Melanin production at 37°C is linked to the high virulent *Cryptococcus gattii* Vancouver Island outbreak genotype VGIIa

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Abstract

Cryptococcus neoformans and *Cryptococcus gattii*, are the most common causes of fungal infection in the central nervous system of humans and other mammals. *C. neoformans* causes disease most frequently in immunocompromised hosts whilst *C. gattii* is a primary pathogen infecting mainly immunocompetent hosts. *C. gattii* has gained particular importance since it was identified as the cause of an outbreak on Vancouver Island, BC, Canada in 1999. M13 PCR fingerprinting, *URA5*-RFLP and AFLP analysis identified the outbreak isolates as molecular type VGII/AFLP6 and recognizing two subtypes, VGIIa and VGIIb; with the former being more virulent in a mice virulence model. However, the correlation of the molecular subtype with virulence has not yet been defined. From the three major cryptococcal virulence factors, polysaccharide capsule, growth at 37°C and melanin production, the melanin production of 23 VGIIa and 17 VGIIb globally selected strains was quantified using dopamine or epinephrine as a substrate in a colorimetric assay. A mixed VGIIa/VGIIb population has until now only been found on Vancouver Island, supporting the theory, that the two outbreak genotypes were independently introduced to Vancouver Island. At 37°C, VGIIa strains produced more melanin (OD VGIIa/VGIIb = 0.25 ± 0.18/0.14 ± 0.2, $p < 0.001$) than VGIIb strains, which is the opposite to the results obtained when tested at 30°C (OD VGIIa/VGIIb = 0.62 ± 0.42/0.80 ± 0.46, $p < 0.014$) using dopamine as a substrate. The results indicate that VGIIa strains have a greater ability to produce melanin at 37°C, offering more protection, making those strains potentially more virulent when infecting mammalian hosts. Further studies of the correlation between melanin production, molecular subtype of *C. gattii* and *in vivo* virulence in animal models are warranted.

Introduction

The *Cryptococcus* species complex, is composed of two basidiomycetous yeast species, *Cryptococcus neoformans* (Benham 1956) and *Cryptococcus gattii* (Kwon-Chung *et al.* 2002). These yeasts have long been known as the cause of the most common fungal infections of the central nervous system in humans and other mammals (Casadevall & Perfect 1998). Based on molecular, epidemiological and clinical differences, *C. gattii* was recently raised to species level (Kwon-Chung *et al.* 2002; Kwon-Chung & Varma 2006). *C. neoformans* is an opportunistic pathogen, causing infections mainly in immunosuppressed hosts worldwide (Casadevall & Perfect 1998), compared to *C. gattii*, which is a primary pathogen, infecting mainly immunocompetent hosts and was till recently thought to be restricted to tropical and subtropical regions (Sorrell 2001).

Numerous molecular typing techniques have been used to study the genetic diversity within the *Cryptococcus* species complex, among them, M13 PCR fingerprinting, Restriction Fragment Length Polymorphism (RFLP) analysis of the orotidine monophosphate pyrophosphorylase (*URA5*) and Amplified Fragment Length Polymorphism (AFLP) analysis recognized eight major molecular types within the *Cryptococcus* species complex: VNI/VNB and VNII for *C. neoformans* var. *grubii*, serotype A; VNIV for *C. neoformans* var. *neoformans*, the serotype D; VNIII for the hybrid serotype AD, and VGI, VGII, VGIII and VGIV for *C. gattii*, serotypes B and C (Boekhout *et al.* 2001; Litvintseva *et al.* 2006; Meyer *et al.* 2003).

Three major virulence factors have been shown to be essential for the pathogenesis of members of the *Cryptococcus* species complex. These include melanin synthesis, capsule production and the ability to grow at 37ºC (Casadevall & Perfect 1998). Melanin and capsule production are stress protectants, including the human immune system, for the members of the *Cryptococcus* species complex. In addition, growth at 37ºC is critical for the yeasts to grow at mammalian physiological temperature. Over the last few years, several additional virulence factors have been discovered, those include, phospholipase B (Chen *et al.* 1997; Cox *et al.* 2001), calcineurin (Odom *et al.* 1997), trehalose synthases (Petzold *et al.* 2006), superoxide dismutase (Cox *et al.* 2003), adenyl cyclases (Alspaugh *et al.* 2002) and mitogen-activated protein kinases (Kraus *et al.* 2003).

Melanin — a major cryptococcal virulence factor—Most cryptococcal isolates can metabolise diphenolic compounds to form melanin (Williamson 1997). However, the members of the *Cryptococcus* species complex are not the only species in the genus *Cryptococcus,* which can produce melanin. *C. albidus*, *C. laurentii* and *C. curvatus* were reported of having also the ability to produce melanin (Ikeda *et al.* 2002). Unlike other fungi that produce melanin, melanin production in *Cryptococcus* spp. needs exogenous sources of dephenolic compounds and catecholamines, such as 3-4 dihydroxyphenyl alanine (DOPA), norepinephrine, epinephrine and dopamine (which function as neurotransmitters in the CNS) (Williamson 1997). Indeed, the neurotropism of the members of the *Cryptococcus* species complex is attributed to the availability of diphenolic compounds, such as L-3,4-

Table 1 *C. gattii* strains representing each of the two Vancouver Island outbreak genotypes VGIIa and VGIIb examined in this study.

Strain Name/No.	Mating type	Country	Source	Sub-type	Reference
129A-1	alpha	Canada	Environment	VGIIa	Kidd *et al.* 2004
152A-6	alpha	Canada	Environment	VGIIb	Kidd *et al.* 2004
47-5061	alpha	Thailand	Clinical	VGIIb*	This study
ARN001	alpha	Australia	Environment	VGIIb*	Chen *et al.* 2000
Bandiaga	alpha	Australia	Clinical	VGIIb*	Chen *et al.* 2000
CBS7750	alpha	USA	Environment	VGIIa	Kidd *et al.* 2005
DMST20765	alpha	Thailand	Clinical	VGIIb*	Poonwan *et al.* 1997
DMST20767	alpha	Thailand	Clinical	VGIIb*	Poonwan *et al.* 1997
E113	alpha	Canada	Environment	VGIIa	Kidd *et al.* 2004
ENV129	alpha	Canada	Environment	VGIIb	Kidd *et al.* 2004
F1623	alpha	Brazil/Japan**	Clinical	VGIIa	This study
F2596	alpha	Canada	Veterinary	VGIIa	Kidd *et al.* 2004
F3179	alpha	Canada	Clinical	VGIIa	Kidd *et al.* 2004
LA295	alpha	Argentina	Clinical	VGIIa*	Meyer *et al.* 2003
McBride	alpha	Australia	Veterinary	VGIIb*	Sorrell *et al.* 1996
NIH444	alpha	USA	Clinical	VGIIa	Kwon-Chung 1976
R265	alpha	Canada	Clinical	VGIIa	Kidd *et al.* 2004
R272	alpha	Canada	Clinical	VGIIb	Kidd *et al.* 2004
R273	alpha	Canada	Clinical	VGIIa	Kidd *et al.* 2004
R360	alpha	Canada	Clinical	VGIIa	Kidd *et al.* 2004
R368	alpha	Canada	Clinical	VGIIa	Kidd *et al.* 2004
R406	alpha	Canada	Clinical	VGIIa	Kidd *et al.* 2004
R507	alpha	Canada	Clinical	VGIIa	Kidd *et al.* 2004
R540	alpha	Canada	Clinical	VGIIa	Kidd *et al.* 2004
R634	alpha	Canada	Clinical	VGIIa	Kidd *et al.* 2004
RAM002	alpha	Australia	Environment	VGIIb*	Chen *et al.* 2000
RAM005	alpha	Australia	Environment	VGIIb	Chen *et al.* 2000
RAM015	alpha	Australia	Environment	VGIIb*	Chen *et al.* 2000
RB14	alpha	Canada	Environment	VGIIa	Kidd *et al.* 2004
RB15	alpha	Canada	Environment	VGIIa	Kidd *et al.* 2004
RB17	alpha	Canada	Environment	VGIIa	Kidd *et al.* 2004
RB19	alpha	Canada	Environment	VGIIa	Kidd *et al.* 2004
RB28	alpha	Canada	Environment	VGIIb	Kidd *et al.* 2004
RB31	alpha	Canada	Environment	VGIIb	Kidd *et al.* 2004
RB39	alpha	Canada	Environment	VGIIa	Kidd *et al.* 2004
RB45	alpha	Canada	Environment	VGIIa	Kidd *et al.* 2004
RB52	alpha	Canada	Environment	VGIIb	Kidd *et al.* 2004
RB57	alpha	Canada	Environment	VGIIb	Kidd *et al.* 2004
RB59	alpha	Canada	Environment	VGIIa	Kidd *et al.* 2004
RB67	alpha	Canada	Environment	VGIIb	Kidd *et al.* 2004

* = VGII subtype was designated by MLST (Meyer W, unpublished data), ** = Isolated from a Brazilian patient living in Japan

dihydrophenylalanine (L-DOPA), in the brain, which can be used as substrates for melaninogenesis (Polacheck *et al.* 1982). Areas of the brain that are rich in catecholamines e.g., the basal ganglia, are most often affected (Casadevall *et al.* 2000). Further evidence comes from the fact that in the absence of the glucose, laccase production is up-regulated, which may contribute to the neurotropism of *Cryptococcus* spp. due to the low glucose concentrations in the CNS (Polacheck *et al.* 1982). The initial step of melanogenesis is the oxidation

of DOPA by a phenol oxidase (Williamson 1997) followed by spontaneous polymerization to melanin (Casadevall *et al.* 2000). Melanins are produced in the cytoplasm and subsequently deposited in the cell wall (Wang *et al.* 1996). The phenol oxidase of *Cryptococcus* spp. is a laccase (Williamson 1994) and is cell wall-associated (Zhu *et al*. 2001).

The first recognition of melanin as a virulence factor of *Cryptococcus* came from classical genetic studies, in which where melanin mutants (Mel$^-$) were less virulent than the wild-type strains (Mel$^+$) in an animal model (Kwon-Chung *et al.* 1992). This was followed by the discovery of the laccase gene (*CNLAC1*) (Williamson 1994). The *CNLAC1* mutant, in which the laccase gene was disrupted via homologous recombination, lost its melanin synthesis ability and was less virulent than the wild-type strain in mice, despite causing morbidity and mortality in animals in the long term (Salas *et al.* 1996). Subsequently, the second laccase gene, *LAC2*, were identified and proved to have a minimal impact on the melanin production in *Cryptococcus* (Pukkila-Worley *et al.* 2005). In fact several genes other than the laccase gene are known to be involved in melanization of cryptococcal *cells,* these include genes in the protein kinase C (PKC) signalling pathway (Gerik *et al.* 2005), copper transporter (*CCC2*), the copper chaperone (*ATX1*), the chitin synthase (*CHS3*), the transcriptional coactivator (*MBF1*) and the chromatin-remodelling enzyme (*SNF5*) (Walton *et al.* 2005). Additional studies proved that melanin is a virulence factor, by showing that mice can survive longer when treated with an inhibitor of the melanin production, glyphosate (Nosanchuk *et al.* 2001) and when immunized with a melanin-binding monoclonal antibody (Rosas *et al.* 2001).

Melanin has been thought to have a protective ability against both nitrogen and oxygen derived oxidants (Wang *et al.* 1995), to impede phagocytosis (Wang *et al.* 1995), to shield against microbicidal peptides of the defensin, protegrin and magainin families and to down-regulate the afferent phase of the T-cell mediated inflammatory response (Huffnagle *et al.* 1995). Melanin may also play a role in maintaining the cell wall structure, protecting cryptococcal cells from UV light (Wang & Casadevall 1994b), X-rays or gamma radiation (Polak 1990), heat and cold (Rosas & Casadevall 1997) and Amphotericin B (Wang & Casadevall 1994a). When cultured in pigeon excreta, melanization was observed (Nosanchuk *et al.* 1999a). Both melanin and capsule are responsible for the overall negative charge of cryptococcal cells, which impede phagocytosis by macrophages (Nosanchuk & Casadevall 1997).

Though many reports have proven that melanin is a virulence factor, some reports have questioned the production of melanin *in vivo*. Phenoloxidase activity has been shown to be decreased at physiological temperature at 37°C (Jacobson & Emery 1991), while anothers reported an optimal activity of the enzyme at this temperature (Polacheck *et al.* 1982). An additional study concluded, that cryptococcal melanin may not be synthesized *in vivo* and, thus, is not involved in virulence (Jacobson & Emery 1991). One study detected degradation products of catecholamines, but failed to detect melanin in mice (Liu *et al.* 1999). However, evidence that polymerized melanin is produced *in vivo,* has been shown by using melanin-binding peptides and antibodies to melanin (Nosanchuk *et al.* 1999b). Monoclonal antibodies to melanin enhanced the protective immunity in mice challenged with wild-type cells and prolonged survival (Rosas *et al.* 2001). Moreover, melanin "ghosts" were recovered from infected tissues and tissue homogenates, providing further evidence for melanin synthesis in the lung and brain (Nosanchuk *et al.* 2000).

In vitro melanin production of cryptococcal strains can be induced when grown on agar containing diphenolic compound either dopamine (Fig. 1A), caffeic acid (Fig. 2B) or niger seed (*Guizotia abyssinica*) extract (Fig. 1C) (Casadevall *et al.* 2000). Quantification of melanin production was firstly described in 1994 by Williamson *et al.* as a laccase activity test (Williamson 1994), However, the method was complicated and labor intensive thus a simplified quantification method was proposed in 2005 (Pukkila-Worley *et al.* 2005) by measuring the optical density from the cell supernatant when the yeast is grown in melanin inducing media.

Since the recent outbreak of cryptococcosis on Vancouver Island, British Columbia, Canada in 1999, due to *C. gattii*, the importance of this species as a primary pathogen has drastically increased (Kidd *et al.* 2004). *C. gattii*, which was till now believed to be restricted to tropical and subtropical climates, has since expanded his ecological niche into temperate regions and has been found in large numbers not only on Vancouver Island, BC, Canada but also in high altitude regions in Columbia (Escandon *et al.* 2006; Kidd *et al.* 2004). Unlike elsewhere the Vancouver Island outbreak isolates were found to belong to molecular type VGII, with two subtypes being identified VGIIa (90% of all strains) and VGIIb (10% of all strains) (Kidd *et al.* 2004). Fraser *et al.* 2005 have further shown using a immunosuppressed A/Jcr mice model that a representative strain of the major genotype VGIIa, strain R265, was more virulent than a representative strain of the minorgenotype VGIIb, strain R272. However, this study was based on only a single isolate of each subtype. In order to verify these limited findings, the current study was undertaken, focusing on the ability to synthesize melanin, a major virulence factor of the *Cryptococcus* species complex, as an indicator of differences in virulence in a global selection of strains representing both Vancouver Island outbreak genotypes.

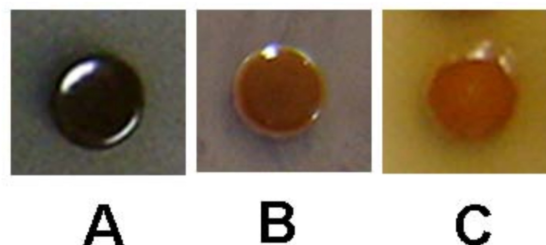

Fig. 1 Melanin production of *C. gattii*, strain R265, on different sources of diphenol compounds; A) dopamine, B) caffeic acid and C) extract from Niger seed (*Guizotia abyssinica*).

Materials and Methods

Strains and media—Forty VGII strains from a wide range of geographic locations were studied. All strains were obtained

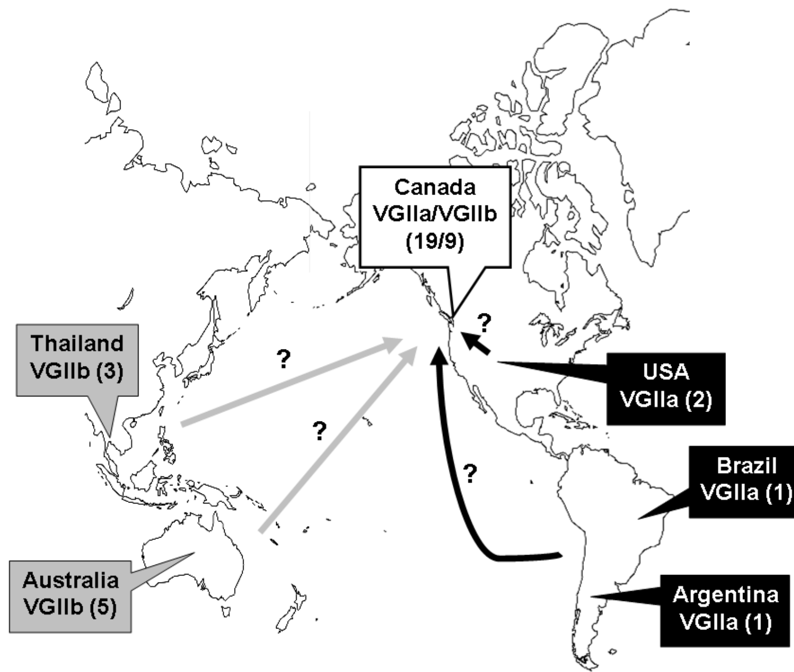

Fig. 2 Global distribution of VGIIa and VGIIb subtypes studied.

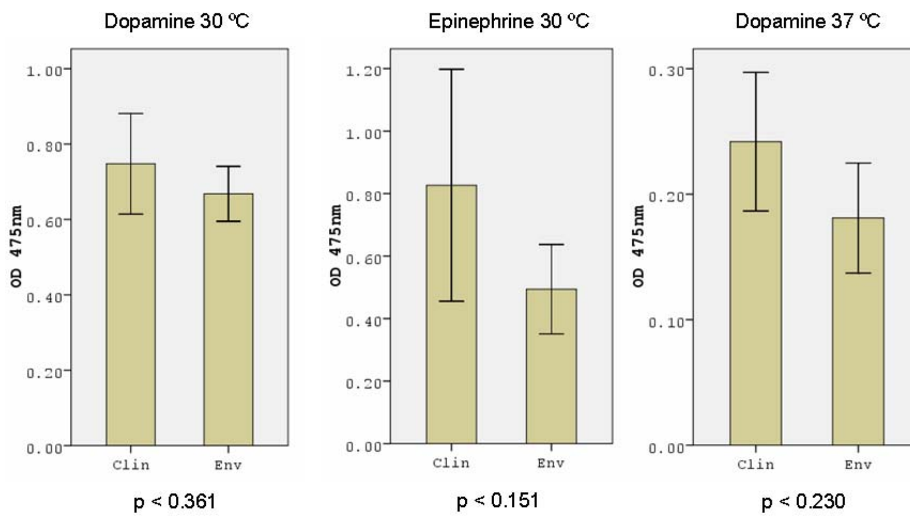

Fig. 3 Melanin assay comparing clinical (Clin) and environmental (Env) isolates under three different test conditions. Veterinary isolates were excluded from the analysis due to the low numbers. No statistically significant differences were observed. (Error bars = ± 2 standard errors).

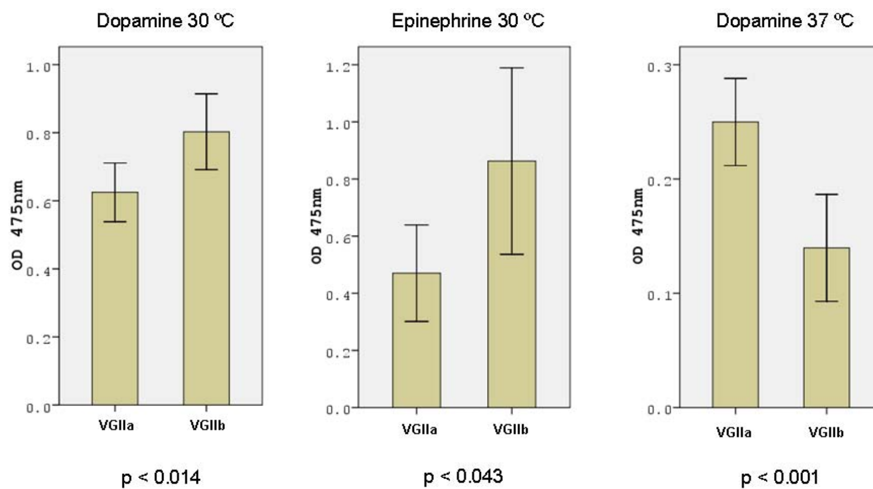

Fig. 4 Melanin quantification tests carried out at 30°C ad 37°C in two different melanin-inducing media. revealed opposite results in 30°C comparing and 37°C for dopamine. Statistically significant differences were observed in all conditions shown. (Error bars = ± 2 standard errors).

from the Molecular Mycology Research Laboratory Culture Collection at Westmead Hospital, University of Sydney, Australia (Table 1). All isolates were grown on Saboraud's Dextrose Agar (2% glucose, 2% peptone and 2% agar) for 2-3 days at 30°C before conducting the melanin quantification assay.

Determination of the VGII subtypes—The VGII subtype of each strain was designated according to the results obtained previously, using either M13 fingerprinting or Multi-Locus Sequence Typing (MLST) (Fraser *et al.* 2005; Kidd *et al.* 2004; Kidd *et al.* 2005; Meyer *et al*. unpublished data).

Melanin quantification assay—The modified laccase activity test was performed as previously described (Pukkila-Worley *et al.* 2005) with minor modifications. Briefly, each cryptococcal strain was grown in YPD broth (2% glucose, 2% peptone and 1% yeast extract) to saturation for 24 hr at 30°C. 100 µl (~ 10^7 CFU) of the saturated culture were then added in 10 ml of melanin induction media (1% glucose YNB without amino acid and ammonium sulfate) containing either 10 mM dopamine or 10 mM epinephrine and incubated at 30 ºC. Concentration of the initial culture of each strain were determined conducting plate counts. Supernatants of the culture were taken at 48 hr and their optical density (OD) at 475 nm was measured. To determine the effect of the growth temperature on melanin production the melanin assay was also carried out at 37°C and the OD was measured as described above after 48 hr incubation.

Statistical analysis—Statistical analysis of quantitative melanin production was undertaken based on comparison of the OD values obtained for the isolates using SPSS software version 15.0.0 (LEAD Technologies, Inc., IL, USA) using t-test. Fisher's exact test was used for the cross tab data. Significance was defined at a p value of < 0.05.

Results

Demographics of the studied strains—Of the 40 strains studied, 23 strains represented the major Vancouver Island genotype VGIIa and 17 strains represented the minor Vancouver Island genotype VGIIb. VGIIa strains were obtained from Argentina, Brazil, Canada and the USA, while VGIIb strains were obtained from Australia, Canada and Thailand (Fig. 2). Isolates of both subtypes form the same location were only obtained from Vancouver Island, BC, Canada.

Of the 40 isolates studied, 17, 2 and 21 strains were isolated from clinical, veterinary and environmental samples, respectively. No significant differences in the proportion of the molecular subtypes, VGIIa and VGIIb, were observed according to the different sources of the isolates (p < 0.383). All isolates were mating type alpha (Tables 1 and 2) as previously identified (Ngamskulrungroj *et al.* 2008).

Table 2 Source of strains

Source	VGIIa	VGIIb
Clinical	12 (75%)	4 (25%)
Veterinary	1 (50%)	1 (50%)
Environmental	10 (45%)	12 (55%)
Total	*23*	*17*

Note: p value < 0.383

Melanin quantification—The melanin production of clinical and environmental isolates, as measured by OD at 475 nm, was similar for all three melanin-inducing media used (p < 0.361, 0.151 and 0.230 in 30ºC dopamine-, 30ºC epinephrine- and 37ºC dopamine-containing media, respectively) (Fig. 3). At 30ºC, VGIIb strains produced more melanin than the VGIIa strains in both dopamine- (OD VGIIa/VGIIb = 0.62 ± 0.42/0.80 ± 0.46, p < 0.014) and epinephrine-containing media (OD VGIIa/VGIIb = 0.47 ± 0.8/0.86 ± 1.34, p < 0.043). Interestingly, this trend was reversed when the strains were grown at 37°C (OD VGIIa/VGIIb = 0.25±0.18/0.14 ± 0.2, p < 0.001), with the VGIIa strains now producing more melanin than the VGIIb strains (Fig. 4).

Discussion

Although the Vancouver Island outbreak has been extensively studied, the origin of the outbreak is still debatable. Several theories have been proposed, including the introduction of two independent clonal lineages (Kidd *et al.* 2005) and same sex mating (Fraser *et al.* 2005). Based on the fact that only one of the two Vancouver Island outbreak subtype was found in each of the studied counties, with the exception of the Vancouver Island outbreak area itself, our findings support the theory, that both subtypes evolved independently and than spread to the outbreak area (Fig. 2).

The herein reported melanin studies revealed initially, that the VGIIb strains produced more melanin when grown at 30°C compared to the VGIIa strains, suggesting a higher virulence of the VGIIb strains, which is in opposition to the previously reported findings (Fraser *et al.* 2005), which had reported that strain R265, representing the major Vancouver Island genotype VGIIa, had a higher virulence than strain R272, representing the minor Vancouver Island genotype, VGIIb. However, when the experiments were repeated at human physiological temperature, 37°C, our results confirmed the previously published findings. The VGIIa strains produced more melanin at 37°C than the VGIIb strains. The herein reported melanin studies confirmed, based on a larger and more global selection of strains that VGIIa strains produce more melanin and hence may be more virulent than the VGIIb strains, which produce less melanin. The higher production of melanin *in vitro* by the VGIIa strains was related to the change from 30 to 37°C, which was not the case for the VGIIb strains. The different expression of melanin may suggest different adaptive strategies of each genotype (VGIIa and VGIIb) to survive and reproduce in higher temperatures and may be related to different survival advantages during the mammalian infection as has previously been suggested as a general concept for *Cryptococcus neoformans* (Casadevall *et al.* 2003).

However, similar levels of melanin production were found in clinical, veterinary and environmental strains, showing that the environmental sources are good reservoirs of the infectious propagules of these yeasts, which are then inhaled as has been suggested as mode of infection (Casadevall & Perfect 1998). This suggests that both genotypes have the same ability to be present in the environment and subsequently to cause disease in humans and other mammals. If this is the case, why is it not like this? The herein described findings suggest that the VGIIa strains have a better protection against the host immune response since they have a greater ability to produce melanin at human physiological temperature,

which may count for the greater prevalence of VGIIa in human and other mammalian infections.

In conclusion, the current study has for the first time given broader indirect evidence, based on the analysis of one of the three major cryptococcal virulence factors, the melanin production from a large globally collected set of strains, that the major Vancouver Island outbreak genotype, VGIIa, produces more melanin at physiological temperature, which contributes to the fact that those strains are more virulent than the ones of the minor Vancouver Island outbreak genotype, VGIIb. It is now warranted to expend those studies and to conduct virulence experiments *in vivo*.

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