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Clinical and microbiological characteristics of cryptococcosis in Singapore: predominance of *Cryptococcus neoformans* compared with *Cryptococcus gattii*☆

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SUMMARY

Objectives: To describe the clinical features, treatments, outcomes, and subtype prevalence of cryptococcosis in Singapore.

Methods: All patients with laboratory confirmed cryptococcal infections admitted from 1999 to 2007 to a teaching hospital in Singapore were reviewed retrospectively. Identification and molecular types of *Cryptococcus neoformans* variants and *Cryptococcus gattii* were determined by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP). Serotypes were inferred with a multiplex PCR method.

Results: Of 62 patients with cryptococcosis, *C. neoformans* var. *grubii* was the predominant subtype (in 95%), affecting mainly immunocompromised hosts (91%) with HIV infection (80%). Patients with HIV were younger (median age 36.5 vs. 49.5 years, $p = 0.006$) and less likely to present with an altered mental status (14% vs. 50%, $p = 0.013$). In contrast, delayed treatment (median 7 days vs. 2 days, $p = 0.03$), pulmonary involvement (58% vs. 14%, $p = 0.03$), and initial treatment with fluconazole (25% vs. 2%, $p = 0.02$) were more common in HIV-negative patients. *C. gattii* was uncommon, affecting only three patients, all of whom were immunocompetent and had disseminated disease with pulmonary and neurological involvement. All *C. gattii* were RFLP type VG II, serotype B and all *C. neoformans* var. *grubii* were RFLP type VN I, serotype A, except for one that was RFLP type VN II.

Conclusion: *C. neoformans* var. *grubii*, subtype VN I, was the predominant subtype in Singapore, infecting younger, mainly immunocompromised hosts with HIV. *C. gattii* was uncommon, causing pulmonary manifestations in older, immunocompetent patients and were RFLP type VG II.

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1. Introduction

Cryptococcus neoformans is a basidiomycetous encapsulated yeast with worldwide distribution. After inhalation from environmental sources, this pathogen may result in life-threatening infections in humans commonly affecting the central nervous system or respiratory system.[1]

C. neoformans was previously subclassified into three varieties based upon biochemical differences and into four non-hybrid serotypes according to capsular agglutination reactions: *C. neoformans* var. *grubii* (serotype A), *C. neoformans* var. *neoformans* (serotype D), and *C. neoformans* var. *gattii* (serotype B and C).[2,3]

C. neoformans is the commonest cause of fungal meningitis worldwide.[4,5] *C. neoformans* var. *grubii* is an opportunistic pathogen of immunocompromised patients, with HIV infection, corticosteroid therapy, haematological malignancies, and solid-organ transplantation identified as major risk factors.[6,7] Cryptococcal meningitis is the fourth most common opportunistic infection in patients with HIV, with an estimated one million HIV-associated cryptococcosis cases diagnosed annually worldwide.[5] In Southeast Asia, cryptococcosis is common amongst HIV-infected individuals, with an estimated 120 cases per 1000 HIV-infected individuals per year.[4] The growing size of the immunocompromised patient population from treatment with chemotherapy and biological agents will likely further contribute to the medical importance of cryptococcosis.[6]

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Cryptococcus gattii is now recognized as a separate species from *C. neoformans* due to significant differences in genetic, biochemistry, ecology, and clinical characteristics.[8,9]

C. gattii is associated with the unique environmental source of Australian River and Forest Red Gum trees (*Eucalyptus camaldulensis* and *Eucalyptus tereticornis*), although it has also been cultured from other trees, bird excreta, and soil.[9–11] Mainly restricted to tropical and subtropical regions, a recent large outbreak of *C. gattii* in British Columbia and surrounding areas has highlighted a shift in the geographical distribution.[12,13] Unlike *C. neoformans*, disease due to *C. gattii* is usually observed in patients without significant immunosuppression. Characteristics of *C. gattii* include a higher rate of brain mass lesions, more refractory response to antifungal chemotherapy, and more aggressive and prolonged treatment with increased long-term sequelae and higher mortality.[13–15]

In Singapore, identification to the species level has only recently been implemented in clinical laboratories so the historic distribution of the various serotypes and species is unknown. In addition, no recent data exist on patient characteristics, treatments, and outcomes of cryptococcosis since the onset of the HIV epidemic. To provide this information, our study aimed to describe the epidemiological, clinical, and outcome aspects of patients with cryptococcosis admitted to a teaching hospital from 1999 to 2007.

2. Methods

Tan Tock Seng Hospital is a 1400-bed university teaching hospital in Singapore. We retrospectively reviewed the medical and microbiological records of all patients aged 16 years or older hospitalized from March 1999 to June 2007 with cryptococcal infection. Patient characteristics were extracted from patient records and included demographic characteristics, co-morbidities, clinical presentation, radiological and microbiological evaluation including cerebrospinal fluid (CSF) analysis, antifungal treatment, adjunctive neurosurgery, and clinical outcomes including length of hospital stay, hospital re-admission after discharge, and 30-day mortality.

Cryptococcosis was defined as clinical features consistent with active *Cryptococcus spp* infection and isolation of *Cryptococcus spp* from a normally sterile site. Cryptococcal meningitis was defined as positive CSF culture for *Cryptococcus spp*. Pulmonary cryptococcosis was defined by a clinically compatible presentation with parenchymal infiltrates or nodules demonstrated on chest radiography or computed tomography for which no other cause was apparent and *Cryptococcus spp* isolated from a normally sterile site. Cryptococcaemia was defined as blood cultures positive for *Cryptococcus spp*. Disseminated cryptococcosis was defined as involvement of two or more non-contiguous sites according to the criteria above.

All isolates were originally identified as *C. neoformans* with the API20AUX system (bioMérieux), which could not distinguish *C. neoformans* from *C. gattii*. In this study, polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) was used to distinguish *C. neoformans* variants from *C. gattii*[16] and to place isolates into one of eight possible molecular types: VN I, II, III, and IV, and VG I, II, III, and IV. A multiplex PCR was used to infer the serotype as serotyping reagents are no longer manufactured.[17] The presence of cryptococcal antigen in the blood and CSF was determined with the use of a cryptococcal latex agglutination system (CALAS; Meridian Diagnostic Inc., Cincinnati, OH, USA). Blood cultures were collected and processed with the use of BACTEC and BacT/ALERT systems. The collection and publication of these data was approved by the institutional ethics review board.

2.1. Statistical analysis

Descriptive statistics (means, standard deviations, median, range, frequency counts) were used to describe the distribution of the variables in the study population of HIV-positive and HIV-negative patients. A Chi-square test or Fisher's exact test was used accordingly to evaluate differences in categorical variables. Crude odds ratios (ORs) and 95% confidence intervals (CIs) were calculated for categorical variables. Crude ORs for continuous variables were obtained using simple logistic regression. Stratified analyses were used for death within 30 days after diagnosis and CSF parameters (white blood cell count (WBC), glucose, and protein). All tests were two-tailed and p-values less than 0.05 were considered statistically significant. The statistical analysis was performed using Stata version 10 (StataCorp LP, College Station, TX, USA).

3. Results

A total of 77 cryptococcal isolates were recovered during the 9-year study period. Medical records were available for 62 patients with 69 positive cultures (Table 1). The mean age was 42 ± 12 years (range 17–76 years). Forty-nine patients (79%) were male. The majority (81%) were infected with HIV with CD4 counts <200 cells/mm^3 (median 20 cells/mm^3, range 2–140 cells/mm^3). All four patients with rheumatologic disease had systemic lupus erythematosus (SLE). Three patients had active SLE on chronic steroid therapy, and the remaining patient presented with neuropsychiatric SLE, pulmonary haemorrhage, and cryptococcaemia. None were on immunobiologicals. Five patients had no apparent underlying medical condition, three of whom had *C. gattii*. The most common symptoms were fever (79%), headache (71%), and cough (45%). On admission 45% were febrile.

On physical examination, 12 patients (19%) had meningism. Nine patients had cranial nerve (CN) palsies, the most common being CN VI palsy, which was present in seven patients. Two patients had hemiplegia. Reduced visual acuity was present in four patients and papilloedema in three. Six patients had cutaneous findings including folliculitis (two patients), eczema, pyoderma gangrenosum, Kaposi sarcoma, and herpes zoster (one patient each).

Chest radiographs were abnormal in 28 (45%) patients (Table 2). Of the abnormal chest radiographs, nine showed coin-like opacities, 18 showed infiltrates, and two had pleural effusions. Brain imaging was performed in 61 patients. Meningeal enhancement occurred in eight patients. Multiple space-occupying lesions suggestive of cryptococcoma were present in five patients and single space-occupying lesions in three. Sixty patients had a CSF examination. The median opening pressure on lumbar puncture was 23 cmH_2O (range 4–50 cmH_2O). The median value for the CSF WBC was 4×10^6 cells/l (range 0–342×10^6 cells/l), glucose was 2.5 mmol/l (range 0.1 to 9 mmol/l), protein was 59 mg/dl (range 16–433 mg/dl), and the cryptococcal antigen titre was 512 (range 0–65 536). The median serum cryptococcal antigen titre was 2048 (range 0–262 144). Blood cultures obtained in our hospital were positive for *Cryptococcus spp* in 29 patients, *Salmonella enteritidis* in two patients, and methicillin-sensitive *Staphylococcus aureus* in one patient.

Cryptococcaemia was diagnosed in 34 patients (55%). Five patients had positive blood cultures for *Cryptococcus spp* at the referring institution, prior to transfer to our hospital. Cryptococcal meningoencephalitis was diagnosed in 55 patients (89%), respiratory involvement occurred in 14 (23%), and disseminated disease in 31 (50%).

All 61 patients diagnosed ante-mortem received antifungal treatment for cryptococcosis (Table 3). Delay in treatment was

Table 1
Demographic and clinical features of HIV-positive and HIV-negative patients with cryptococcosis[a]

Variables	HIV-positive ($n=50$)	HIV-negative ($n=12$)	OR (95% CI)	p-Value
Age, years, median (IQR)	36.5 (32–46)	49.5 (42–62.5)	0.9 (0.9–1.0)	0.006
Gender, male	42 (84)	7 (58)	3.8 (0.9–14.8)	0.05
Co-morbidities				
Rheumatologic diseases	0	4 (33.3)	NC	0.001
Diabetes mellitus	3 (6)	2 (16.7)	0.3 (0.04–2.2)	0.246
Liver cirrhosis	1 (2)	1 (8.3)	0.2 (0.01–3.9)	0.352
Renal diseases	0	2 (16.7)	NC	0.035
Corticosteroid (>10 mg/day for 3 months)	0	5 (41.7)	NC	<0.001
Presenting symptoms				
Duration of symptoms before presentation, days, median (IQR)	14 (7–21)	21 (5–30)	1.0 (1.0–1.01)	0.450
Fever	42 (84)	9 (75)	1.8 (0.39–7.92)	0.432
Cough	24 (48)	4 (33.3)	1.8 (0.50–6.93)	0.521
Headache	37 (74)	8 (66.7)	1.4 (0.37–5.52)	0.721
Vomiting	24 (48)	3 (25)	2.8 (0.67–11.45)	0.202
Altered mental status	7 (14)	6 (50)	0.2 (0.04–0.65)	0.013
Neck stiffness	8 (16)	3 (25)	0.6 (0.13–2.59)	0.432
Presenting signs				
Respiratory	9 (18)	2 (16.7)	1.1 (0.20–5.89)	1.000
Neurologic (meningism)	11 (22)	1 (8.3)	3.1 (0.36–26.73)	0.431
Neurologic (other)	10 (20)	0	NC	0.186
Ophthalmologic	6 (12)	1 (8.3)	1.5 (0.16–13.78)	1.000

OR, odds ratio; CI, confidence interval; IQR, interquartile range; NC, non-calculable.
[a] Data are in number (%), unless otherwise specified.

significantly increased in HIV-negative patients ($p = 0.03$). The majority (93%) received intravenous amphotericin B with half of these patients receiving additional flucytosine (45%). Fluconazole for initial therapy was significantly more common in HIV-negative patients ($p = 0.02$). Nine patients (15%) required neurosurgical shunt insertion. Two patients were repatriated to Thailand and one patient each to Indonesia and Malaysia. The outcome was not known for these four patients. For the remaining 58 patients, the 30-day mortality was 17%.

Only three patients (5%) were infected with *C. gattii*. All were men with no underlying conditions (Table 4). All had disseminated disease involving the respiratory and central nervous systems.

Molecular typing of 77 isolates showed three to be C. *gattii*, RFLP type VG II, serotype B. Of the remaining 74, all were *C. neoformans* var. *grubii* RFLP type VN I, serotype A, except for one that was RFLP type VN II.

4. Discussion

In this study, *C. neoformans* var. *grubii* was the most common infecting species, affecting mainly patients with HIV. From the identification of the first case of HIV infection in Singapore, which was reported in 1985, the cumulative number of Singapore residents diagnosed with HIV reached 5775 by the end of 2012 with the majority (93%) of new cases being male.[18] Our finding that HIV infection was the underlying condition in the majority (81%) of cryptococcosis reflects the rise in HIV infection locally. In contrast, two older studies on Singaporean patients with cryptococcal meningitis published in 1972 (30 patients) and 1985 (25 patients) reported few patients with pre-existing chronic disease and the majority had no underlying immune defect.[19,20]

The male predominance (79%) is similar to other studies, likely due to the gender composition of the HIV population locally,

Table 2
Laboratory and radiologic findings in HIV-positive and HIV-negative patients with cryptococcosis[a]

Variables	HIV-positive ($n=50$)	HIV-negative ($n=12$)	OR (95% CI)	p-Value
CSF parameters				
Opening pressure >20 cmH_2O	29 (58)	5 (41.7)	1.9 (0.54–6.94)	0.349
WBC $\geq 5 \times 10^6$ cells/l	21 (42)	6 (50)	0.7 (0.20–2.56)	0.616
Glucose <2.5 mmol/l	23 (46)	5 (41.7)	1.2 (0.33–4.27)	1.000
Protein >0.45 g/l	26 (52)	8 (66.7)	0.5 (0.14–2.03)	0.521
India ink positive	40 (80)	6 (50)	4.0 (1.06–15.08)	0.033
Culture positive	45 (90)	9 (75)	3.0 (0.61–14.86)	0.177
Abnormal chest radiograph	20 (40)	8 (66.7)	0.3 (0.09–1.26)	0.117
Brain images (CT or MRI)				
Normal	35 (70)	9 (75)	0.8 (0.18–3.28)	1.000
Mass lesion	7 (14)	1 (8.3)	1.8 (0.20–16.12)	1.000
Hydrocephalus	4 (8)	0	NC	0.578
Blood culture positive	24 (48)	5 (42)	1.3 (0.30–5.88)	0.693
Diagnosis				
CNS	46 (92)	9 (75)	3.8 (0.73–20.13)	0.125
Pulmonary	7 (14)	7 (58.3)	0.1 (0.03–0.47)	0.003
Cryptococcaemia	29 (58)	5 (41.7)	1.9 (0.54–6.94)	0.349
Disseminated (more than 1 site)	25 (50)	6 (50)	1.0 (0.28–3.53)	1.000

OR, odds ratio; CI, confidence interval; CSF, cerebrospinal fluid; WBC, white blood cell count; CT, computed tomography; MRI, magnetic resonance imaging; NC, non-calculable; CNS, central nervous system.
[a] Data are in number (%).

Table 3
Treatments, complications, and outcomes in HIV-positive and HIV-negative patients with cryptococcosis[a]

Variables	HIV-positive ($n=50$)	HIV-negative ($n=12$)	OR (95% CI)	p-Value
Treatment				
Delay to treatment, days, median (IQR)	2 (1–4)	7 (2–11)	0.8 (0.63–0.92)	0.003
Amphotericin B alone	26 (52)	3 (25)	3.2 (0.78–13.44)	0.116
Amphotericin B and 5-flucytosine	23 (46)	5 (41.7)	1.2 (0.33–4.27)	0.787
Fluconazole alone	1 (2)	3 (25)	0.1 (0.006–0.66)	0.021
Amphotericin B then fluconazole	42 (84)	8 (66.7)	2.6 (0.64–10.84)	0.223
Duration amphotericin B, days, median (IQR)	14 (9–17)	22 (14–33)	0.9 (0.87–0.99)	0.016
Total duration of antifungals, months, median (IQR)	17 (3–43)	15 (1–19)	1.0 (0.99–1.07)	0.414
Neurosurgery	8 (16)	1 (8.3)	2.1 (0.24–18.58)	0.675
Complications				
Raised ICP needing shunts	9 (18)	0	NC	0.185
Blindness	6 (12)	0	NC	0.586
Acute renal impairment	14 (28)	7 (58.3)	0.3 (0.08–1.02)	0.086
Outcome				
Length of stay, days, median (IQR)	18.5 (13–33)	31 (17.5–44.5)	0.99 (0.97–1.01)	0.192
Readmission related to Cryptococcus ($n=34$)	12/27	3/7	1.1 (0.20–5.71)	0.940
Death ($n=57$)	15/45	1/12	5.5 (0.65–46.69)	0.118
Death at ≤30 days after diagnosis ($n=58$)	9/46	1/12	2.68 (0.30–127.77)	0.670

OR, odds ratio; CI, confidence interval; IQR, interquartile range; ICP, intracranial pressure; NC, non-calculable.
[a] Data are in number (%), unless otherwise specified.

although increased environmental exposure, hormonal influences, and genetic predisposition have been suggested as contributing factors.[21] The median age of HIV-positive patients was lower compared with HIV-negative patients, likely due to the younger age of HIV-positive patients locally.[18]

The majority of HIV-positive patients (92%) had cryptococcal meningoencephalitis, thus fever, headache, and vomiting were the most common presenting symptoms. Altered mental state was less common in HIV-positive patients, possibly as a result of a relatively poor CSF inflammatory response.[22] In contrast, neurologic deficits, in particular CN VI palsy, were present in HIV-positive patients, likely reflecting raised intracranial pressure associated with cryptococcal meningoencephalitis. HIV-positive patients were more likely to have positive CSF India ink staining ($p = 0.033$), likely due to the presence of a higher fungal burden. Despite positive CSF cultures in the majority (87%), CSF WBC, protein, and glucose findings were normal in 33–58%, similar to other studies.[23]

The diagnosis and treatment in HIV-positive patients were significantly earlier than in HIV-negative patients due to the early consideration of and testing for cryptococcosis in HIV-positive patients. Fluconazole was more likely to be used in HIV-negative patients for initial treatment, likely due to the predominance of respiratory involvement compared with central nervous system infection, which requires treatment with amphotericin B.[5]

Despite improvements in medical care and access to highly active antiretroviral therapy (HAART), there was no significant decrease in mortality over the study time period. The overall 30-day mortality of 16% is comparable to other studies.[5] Comparing HIV-positive to HIV-negative patients, mortality and morbidity were not significantly different between the two groups. This contrasts with other studies, which have reported increased morbidity, mortality, and surgical interventions in HIV-negative patients.[8,12,14]

C. gattii, now recognized as a separate species from *C. neoformans*, was first reported in Singapore in 2002.[24] Mostly restricted to tropical and subtropical regions of Australia and Papua New Guinea,[13,14] a large outbreak in Vancouver Island and surrounding areas within Canada, with spread into the Pacific

Table 4
Baseline characteristics, clinical presentation, laboratory findings, and treatment of three patients with *Cryptococcus gattii*

Characteristic	Patient 1	Patient 2	Patient 3
Age, years	47	39	65
Sex	Male	Male	Male
Days of illness	30	21	30
Presenting symptoms	Fever, cough, headache, anorexia, mental status change	Headache, mental status change	Fever, anorexia
Co-morbidities	Nil	Nil	Nil
Investigations			
CXR	Lung mass	Lobar consolidation	Lung mass
Brain imaging	Focal brain mass	Normal	Normal
Serum CLA, titre	262 144	8192	256
CSF CLA, titre	1024	512	4096
CSF India ink	Positive	Positive	Positive
CSF culture	Positive	Positive	Positive
Blood culture	Negative	Negative	Negative
Treatment			
Delay to treatment, days	12	2	9
Treatment	AmB × 28 days + 5FC × 24 days then fluconazole 800 mg	Fluconazole 800 mg	AmB × 14 days + 5FC × 14 days then fluconazole 400 mg
LOS, days	28	6	37
Surgery	Yes	No	No
Follow-up, years	5	1.5	1.5

CXR, chest radiograph; CLA, cryptococcal latex agglutination; CSF, cerebrospinal fluid; AmB, amphotericin B; 5FC, 5-flucytosine; LOS, length of stay.

Northwest region of the USA has widened the global distribution of this pathogen.[12,25,26] In neighbouring Malaysia, *C. gattii* comprised 11.5% of cryptococcal infections, although notably no isolates originated from the southern states adjacent to Singapore.[27] Despite infecting hosts with normal immune status, *C. gattii* behaves more aggressively than *C. neoformans* with higher rates of brain mass lesion, long-term neurologic and pulmonary sequelae, and increased need for prolonged treatment and surgery.[12,14,21] In our study, *C. gattii* was uncommon with only three cases (3.9%) identified. Similar to previous studies, all patients had a normal immune status.[12,21] All patients had lung involvement, whereas only one patient had abnormal brain imaging. Despite the small numbers, one patient required neurosurgery and all survived.

Molecular typing techniques, such as RFLP, have become essential tools in tracking the epidemiology of *C. neoformans*.[16] For *C. gattii*, RFLP VG I, VG II, VG III, and VG IV appear to have distinct distribution zones according to geographical and climactic influences.[28–30] Most clinical isolates from Australia and eucalyptus-associated *C. gattii* isolates belong to RFLP VG I.[31] In contrast, in the outbreak on Vancouver Island and mainland British Columbia, most human, veterinary, and environmental *C. gattii* isolates belonged to the genotype VG II, with its two molecular subtypes VG IIa being the predominant genotype in 90% and VG IIb in 10%.[26,30,32]

In our study, all *C. gattii* isolates were VG II. Epidemiological studies from neighbouring countries have similarly shown the presence of molecular type VG II in the region. In Malaysia, of eight clinical isolates of *C. gattii*, half were VG I and the remaining were VG II.[27] Elsewhere in Southeast Asia, the majority of 13 clinical isolates from Thailand were VG II, with only a single isolate of VG I.[33] This is in direct contrast to Vietnam, where VG I was the predominant type and only one of 13 isolates was found to be VG II.[34]

For *C. neoformans* var. *grubii*, VN I accounted for the vast majority of isolates in our study. Similarly, all *C. neoformans* var. *grubii* isolates were VN I in studies from Malaysia and Vietnam,[27,34] and this was the predominant molecular type (94.6%) in clinical isolates from Thailand.[33] This predominance of VN I molecular type is in concordance with other studies reported worldwide.[35–37]

Cryptococcosis is not a notifiable disease in Singapore. The data presented in this study represent a single institution record. Our institution supports a limited haematology and transplant service, thus cryptococcal disease related to associated immunosuppression is lacking in this study. However, being the national referral centre for HIV in Singapore, this study is expected to capture the majority of HIV-related cryptococcosis. Since this was a retrospective study, not every patient had their HIV status checked. Of the four patients who did not have HIV testing, three were diagnosed with SLE and one had CD4 534 cells/mm^3. None of the 12 patients classified as HIV-negative had other AIDS-defining illnesses before or after the episode of cryptococcaemia. Thus, the likelihood of undetected HIV infection was low in these patients.

This study is the first to describe the clinical characteristics and report on the distribution of molecular serotypes of Cryptococcus in Singapore. In conclusion, *C. neoformans* var. *grubii* VN I is the predominant subtype in Singapore, infecting younger, mainly immunocompromised hosts with HIV infection. *C. gattii* is uncommon, of VG II serotype, causing pulmonary manifestations in older, immunocompetent patients.

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Conflict of interest: All authors declare no financial interest in the subject matter and declare no conflict of interest related to this article.

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