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 \( a \) 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 MIC₉₀ 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 MAT\( \alpha \) cells [4,9]. MAT\( \alpha \) 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, x 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 x (MATx1–MATx2) and a (MATa1–MATa2), respectively, were performed [5]. Cryptococcus neoformans H99 (serotype A, MATx) and C. neoformans KN99a (serotype A, MATa) were used as positive controls.

Genotyping by PCR fingerprinting and URAS 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]. URAS 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. $5 \times 10^6$ 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 col-
ony 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 (10⁴ 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,
then suspended in RPMI 1640 medium (Gibco) containing
5% FBS and resuspended with 10% fetal bovine serum (FBS).
A549 cells were then trypsinized, washed with water and the resulting suspension plated onto
SDA plates. Colonies were counted after incubation at 37°C
for 2 days, onic for removal of yeast cells and assayed for the release of
lactate dehydrogenase (LDH) by the LDH kit (Cyto-
Tox 96® Non-radioactive Cytotoxicity Assay: Promega, Madison, WI, USA) [34]. All experiments were performed in
triple 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 × 10⁵ cells/mL. Cell counts were determined with a haem-
atometer. 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 incu-
bation at 37°C for 2 h, aliquots were plated on SDA to
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 tissue swab (n = 1). Forty-one patients had
underlying diseases, including 27 patients with AIDS and
14 with other diseases, including acute lymphoblastic leu-
kaemia (n = 1), adult T-cell leukaemia (n = 1), chronic lym-
phocytic 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 sero-
type A and one was serotype B (example profiles in
Fig. 1a,b), which tested positive on the CGB plate. M13 PCR
fingerprinting and URAS-RFLP typing were used to investigate
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 MATα and MATβ 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 MIC₉₀ and MIC₅₀ 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.

### 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

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. (%) of clinical isolates (N = 100)</th>
<th>No. (%) of environmental isolates (N = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>74 (74)</td>
<td>8 (100)</td>
</tr>
<tr>
<td>Female</td>
<td>26 (26)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Specimens</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood</td>
<td>40 (40)</td>
<td>8 (100)</td>
</tr>
<tr>
<td>Bronchial washing</td>
<td>2 (2)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Bronchoalveolar lavage</td>
<td>3 (3)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Cerebrospinal fluid</td>
<td>43 (43)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Lung abscess</td>
<td>1 (1)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Pleural effusion</td>
<td>6 (6)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Sputum</td>
<td>3 (3)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Surgical wound</td>
<td>1 (1)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Throat swab</td>
<td>1 (1)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Pigeon droppings</td>
<td>0 (0)</td>
<td>8 (100)</td>
</tr>
<tr>
<td>Underlying diseases</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AIDS</td>
<td>27 (27)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Others</td>
<td>14 (14)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>None</td>
<td>59 (59)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Serotypes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>99 (99)</td>
<td>8 (100)</td>
</tr>
<tr>
<td>B</td>
<td>1 (1)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Molecular types</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VNI (VN6b)</td>
<td>98 (98)</td>
<td>8 (100)</td>
</tr>
<tr>
<td>VNII</td>
<td>1 (1)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>VGI</td>
<td>1 (1)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Pz (phospholipase activity)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.26–0.5 (high)</td>
<td>59 (59)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>0.51–0.75 (intermediate)</td>
<td>38 (38)</td>
<td>5 (42.5)</td>
</tr>
<tr>
<td>0.75–1 (low)</td>
<td>3 (3)</td>
<td>3 (37.5)</td>
</tr>
<tr>
<td>Melanin production</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4+</td>
<td>49 (49)</td>
<td>4 (40)</td>
</tr>
<tr>
<td>3+</td>
<td>7 (7)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>2+</td>
<td>36 (36)</td>
<td>4 (50)</td>
</tr>
<tr>
<td>1+</td>
<td>53 (53)</td>
<td>4 (50)</td>
</tr>
<tr>
<td>Urease activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3+</td>
<td>23 (23)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>2+</td>
<td>26 (26)</td>
<td>3 (37.5)</td>
</tr>
<tr>
<td>1+</td>
<td>51 (51)</td>
<td>5 (42.5)</td>
</tr>
</tbody>
</table>

*a* By PCR fingerprinting using M13 primer and URA5-RFLP.  
*b* By PCR fingerprinting using (GACA)_4 primer.
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 serotype 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, URAS5-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.

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 serotype 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, URAS5-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.
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 \( \alpha \) 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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