Microbiological characteristics of clinical isolates of *Cryptococcus neoformans* in Taiwan: serotypes, mating types, molecular types, virulence factors, and antifungal susceptibility

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Abstract

This study investigated the microbiological characteristics of 100 clinical isolates of *Cryptococcus neoformans* species complex, including serotypes, mating types, molecular types, antifungal susceptibility and virulence. The isolates were collected at National Taiwan University Hospital from 1999 to 2004. Eight isolates of *C. neoformans* from pigeon droppings were also evaluated. Among these isolates, 99 were *C. neoformans* var. *grubii* serotype A and one was *C. neoformans* var. *gattii* serotype B. All of these isolates were α mating types. PCR fingerprinting, generated by primers M13 and $(GACA)_4$, and *URA5* gene restriction fragment length polymorphism analysis revealed that *C. neoformans* var. *grubii* isolates belonged to the VNI (98 isolates) and the VNII (one isolate) types, and the single *C. neoformans* var. *gattii* was VGI type. The similar profiles of clinical and environmental isolates suggest that patients might acquire these yeasts from the environment. The MIC_{90} for fluconazole, itraconazole, 5-flucytosine, voriconazole and amphotericin B against all *C. neoformans* isolates were 8, 0.5, 4, 0.125 and 0.5 mg/L, respectively. All clinical isolates produced urease, phospholipase, capsule and melanin, but these activities varied with individual isolates. Analysis of six clinical and two environmental isolates with various levels of phospholipase activity indicated a correlation between phospholipase activity and the ability to adhere to the lung epithelial cell line, A549. The extent of cell damage, as indicated by lactate dehydrogenase release, also paralleled the phospholipase activity of these isolates. In addition, production of melanin contributed significant protection against amphotericin B killing of the isolates tested.

Keywords: Antifungal susceptibility, *Cryptococcus neoformans*, mating types, molecular types, serotypes, virulence
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Introduction

Cryptococcus neoformans is an encapsulated, ubiquitous environmental yeast that causes cryptococcosis, a potentially serious disease that affects healthy and immunocompromised individuals, especially patients with AIDS [1]. The aetiological agent of cryptococcosis is classified into two species [2]; they are *C. gattii* (serotypes B and C) and *C. neoformans*, with two varieties: *C. neoformans* var. *grubii* (serotype A) [3] and *C. neoformans* var. *neoformans* (serotype D) [4,5] as well as an AD hybrid [1]. These species and varieties may have different ecological characteristics, as well as a different epidemiology and pathogenicity [6–8].

Cryptococcus neoformans is a heterothallic basidiomycetous yeast with a sexual cycle involving mating between haploid MATa and MATα cells [4,9]. MATα strains have been shown to be much more common and more virulent than congenic MATa strains [10,11].

PCR fingerprinting based on M13 DNA has identified two major genotypes (VNI and VNII) among the strains of sero-

type A, the VNI genotypes being the most commonly found worldwide [12,13].

Amphotericin B, alone or combined with 5-flucytosine and azoles, including fluconazole, itraconazole or voriconazole, has been the treatment of choice for cryptococcal infections, although alternative agents with activities against *C. neoformans* have recently become available [14]. Periodic surveillance in Taiwan hospitals is needed to monitor trends of resistance to commonly used antifungal agents including amphotericin B and azoles within *C. neoformans* [15,16].

Several factors have been identified that contribute to the virulence of *C. neoformans* strains. Among the best characterized are the presence of a capsule, and the production of melanin, urease and phospholipase [1,17–19]. Studies of cryptococcosis in animal models infected with different strains of *C. neoformans* have indicated that there is considerable variation in the virulence of individual isolates [20,21]. Blackstock *et al.* [21] showed that the more virulent isolates had higher expression of several virulence factors. Although secretory phospholipase has been demonstrated to be a virulence factor for *C. neoformans* [19,22–24], no conclusive correlations were made between virulence and phospholipase activities in clinical isolates [5,20,25,26].

The purposes of this study were: (i) to identify the serotypes; (ii) to identify the mating type; (iii) to assess the antifungal susceptibility; (iv) to identify the genotypes; (v) to analyse virulence factors in *C. neoformans* isolates, and to assess the relative importance of particular virulence factors.

Materials and Methods

Isolates

One hundred randomly selected, consecutive, and non-duplicate clinical isolates from patients admitted to NTUH, a 2000-bed teaching hospital in northern Taiwan, and eight environmental isolates (E1-E8 from pigeon guano in Taipei) of *C. neoformans* collected between 1999 and 2004 were studied. All clinical isolates were recovered from patients with clinically significant infections, including meningitis, cryptococcaemia, and pneumonia. These isolates were identified to the species level by conventional methods, based on growth appearance on Sabouraud dextrose agar (SDA; Becton Dickinson, Franklin Lakes, NJ, USA) at 37°C, assimilation of carbohydrates, production of urease, and the presence of a capsule [27] and confirmed using the API 20C and Vitek YBC systems (bioMerieux Vitek, St Louis, MO, USA). L-canavanine-glycine-bromothymol blue (CGB) agar was used to screen for *C. gattii*. Reference strains, ATCC 90112 (serotype A), ATCC 32269 (serotype B), ATCC 34873 (serotype D), ATCC 32608 (serotype C), H99 (serotype A, α mating type) [11] and KN99a (serotype A, a mating type) [11] were included in individual assays for comparison, when needed.

DNA isolation

Genomic DNA was prepared using a commercial kit (PureGene Yeast and Gram Positive DNA Isolation Kit; Minneapolis, MN, USA) following the manufacturer's protocol [28].

Molecular identification of serotypes

CAP59 gene restriction enzyme analysis (*CAP59*-REA) [29] and multiplex PCR using six primers for the *LAC1* gene and the *CAP64* gene [30] were performed to differentiate the serotypes of *C. neoformans*.

Determination of mating types by PCR

PCR using primer pairs, specific for mating type α (MATα1–MATα2) and a (MATa1–MATa2), respectively, were performed [5]. *Cryptococcus neoformans* H99 (serotype A, MATα) and *C. neoformans* KN99a (serotype A, MATa) were used as positive controls.

Genotyping by PCR fingerprinting and *URA5* gene restriction fragment length polymorphism analysis

Oligonucleotides of the minisatellite-specific sequence of phage M13 and of the microsatellite-specific sequence $(GACA)_4$ were used as single primers in the PCR fingerprinting reactions [13]. *URA5* gene restriction fragment length polymorphism (RFLP) analysis was performed as described [12].

Antifungal susceptibility testing

Antifungal susceptibility testing of *C. neoformans* isolates was performed according to the protocol described in the NCCLS document M27-A2 [31]. The following antifungal agents were used: fluconazole and voriconazole (Pfizer, Inc., New York, NY, USA), itraconazole (Janssen, Titusville, NJ, USA), flucytosine (Sigma Chemical Co., St Louis, MO, USA), and amphotericin B (Bristol-Myers Squibb, Princeton, NJ, USA). The tested concentrations of these agents ranged from 0.03 to 64 μg/mL. *Cryptococcus neoformans* ATCC 90112 was used as the control.

In vitro melanin production

Cryptococcus neoformans cells were grown on SDA medium at 37°C for 2 days. Cell suspension was made equivalent to

4 McFarland standard scale (*c.* 5×10^6 cells/mL) and 3 μL was inoculated onto the minimal medium agar plates with the addition of 1 mM L-dopa [32]. After incubation at 37°C for 7 days, the extent of brown pigment formation in each spot was graded as 1+ to 4+ indicating the increased pigment production.

Urease activity

Cell suspension at McFarland 2 standard scale was prepared as described above. One hundred microlitres were inoculated onto the Christensen's agar slant to test for urease activity [18]. Activity was graded as 1+ to 3+ indicating the increased pink colour intensity after incubation at 37°C for 24 h.

Phospholipase activity

Each sample was examined for extracellular phospholipase production by the method of Chen *et al.* [25]. The ratio of the diameter of the colony to the total diameter of the colony plus precipitation zone (Pz) was measured as an index of phospholipase activity. A Pz value of 1.0 indicated that the test sample was phospholipase negative.

Adherence to A549 cells

Adhesion assays were performed as described [20] with some modifications. Cells of the A549 human lung epithelial line were grown to confluence (10^4 cells/well) in 96-well plates in RPMI 1640 medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS). A549 monolayers were washed with Hank's balanced salts (Gibco) containing 5% FBS (HBS–FBS). Yeast cells were grown overnight in Sabouraud Dextrose Broth at 37°C, washed with phosphate-buffered saline (PBS) and resuspended in RPMI 1640 medium to generate a ratio of 20 yeast cells per host cell. After co-incubation at 37°C in a CO_2 incubator for 6 h, non-adherent fungi were removed by washing with HBS–FBS. A549 cells were then trypsinized, lysed with water and the resulting suspension plated onto SDA plates. Colonies were counted after incubation for 48 h. Adherence was calculated as a percentage of the original inoculum recovered. All experiments were performed in triplicate sets and statistically analysed using Student's *t*-tests. For microscopic observation, after removal of non-adherent fungi by washing, the cells were trypsinized (0.05%), washed again with PBS, resuspended in HBS–FBS and cytospun onto slides [33]. Interaction between A549 cells and fungi was observed by microscopy after Gram staining. A549 cells were unable to kill cryptococci, which was concluded from the observation that no significant differences in the number of viable yeasts were found before and after exposure to A549 cells.

Analysis of host cell damage by lactate dehydrogenase assay

To evaluate host cell damage induced by the presence of *C. neoformans*, confluent A549 cells were incubated with cryptococci for 24 h under the conditions described above. After incubation, supernatants were collected, centrifuged for removal of yeast cells and assayed for the release of lactate dehydrogenase (LDH) by the LDH assay kit (Cyto-Tox 96® Non-radioactive Cytotoxicity Assay; Promega, Madison, WI, USA) [34]. All experiments were performed in triplicate sets and statistically analysed using Student's *t*-tests.

Amphotericin B killing assay

Melanization was induced by growing yeast cells on defined minimal medium agar plates with 1 mM L-dopa for 7 days as described above [35]. Melanized or non-melanized yeast cells were suspended in normal saline at a density of 2×10^3 cells/mL. Cell counts were determined with a haemocytometer. Microcentrifuge tubes containing 0.1-mL aliquots of an antifungal at ten times the final concentration were inoculated with 0.9 mL of the yeast suspensions. After incubation at 37°C for 2 h, aliquots were plated on SDA to determine their survival as measured by determination of the number of CFUs. The survival rate was compared with that of fungal cells incubated in PBS.

Results

Patients

Men predominated (male 74% vs. female 26%) among the 100 patients (Table 1). The majority of isolates were from cerebrospinal fluid (n = 43), followed by blood (n = 40) and pleural effusion (n = 6). Other specimens included bronchial washing (n = 2), bronchoalveolar lavage (n = 3), lung abscess (n = 1), sputum (n = 3), surgical wound (n = 1) and throat swab (n = 1). Forty-one patients had underlying diseases, including 27 patients with AIDS and 14 with other diseases, including acute lymphoblastic leukaemia (n = 1), adult T-cell leukaemia (n = 1), chronic lymphocytic leukaemia (n = 1), non-small cell lung cancer (n = 1), cervical cancer (n = 1), melanoma (n = 1), end-stage renal disease (n = 2), and systemic lupus erythematosus (n = 4).

Serotypes and genotypes

Among the 100 clinical isolates tested, 99 isolates were serotype A and one was serotype B (example profiles in Fig. 1a,b), which tested positive on the CGB plate. M13 PCR fingerprinting and *URA5*-RFLP typing were used to investigate

TABLE 1. Characteristics of *Cryptococcus neoformans* isolates tested in this study, including host patient, sources, serotypes, mating types, molecular types, melanin production, urease and phospholipase activities

	No. (%) of clinical isolates (*N* = 100)	No. (%) of environmental isolates (*N* = 8)
Gender		
Male	74 (74)	
Female	26 (26)	
Specimens		
Blood	40 (40)	
Bronchial washing	2 (2)	
Bronchoalveolar lavage	3 (3)	
Cerebrospinal fluid	43 (43)	
Lung abscess	1 (1)	
Pleural effusion	6 (6)	
Sputum	3 (3)	
Surgical wound	1 (1)	
Throat swab	1 (1)	
Pigeon droppings	0 (0)	8 (100)
Underlying diseases		
AIDS	27 (27)	
Others	14 (14)	
None	59 (59)	
Serotypes		
A	99 (99)	8 (100)
B	1 (1)	0 (0)
Mating types		
α	100 (100)	8 (100)
a	0 (0)	0 (0)
Molecular types		
VNI[a] (VN6[b])	98 (98)	8 (100)
VNII[a]	1 (1)	0 (0)
VGI[a]	1 (1)	0 (0)
Pz (phospholipase activity)		
0.26–0.5 (high)	59 (59)	0 (0)
0.51–0.75 (intermediate)	38 (38)	5 (62.5)
0.75–1 (low)	3 (3)	3 (37.5)
Melanin production		
4+	4 (4)	0 (0)
3+	7 (7)	0 (0)
2+	36 (36)	4 (50)
1+	53 (53)	4 (50)
Urease activity		
3+	23 (23)	0 (0)
2+	26 (26)	3 (37.5)
1+	51 (51)	5 (62.5)

[a]By PCR fingerprinting using M13 primer and URA5-RFLP.
[b]By PCR fingerprinting using $(GACA)_4$ primer.

the genetic diversity and relationships among the isolates. We found that the single var. *gattii* isolate (CB) belonged to the VGI genotype and the var. *grubii* serotype A isolates exhibited VNI (98 isolates) and VNII (one isolate, C9) patterns (example profiles in Fig. 2a,b). All eight environmental isolates were serotype A and belonged to VNI (example profiles in Figs 1a and 2a,c). Fig. 2c shows the representative $(GACA)_4$ fingerprinting of two clinical and four environmental isolates with the patterns of serotypes A, B, C and D reference strains shown in parallel.

Mating types

Direct PCR of MATa and MATα pheromones revealed that all clinical and environmental isolates were positive for MATα as indicated by a 101-bp PCR product.

Antifungal susceptibility testing

In vitro susceptibility testing of all clinical isolates of *C. neoformans* to amphotericin B, flucytosine, fluconazole, itraconazole and voriconazole was performed. The MIC_{90s} and MIC_{50s} for fluconazole, itraconazole, 5-flucytosine, voriconazole and amphotericin B were 8, 0.5, 4, 0.125 and 0.5 mg/L, and 2, 0.25, 1, 0.06 and 0.25 mg/L, respectively.

Virulence

We examined differences in the production of melanin and the activities of phospholipase and urease in these isolates. The expression of these three virulence traits was highly divergent among the isolates. Whereas 97 isolates expressed intermediate to high phospholipase activity, urease activities were low to intermediate in 77 isolates and the level of melanin production was low to intermediate in 89 isolates (Table 1). All isolates could produce capsules (data not shown), but there was no significant correlation of capsule size with any of the three virulence traits. The intermediate to high expression of phospholipase but not urease activity or melanin production among these clinical isolates implies that phospholipase may contribute to the virulence of these isolates. In order to investigate further the role of phospholipase in these isolates, we performed adhesion assays using the high and the low phospholipase-producing isolates (the growth rate of all isolates was comparable and RT-PCR was used to confirm the level of phospholipase expression). Fig. 3a shows that adherence of the low phospholipase-producing isolates to the A549 cells was about half that of the high phospholipase-producing isolates, whereas adhesion of the two environmental isolates was intermediate between the low and high phospholipase-producing isolates. Light microscopy of adhesion between *C. neoformans* and the A549 cells after trypsin treatment showed that more cells of the high phospholipase-producing isolate were associated with A539 cells compared with the low phospholipase-producing isolate (Fig. 3c), reconfirming the importance of phospholipase activity in adherence to the A549 cells. Interaction of high phospholipase-producing *C. neoformans* with A549 cells also resulted in significant A549 cell damage compared with the low phospholipase-producing isolates (Fig. 3b).

Amphotericin B killing assay of melanized and non-melanized cells

We evaluated the effect of melanin production on the survival of clinical *C. neoformans* isolates after amphotericin B exposure. As shown in Fig. 4, melanized cells were significantly less susceptible to amphotericin B than non-melanized cells at the drug concentrations of 0.5 and 0.25 mg/L.

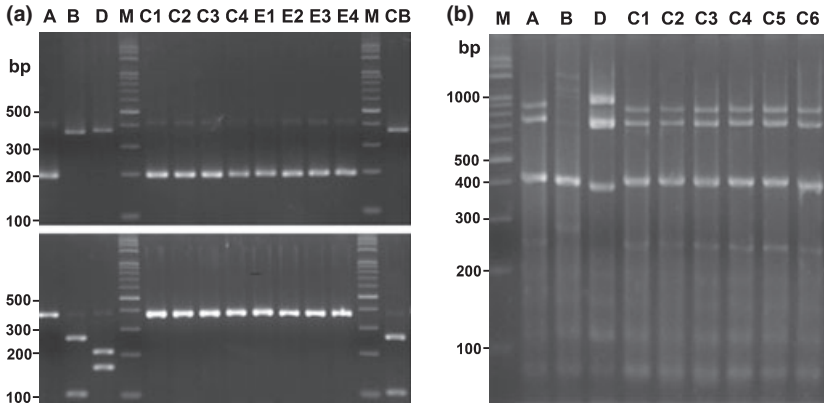

FIG. 1. (a) Agarose gel electrophoresis of the *CAP59* gene restriction profiles of *Cryptococcus neoformans* serotypes. Upper panel, *Bsm*FI-restricted profile; lower panel, *Hpa*II-restricted profile. C1–C4, serotype A clinical isolates; E1-E4, environmental isolates; CB, serotype B clinical isolate; A, ATCC 90112, serotype A; B, ATCC 32269, serotype B; D, ATCC 34873, serotype D; M, 100-bp marker. (b) Amplified fragments of isolates obtained in multiplex PCR with the primers for *LAC1* and *CAP64*. C1-C6, serotype A clinical isolates; A, B, D and M are as in (a).

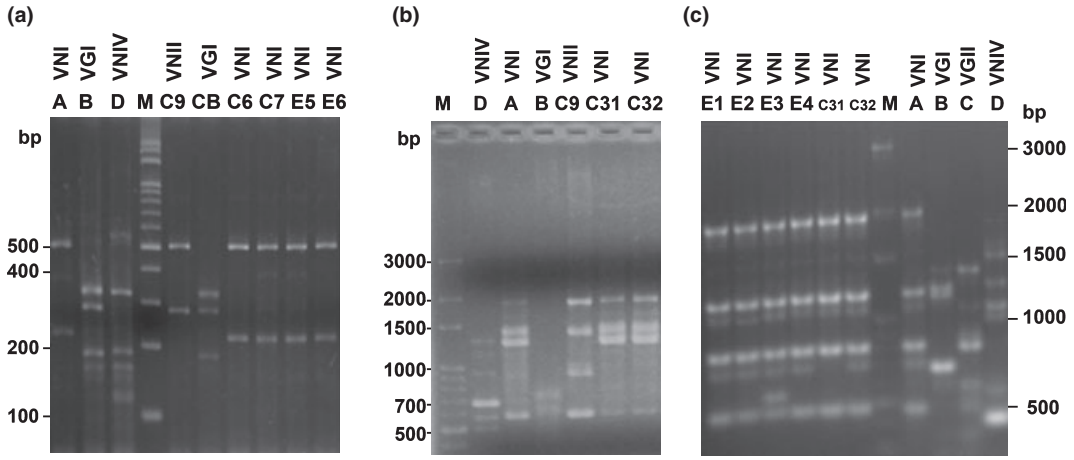

FIG. 2. (a) Examples of *URA5* gene restriction fragment profiles of clinical and environmental isolates of *Cryptococcus neoformans*. C6-C7, C9 and CB, clinical isolates; E5-E6, environmental isolates; A, B, D, and M are as in Fig. 1. (b) Examples of PCR fingerprinting patterns obtained with the primer M13. C9, C31-C32, clinical isolates of *C. neoformans*; A, B, D, and M are as in Fig. 1. (c) Examples of PCR fingerprinting patterns obtained with the primer $(GACA)_4$. C31-32, clinical isolates; E1-E4, A, B, D, M are as in Fig.1; C, ATCC 32608, serotype C.

Discussion

Cryptococcosis is one of the most prevalent and serious mycoses. In Taiwan, only a few sporadic studies of *C. neoformans* have been reported [15,16,36]. In this study, we add important information about the characteristics of *C. neoformans* isolates from northern Taiwan. We found that 99% of clinical isolates were var. *grubii* serotpe A. This finding is consistent with previous studies worldwide, which found that var. *grubii* is the most prevalent cryptococcal isolate [5,12,13,37,38]. The low incidence of var. *gattii* compared with previous reports from Taiwan [16,36] (1% vs. 35.6% and 14.7%) may be due to the differences in patients, the time period of sample collection and the randomness introduced by including non-duplicate samples. For the first time in Taiwan, two molecular methods, *URA5*-RFLP and PCR fingerprinting using primer M13 and primer $(GACA)_4$, were applied simultaneously to unravel the genotypes of *C. neoformans* isolates. Previous analysis of *C. neoformans* isolates by $(GACA)_4$ fingerprinting revealed four different groups [39]. One of them includes serotype A isolates (VN6), another includes serotype D isolates (VN1) and the remaining two (VN3 and VN4) include AD strains. All of our isolates were revealed to be of the VNI (VN6) type. These results are in agreement with studies from other countries,

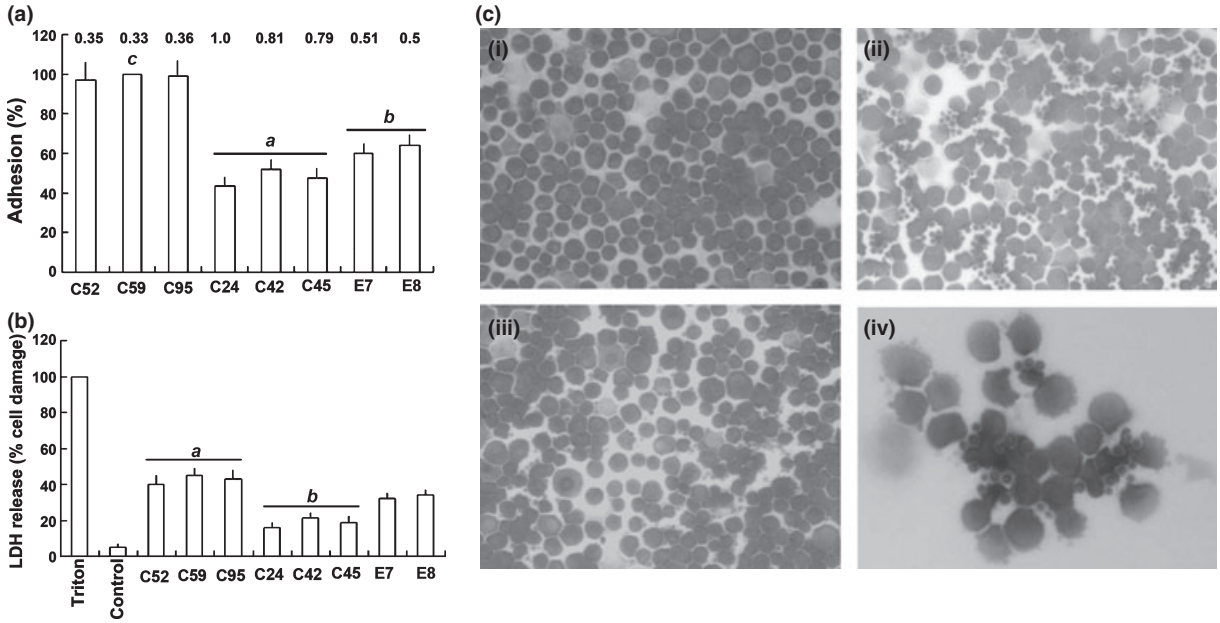

FIG. 3. Adhesion of phospholipase-producing *Cryptococcus neoformans* to A549 cells. (a) C24, 42, 45, low producers; C52, 59, 95, high producers; E7, 8, environmental isolates. The Pz value is shown above each bar. Results obtained for C59 cells were defined as 100%. The data are averages and standard deviations of three independent experiments. *a*, *b* significantly different from *c* ($p < 0.01$, by Student's *t* test). (b) Lactate dehydrogenase (LDH) release of A549 cells after interaction with phospholipase-producing *C. neoformans*. C24, 42, 45, 52, 59, 95, E7-8, are as in (a). Supernatants, after incubation, were assayed for LDH release using colorimetry described in Materials and Methods. Data are expressed as a percentage of LDH release from unstimulated cells treated with Triton X-100 and represent the mean ± SD of triplicate measurements from two independent experiments. Control, medium only background. *a* vs. *b*, $p < 0.01$ by Student's *t* test. (c) Light microscopy of *C. neoformans* adhering to A549 cells. A549 cells were stimulated for 6 h at 37°C with low (C24) or high (C59) phospholipase-producing isolates of *C. neoformans*, followed by washing, trypsinization, cytospin to slides and observation. a, no yeast control, 400×; b, A549 incubated with C59, 400×; c, A549 incubated with C24, 400×; d, A549 incubated with C59, 650×. Data are representative of three independent experiments.

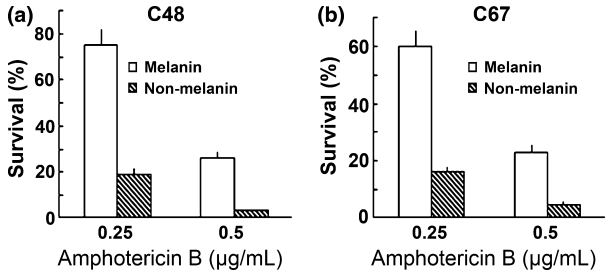

FIG. 4 Amphotericin B killing assay of melanized and non-melanized *Cryptococcus neoformans*. The survival rates of melanized vs. non-melanized yeast cells (clinical isolates, C48 and C67) after exposure to 0.25 and 0.5 mg/L of amphotericin B for 2 h were compared with those of fungal cells incubated in phosphate-buffered saline. The data represent averages of four independent experiments with standard deviations. $p < 0.05$ as determined by Student's *t* test for a comparison between non-melanized and melanized cells.

which reported that VNI is the most common molecular type among *C. neoformans* isolates [5,12,13,37,38].

We found that expression of the various virulence traits was not correlated with each other. This is similar to a study by Clancy *et al.* [20], who found no correlation among capsule size, phospholipase activities and melanin formation. These results suggest that host factors contribute significantly to the pathogenesis of *C. neoformans*. Interestingly, we found three lines of evidence that suggest that phospholipase activity is an important causative factor of cryptococcosis. First, the majority of isolates exhibited high phospholipase activity instead of urease or melanin production. Second, high phospholipase activity was correlated with increased adherence to lung epithelial A549 cells. Third, cell damage indicated by LDH release was more frequent in the high phospholipase-producing isolates. Further fungal–macrophage interaction assay and animal experiments are needed to confirm the importance of phospholipase.

Previous studies have revealed that melanin affects the susceptibility of *C. neoformans* to certain compounds, such as amphotericin B [40,41]. It has been suggested that cryptococcal melanin deposited in the cell wall appears to capture drugs, preventing them from reaching their active sites [42]. In this study, we found that melanin contributes

significant protection against amphotericin B killing of *C. neoformans*.

In conclusion, the majority of the clinical isolates in this study belonged to var. *grubii*, serotype A, were mating type α and could be grouped into the molecular type VNI. We re-identified phospholipase as a potential virulence factor and a cause of cryptococcosis.

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Transparency Declaration

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