In vitro*, isolates were assessed for growth at 30 and 37°C, melanin production, capsule size, resistance to H_2O_2, and antifungal susceptibility. A mediator model was used to assess molecular type and virulence characteristics as predictors of survival in the fly model. VGIII was the most virulent molecular type in flies ($P < 0.001$). At 30°C, VGIII isolates grew most rapidly; at 37°C, VNI isolates grew best. *C. gattii* capsules were larger than those of *C. neoformans* ($P < 0.001$). Mediator model analysis found a strong correlation of *Drosophila* survival with molecular type and with growth at 30°C. We found molecular-type-specific differences in *C. gattii* in growth at different temperatures, melanin production, capsule size, ability to resist hydrogen peroxide, and antifungal susceptibility, while growth at 30°C and the VGIII molecular type were strongly associated with virulence in a *Drosophila* model of infection.

Cryptococcosis is an invasive fungal infection most commonly caused by one of two species of encapsulated yeast, *Cryptococcus neoformans* and *Cryptococcus gattii*. *C. neoformans* is ubiquitous with a worldwide distribution, while *C. gattii* has been traditionally associated with tropical and subtropical regions (1, 2). However, the geographic distribution of *C. gattii* continues to expand, following the emergence of this species within the Pacific Northwest (3). Numerous cases have since been described in New Mexico, California (4), North Carolina (5), Georgia, Florida, and Alabama (6), suggesting that this species is already widely distributed within the United States.

Existing guidelines published by the Infectious Disease Society of America do not define differences in management according to *Cryptococcus* species (7). However, previous reports illustrate significant differences in the epidemiology, susceptibility patterns, and chronicity of infection and a frequency of neurosurgical intervention in *C. gattii* infections higher than the level in *C. neoformans* infections, likely due to the propensity of *C. gattii* to form cryptococcomas (1, 8, 9).

C. gattii has been divided into four distinct molecular types (VGI, VGII, VGIII, and VGIV). VGI and VGII primarily affect immunocompetent patients, while VGIII and VGIV are most frequently found in the immunocompromised (4). Comparative studies between different cryptococcal molecular types have been limited and have primarily focused on the major outbreak strain associated with the Pacific Northwest (VGIIa) and have found VGIIa strains more lethal than VGIIb isolates (10, 11). More recently, VGIIIa and VGIIIb subtypes recovered from HIV/AIDS patients in California were compared in an intranasal murine model (4), and VGIIIa isolates were found to be more virulent than VGIIIb isolates; however, comparisons of cryptococcal molecular types and levels of virulence on a broader scale have not previously been performed. We analyzed the relationship of *in vitro* production of putative virulence factors with molecular type and virulence in a *Drosophila melanogaster* model of infection.

(This study was presented in part at IDWeek: a Joint Meeting of IDSA, SHEA, HIVMA, and PIDS, San Francisco, CA, 2013 [12].)

MATERIALS AND METHODS

Isolates. Forty-nine *Cryptococcus* isolates obtained from clinical, environmental, and veterinary sources were used (Table 1). Molecular types were identified as described in prior studies (11). Mating type was determined by PCR using two pairs of mating-type-specific primers per cryptococcal species as described previously (13, 14).

***In vivo* assessment of virulence in *Drosophila*.** Two- to 4-day-old Oregon-R (OR) flies (60 flies per isolate) were used as wild-type (WT) flies (15). Each isolate was inoculated through the thorax (2×10^9 cells/ml) using a 0.1-μm-inner-diameter needle (approximately 5×10^3 cells per fly). The flies were kept at 29°C and transferred to fresh vials every 2 days.

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TABLE 1 List of isolates and strain characteristics used in the present study

Molecular type (no. of isolates)	Name	Alias	Origin	Source	Mating type
VGI (10)	WM179[a]	CBS 10078/	Human	Australia	α
	10		Eucalyptus tree	CA	α
	88		Human	Washington, DC	**a**
	JS66		Koala	Los Angeles, CA	α
	JS80		Domestic cat	Gainesville, FL	α
	JS55		Koala	Los Angeles, CA	α
	JS53		Domestic cat	Rocklin, CA	α
	JS56		Parrot	Escondido, CA	α
	JS67		Koala	Los Angeles, CA	α
	532		Koala	Sydney, Australia	α
VGIIa (9)	WM02.32[a]	CDC R265	Human	Duncan, Canada	α
	CAT		Domestic cat	Sacramento, CA	α
	JS7		Dog	Sacramento, CA	α
	JS74		Domestic cat	El Cerrito, CA	α
	WM09.144	CBS10485	Human	Herning, Denmark	α
	WM06.13	CBS7750	Environmental	San Francisco, CA	α
	06-3908		Human	San Antonio, TX	α
	JS71		Dog	Sacramento, CA	α
	432		Human	Los Angeles, CA	α
VGIIb (7)	WM06.25[a]	CDC R272	Human	Vancouver Island, BC, Canada	α
	WM1008	99-194-1904	Environmental	Blacktown, Australia	α
	WM09.155	N#10	Koala	Perth, Australia	α
	535		Veterinary	Sydney, Australia	α
	537		Veterinary	Sydney, Australia	α
	50513971		Horse	CA	α
	JS65		Dog	La Mesa, CA	α
VGIIc (4)	08-10290		Domestic cat	OR	α
	09-10082		Ovine	OR	α
	09-11171		Domestic cat	OR	α
	11-7650		Dog	Eugene, OR	α
VGIII (10)	82		Human	Washington, DC	α
	83		Human	Washington, DC	α
	BR		Human	Sacramento, CA	α
	50805443		Horse	CA	α
	B7415		Alpaca	CA	α
	JS54		Domestic cat	Sacramento, CA	α
	JS5		Musk deer	San Diego, CA	α
	JS27		Domestic cat	American Canyon, CA	α
	JS69		Domestic cat	Lomita, CA	α
	JS52		Domestic cat	Davis, CA	**a**
VGIV (4)	WM779[a]	CBS 10101	Cheetah	Johannesburg, South Africa	α
	WM2363	B5742	Human	Punjab, India	α
	WM2364	B5748	Human	Himachal Pradesh, India	α
	JS26		Cheetah	Reno, NV	α
VNI (5)	H99[a]		Human	NC	α
	JS23		Horse	Williams, CA	α
	JS68		Domestic cat	Vancouver, WA	α
	JS73		Dog	San Jose, CA	α
	JS72		Dog	Tiburon, CA	α

[a] American Type Culture Collection reference strain—see text for ATCC reference numbers.

Flies that died <3 h after infection (<5% of the total) were excluded from analysis. Survival to 8 days after infection was assessed. Each experiment was performed in triplicate at different time points.

Quantification of the injection inoculums was performed by transferring cells from the tip of a needle that had been previously dipped into a concentrated *Cryptococcus* solution into 1 ml of 0.85% normal saline solution. Serial dilutions (100 μl each) were then plated onto yeast extract-peptone-dextrose (YPD) plates and incubated at 37°C for 72 h.

Growth at 30°C and 37°C. The *Cryptococcus* isolates used in this study were repopulated in YPD liquid media overnight to mid-log phase. Next, the isolates were washed twice in sterile phosphate-buffered saline (PBS) and resuspended in 2% glucose YNB liquid at a concentration of 1×10^3 yeast cells/ml, and then 100 μl of each isolate was dispensed into 96-well U-bottom plates; medium alone was used as a control. Viability was confirmed by plating a 10-fold dilution of the 1×10^3 yeast cells/ml on a Sabouraud agar plate. The plates were sealed and placed in a plate reader at 30°C or 37°C for 24 h with measurements every hour. Turbidity was measured at 600 nm. Sequential optical density (OD) measurements were used to generate growth curves for each isolate in triplicate.

Susceptibility testing. Susceptibility testing was done by broth microdilution in accordance with the CLSI M27-A3 methodology (16, 17). Final antifungal concentrations ranged from 0.015 to 8 μg/ml for itraconazole, posaconazole, voriconazole, and amphotericin B and from 0.25 to 64 μg/ml for fluconazole and flucytosine. *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were used as controls.

Hydrogen peroxide tolerance. The ability to resist the fungicidal effects of H_2O_2 (Fisher Scientific) was measured by broth microdilution using a concentration of 5×10^3 cells and final H_2O_2 concentrations ranging from 0.1 to 80 mM (18). Isolates were incubated for 3 h at 37°C on a rotary shaker in YPD liquid media, and catalase (Fisher-Scientific) at a concentration of 200 μg/ml was used as an inhibitor of H_2O_2 activity. Isolates were serially diluted and plated on Sabaroud dextrose for assessment of viability.

Melanin production. Melanin synthesis was quantified using a modified laccase activity test as previously described (11, 19). Each isolate was grown in YPD broth for 24 h at 30°C. Saturated cultures were then added to 10 ml of melanin induction media (1% yeast nitrogen base [YNB] without amino acid and ammonium sulfate) containing either 10 mM dopamine or 10 mM epinephrine (Sigma) and incubated at 37°C for 48 h. Aliquots were taken and diluted to ensure optical density (OD) readings within the 0.1 to 0.9 absorbance range, and values at 475 and 600 nm were obtained to control for both the quantity of melanin and cell concentrations.

Capsule production. Isolates were suspended in YPD media, collected in the logarithmic-growth phase by centrifugation, washed twice with PBS, and then placed in six-well plates at a density of 5×10^6 to 5×10^7 cells/ml in RPMI 1640 with MOPS (morpholinepropanesulfonic acid) and HCO_3 (pH 7.3) and incubated for 24 h in 5% CO_2 at 37°C, as described previously (11, 20). The capsule size of at least 30 yeast cells per isolate (10 μl of cell suspension was mixed with India ink) was measured by light microscopy (Olympus BX51) using the ratio of the diameter of the capsule to the diameter of the cell measured in micrometers.

Statistical analysis. To identify the relationship between molecular type and survival, we used a mediator model approach. This is a multistep process to test whether *Drosophila* survival rates differ between molecular types and to investigate the various characteristics that may mechanistically explain differences in survival rates. In the first step, we performed univariate analyses to test if there was a difference in *Drosophila* survival and the various possible candidate mediators (molecular type and subtype, growth at 30°C and 37°C, antifungal susceptibility, H_2O_2 tolerance, capsule size, and melanin production). In the second stage, we included all significant mediators in a multiple-regression model to test which candidates remained significant after controlling for others, excluding molecular type. At this stage, a *P* value of 0.1 or less was considered significant to avoid prematurely removing important explanatory variables.

In the final stage, we examined how *P* values for the significant candidates from stage 2 changed when the molecular type was included in the model. The reason for performing this last step was that if molecular types became nonsignificant when the significant candidate mediators were included, this would indicate that the effect of molecular type was mediated by the candidate. If molecular type remained significant, this would support the hypothesis that the candidates were mediating survival but that there were other mediating characteristics that had not been included in the model.

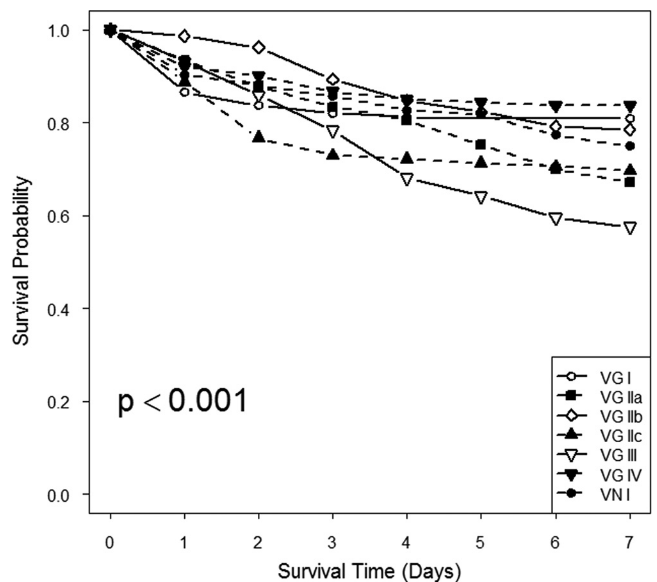

FIG 1 Survival of WT flies infected by injection (5×10^3 cells per fly) of different *Cryptococcus* molecular types and subtypes. VGIII was the most virulent molecular type observed ($P < 0.001$ compared to VNI, VGI, VGIIb, and VGIV). VGIIa also demonstrated significant differences from the other molecular types and subtypes ($P < 0.001$ compared to VGI, VGIIb, and VGIV), and VGIIc demonstrated virulence similar to that of VGIIa ($P < 0.001$ compared to VGI and VGIV).

Welch's analysis of variance (ANOVA) was used to test for differences in survival among molecular types due to nonhomogeneity. The Kruskal-Wallis test was used to determine the relationship between virulence factors and both molecular type and survival. For normally distributed variables, we used Pearson's correlation test for a relationship with survival. Colinearity was assessed using Pearson's or Spearman's correlation. Mediator model analyses were performed using SAS software version 9.3. *P* values of less than 0.05 were considered to be statistically significant.

RESULTS

Strain characteristics. Of the 49 *Cryptococcus* isolates studied, five were molecular type VNI, 10 VGI, 9 VGIIa, 7 VGIIb, 4 VGIIc, and 10 VGIII, and 4 were VGIV (Table 1). The isolates were chosen to represent a diverse global population in attempt to characterize true molecular-type-specific differences that might exist rather than differences apparent merely in regional subpopulations. Well-characterized isolates available as reference strains through the American Type Culture Collection (ATCC) were also chosen for inclusion.

***In vivo* assessment of virulence in *Drosophila*.** Mortality was readily achieved at 5×10^3 cells/fly in our study. The probabilities of flies surviving over time differed significantly among molecular types ($P < 0.001$) (Fig. 1). VGIII was the most virulent molecular type in flies ($P < 0.001$ compared to VNI and VGI [the predominant global molecular types], VGIIb, and VGIV). The major subtype observed in the Pacific Northwest outbreak (VGIIa) was also more virulent than other molecular types and subtypes in the fly model ($P < 0.001$ compared to VGI, VGIIb, and VGIV). Subgroup VGIIc demonstrated virulence similar to that of VGIIa ($P < 0.001$ compared to VGI and VGIV).

Growth at 30°C and 37°C. Logarithmic-phase growth was

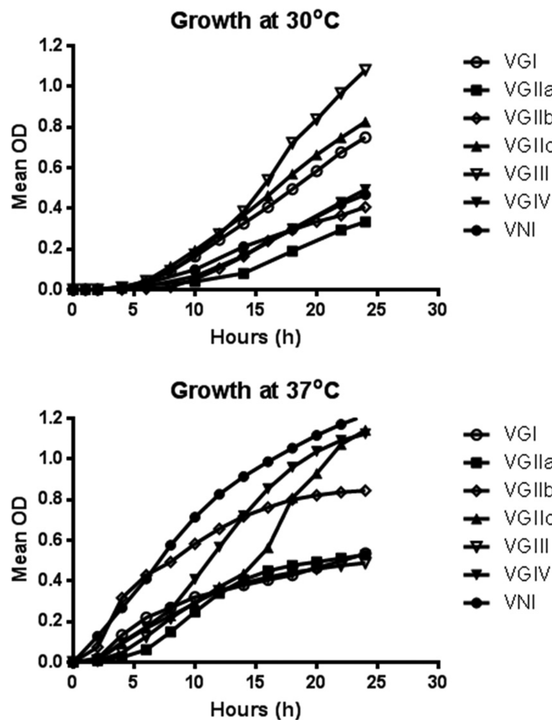

FIG 2 Growth curves of isolates at 30 and 37°C.

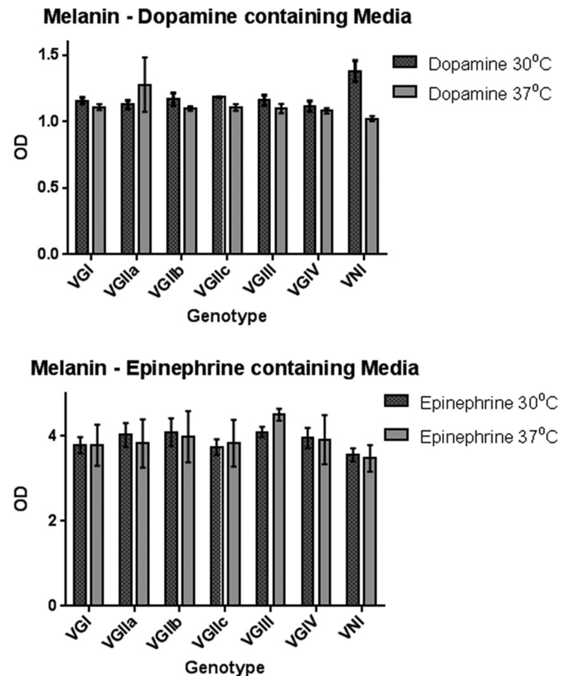

FIG 3 Melanin production of different *Cryptococcus* molecular types under various growth conditions.

reached at different time points. For this reason, doubling time was not used to compare growth curves; instead, overall growth was measured at 24 h as performed in previous studies (11). At 30°C, VGIII isolates grew most rapidly (Fig. 2), with significant increases in growth compared to molecular subtypes VGIIa ($P < 0.0001$), VGIIb ($P = 0.0003$), VGIV ($P < 0.0001$), and VNI ($P < 0.0001$). Differences in growth were also seen in other types, with VGIIc isolates showing significant increases in growth over 24 h compared to VGIIa isolates ($P = 0.027$) and VGI isolates showing significant increases in growth over 24 h compared to VGIIa isolates ($P = 0.003$).

At 37°C, VNI isolates grew most rapidly, with significant differences seen compared to molecular types VGI ($P = 0.007$), VGIIa ($P = 0.04$), and VGIII ($P = 0.014$). At 24 h, VGIV isolates also showed growth that was significantly more rapid than that seen with VGIII ($P = 0.022$) and VGI ($P = 0.015$) isolates. No other significant differences were found between molecular types and subtypes.

Melanin production. At 30°C, VNI isolates in dopamine-containing media produced significantly more melanin than VGI ($P = 0.004$), VGIIa ($P = 0.004$), VGIIb ($P = 0.03$), VGIII ($P = 0.01$), and VGIV ($P = 0.04$) isolates (Fig. 3). Using epinephrine-containing media, no significant differences in melanin production were seen at 30°C. Isolates grown at 37°C in dopamine- or epinephrine-containing media displayed nonsignificant differences in melanin production between molecular types. All isolates produced more melanin using epinephrine than using dopamine ($P < 0.0001$). Others have previously observed changes in melanin production between 30 and 37°C (11); however, no significant differences between isolates grown in either dopamine- or epinephrine-containing media at different temperatures were observed in our study.

Capsule production. Comparison of all *C. gattii* capsule sizes to those of *C. neoformans* revealed highly significant differences, with capsules of *C. gattii* isolates larger than those of *C. neoformans* isolates ($P = 0.0006$). There were also highly significant differences in the capsule ratios between molecular types (Fig. 4)—by ANOVA, VGI isolates produced a capsule larger than that seen with VGIIa isolates ($P = 0.0008$) or with VNI isolates ($P = 0.0001$). VGIII isolates similarly produced a larger capsule than isolates of VGIIa ($P = 0.002$), VGIIc ($P = 0.02$), or VNI ($P = 0.001$). No significant differences were seen between other molecular types.

Hydrogen peroxide tolerance. The ability of different cryptococcal molecular types to resist killing by H_2O_2 was also examined, and at lower H_2O_2 concentrations, similar findings were observed for all isolates. However, when high concentrations (35 mM) were examined, VNI isolates demonstrated significant differences compared to those of other molecular types ($P < 0.05$) (Fig. 5).

Susceptibility testing. Table 2 summarizes the mean MIC ranges and mean fungicidal concentrations (MFC) of each anti-

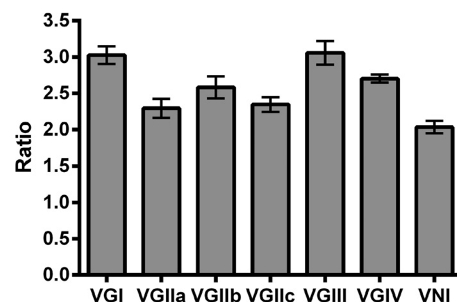

FIG 4 Capsule sizes of different *Cryptococcus* molecular types at 37°C. Ratio, mean capsule-capsule/cell wall-cell wall ratio for each isolate.

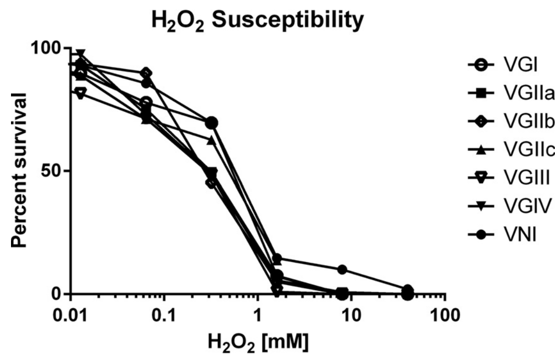

FIG 5 Susceptibility to hydrogen peroxide.

fungal. Fluconazole MICs were higher in VGIIc isolates (geometric mean [GM] = 8) than in the other molecular types and subtypes. VGIIc isolates exhibited significantly higher MICs for fluconazole than those of VGI ($P = 0.008$), VGIII ($P = 0.008$), and VNI ($P = 0.02$). Similarly, itraconazole MICs among VGIIc isolates were significantly higher than those for VGI ($P = 0.02$), VGIIa ($P = 0.04$), and VGIII ($P = 0.001$) isolates. Posaconazole MIC differences were significant only for VGIIc > VNI ($P = 0.01$). Isolates with reduced fluconazole susceptibility exhibited higher posaconazole and voriconazole MICs as well. There were no significant differences in voriconazole, flucytosine, or amphotericin B MICs found between molecular types.

Mediator model analysis. In stage 1 of model selection, we found overwhelming evidence that *Drosophila* survival was related to the molecular type or subtype ($P = 0.0001$). The data provided evidence that survival was positively correlated with enhanced growth at 30°C ($P = 0.0488$), melanin production at 37°C in dopamine-containing media ($P = 0.0627$), and melanin production at 37°C in epinephrine-containing media ($P = 0.0516$) and was negatively correlated with isolate growth at 37°C ($P = 0.033$). The data also provided evidence there were differences between molecular types and subtypes in growth at 30°C ($P = 0.0002$), growth at 37°C ($P = 0.0456$), and capsule size ($P = 0.0002$). In our test for colinearity, we found that no candidate was correlated with any other at 0.6 or higher (results not shown).

In the second stage of model selection, we fitted a multiple-regression model that included voriconazole MIC ($P = 0.36$), itraconazole MIC ($P = 0.22$), growth at 30°C ($P = 0.0049$), growth at 37°C ($P = 0.0989$), capsule size ($P = 0.33$), and melanin production at 37°C in dopamine-containing media ($P = 0.74$) and in epinephrine-containing media ($P = 0.61$). Only growth at 30°C and 37°C were retained for the final stage.

In the final stage of the mediator model analysis, we fitted a general linear model predicting survival from growth at 30°C and a negative correlation for growth at 37°C. Both sets of data were statistically significant, with P values of 0.045 and 0.027, respectively. When molecular type was added to the model, the effect remained significant ($P < 0.0001$), and growth variable data became nonsignificant (0.89 and 0.14, respectively). The analysis therefore indicated there was a strong correlation of *Drosophila* survival with molecular type and that this effect was manifested *in*

TABLE 2 Susceptibility testing against different *Cryptococcus* molecular types and subtypes

Molecular type (no. of isolates)	Parameter	MIC (μg/ml)					
		Fluconazole	Itraconazole	Posaconazole	Voriconazole	Amphotericin B	Flucytosine
VGI (10)	MIC range	1 to 8	1 to 2	0.5 to 2	0.03 to 1	0.25 to 0.5	0.125 to 0.5
	GM MIC	2.30	1.32	0.81	0.06	0.38	0.25
	MFC range	4 to >64	2 to 16	1 to 4	0.125 to 4	0.25 to 0.5	2 to >64
VGIIa (9)	MIC range	0.5 to 8	0.25 to 2	0.125 to 2	0.03 to 0.125	0.125 to 0.5	0.125 to 2
	GM MIC	3.08	1	0.77	0.07	0.39	0.46
	MFC range	4 to >64	2 to >16	1 to 8	0.5 to 16	0.5 to 1	1 to >64
VGIIb (7)	MIC range	1 to 16	1 to 2	0.5 to 1	0.06 to 0.125	0.25 to 0.5	0.125 to 0.5
	GM MIC	4	1.32	0.76	0.08	0.44	0.25
	MFC range	4 to >64	2 to >16	2 to 4	0.25 to 16	0.25 to 1	4 to >64
VGIIc (4)	MIC range	2 to 16	2 to 4	1 to 2	0.06 to 0.5	0.25 to 0.5	0.25 to 0.5
	GM MIC	8	2.38	1.68	0.18	0.42	0.35
	MFC range	64 to >64	2 to 16	4 to 8	0.25 to >16	0.25 to 1	8 to >64
VGIII (10)	MIC range	2 to 8	1 to 2	1	0.06 to 0.5	0.25 to 0.5	0.125 to >64
	GM MIC	3.48	1.07	0.93	0.06	0.47	0.62
	MFC range	16 to 32	2 to 8	2 to 4	0.125 to 1	0.25 to 1	4 to >64
VGIV (4)	MIC range	2 to 8	1 to 2	1	0.06 to 0.5	0.25 to 0.5	0.125 to 0.5
	GM MIC	4	1.52	1	0.11	0.44	0.19
	MFC range	4 to >64	1 to 16	2 to 4	0.25 to >16	0.25 to 0.5	4 to >64
VNI (5)	MIC range	1 to 2	1 to 2	0.25 to 1	0.03 to 0.125	0.5	0.125 to 0.5
	GM MIC	1.74	1.52	0.66	0.05	0.5	0.33
	MFC range	8 to >64	2 to 8	2 to 4	0.25 to >16	0.5	2 to >64

vitro as differences among the isolate groups in growth at 30 and 37°C.

DISCUSSION

The recent outbreak of *C. gattii* infections in the Pacific Northwest illustrates the need to better understand differences in virulence and therapeutic options for infections caused by this emerging pathogen. The majority of isolates in the Pacific Northwest have been of the VGII molecular type (21), which has been found to be more virulent than other cryptococcal molecular type (10, 11) and less susceptible to the triazoles (22, 23). More-recent evidence has suggested that additional *C. gattii* molecular types and subtypes may also be prevalent, may be associated with substantial morbidity (4), and may represent a previously unrecognized and ongoing epidemic (6, 24, 25). Furthermore, it has been proposed that *C. gattii* and *C. neoformans* infections represent distinct clinical syndromes (24, 26), and regimens for treatment of *C. gattii* infections longer than those used in *C. neoformans* infections have demonstrated improved outcomes (27).

The mechanisms responsible for these clinical differences remain incompletely defined. Immunopathology, tissue tropism (26), and the patterns of cytokine and chemokine induction might differ between *C. gattii* and *C. neoformans* (28, 29), and yet the expression of virulence factors responsible for species-specific differences has not previously been characterized. Prior virulence work, conducted primarily in *C. neoformans*, was performed using isogenic gene disruption and reconstitution strains or by comparing strains that differ in the expression of the phenotype of interest. These studies are essential, and yet they represent a reductionist approach (30), and it is difficult to extrapolate results from these tightly controlled studies to clinical isolates where multiple virulence determinants are expressed in various quantities in a coordinated and potentially host-dependent fashion (31).

In our *Drosophila* model of cryptococcal infection, we observed variation in the virulence of isolates within each molecular type, reconfirming the need to evaluate numerous isolates from environmental, veterinary, and human sources. Overall, VGIII isolates were the most virulent, followed by VGIIa, VGIIc, VNI, and VGIIb isolates. These findings are consistent with prior reports using murine models such as the enhanced virulence of VGIIa over VGIIb isolates (11, 32). No mechanism has been proposed for molecular-type-specific virulence differences, and without a known need for a host, *Cryptococcus* spp. have been termed an "accidental pathogen" in humans (30). As such, ecologic factors have likely played a dominant role in molding these different species over time into the disparate organisms we now face. An improved understanding of these differences in virulence that may relate to morbidity and mortality is therefore of paramount importance (33).

Among these ecologic factors, competition with other organisms and their byproducts is likely intermittent, and yet environmental temperature is an ever-present constraint on and requirement for the ability of *Cryptococcus* to grow and reproduce. It is therefore not surprising that enhanced growth at different temperatures was a dominant factor in predicting fly death in our model. This is consistent with the results of prior studies, with growth at body temperature found to be highly correlated with infection (30, 34), and the growth of VGIII isolates at 30°C similarly plays a significant role in *Drosophila*. Further work in other models at different temperatures using these same isolates should provide additional insight.

Few differences in melanin production were seen in our study; at 30°C, however, VNI isolates in dopamine-containing media produced significantly more melanin than the majority of *C. gattii* molecular types and subtypes. The reasons for this are unclear; however, *C. neoformans* is frequently found in pigeon guano, while *C. gattii* is not typically isolated from this source, and pigeon guano has thus been called a "realized ecological niche" for *C. neoformans* (33). Melanin production in this specific environment may provide a competitive advantage to *C. neoformans*, and yet the dependence on temperature for virulence factor production and regulation again seems to be the driving factor (35). Furthermore, as melanin production can protect *Cryptococcus* from a myriad of external insults, including antifungal compounds and xenobiotics, oxidants, UV light, macrophages, and extremes in temperature, this trait is likely to have been selected for environmental survival (36, 37) and thus may account for preferential expression at 30°C rather than 37°C for *C. neoformans*.

Differences in capsule size were also observed in our study and showed significant variation among species, with *C. gattii* isolates producing capsules larger than those of *C. neoformans*. Despite prior reports demonstrating the importance of capsule formation as a protection against fungal recognition and subsequent phagocytosis and as a "sink" for reactive oxygen species (ROS) (38, 39), capsule size did not predict virulence in our model of infection, and yet the larger capsules we noted in *C. gattii* isolates may have direct clinical relevance. Mitchell et al. reported higher serum cryptococcal antigen (CRAG) titers in *C. gattii*-infected patients than in those infected with *C. neoformans*, and this difference was apparent despite the lower incidence of positive cerebrospinal fluid (CSF) cultures in patients with *C. gattii* (78% versus 100%) (40). Animal models have similarly shown that, at the same inoculum of *C. gattii* or *C. neoformans*, CRAG titers are significantly higher in *C. gattii*-infected mice despite similar and, often, significantly lower organ burdens at the end of study (8)—a mechanism potentially explained by our observation that the capsules in *C. gattii* strains are larger than the capsules in *C. neoformans* strains.

Multiple pathways within *Cryptococcus* spp. augment the protective effect of cryptococcal antigen against reactive oxygen species (ROS), suggesting that defense against oxidative damage is critical for survival. We observed few differences between cryptococcal species and molecular types in relation to hydrogen peroxide tolerance, with the exception of VNI strains at H_2O_2 concentrations exceeding those found in phagocytic cell phagosomes (low micromolar range) (41). ROS-induced damage to fungal DNA, lipids, and proteins is lethal to a number of fungal organisms (42) and suggests that these mechanisms may be conserved and fundamental to fungal survival following phagocytosis by either the host or environmental amoebae (43). If these mechanisms are uniformly essential for survival, it is therefore not surprising that no differences were observed.

Susceptibility results found in our study confirm those previously reported with respect to higher fluconazole MICs observed in VGII isolates, particularly those of VGIIc (22). The mechanism behind the elevated fluconazole MICs in these isolates has yet to be elucidated. Investigations of ERG11 have found that neither overexpression nor variations within ERG11 coding play a significant role in the elevated MICs found in Pacific Northwest isolates (44), and it is thus likely that efflux pump overexpression is the pre-

dominant mechanism. Overexpression (or the development of heteroresistance in the environment) may occur due to an as-yet-unknown unique environmental pressure from xenobiotics.

It is possible our *in vitro* results failed to find phenotypic differences that may in fact exist *in vivo*. For example, recent studies have found that *Candida albicans* and *Candida glabrata* actively suppress ROS production in phagocytes (45) and our study assessed resistance to exogenous administration of H_2O_2 and not whole phagocytes. The infection of macrophages by *Cryptococcus* spp. has also been shown to induce the formation of vesicles containing virulence-associated enzymes, including laccase, urease, and phosphatase, and can lead to fungal melanization (46, 47). Thus, *in vivo* virulence factor production may differ from even the optimal conditions used for comparative analysis in our study.

This is the first study to have established a correlation between cryptococcal molecular type and virulence in an invertebrate model of infection. The increased recognition of *C. gattii* infections across the globe accentuates the need to more completely define the epidemiology, susceptibility, and virulence of these organisms, and these differences suggest that identification of all cryptococcal isolates to the species level should be performed on a routine basis. Further work, including validation of molecular-type-specific virulence in murine models of infection, whole-genome evaluation of these organisms, and quantitation of additional virulence factors and their contribution to the predictive model, will need to be undertaken.

In summary, we have conducted a comprehensive assessment of *C. gattii* molecular type differences through *in vitro* testing and an invertebrate model of infection and found VGIII the most virulent molecular type assessed followed by VGIIa and VGIIc, with temperature the dominant influence on virulence. The emergence of these types within discrete geographic regions and observed virulence differences may have implications for treatment and outcomes for patients affected by this emerging pathogen.

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We declare that we have no conflicts of interest.

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