the effect of cytokines such as interleukin 12 and interferon gamma (7,12–14). We therefore conducted morphometric analyses of MGCs to quantitatively elucidate the strength of the recognition function of macrophages and found that the poorest macrophage response occurred in mice infected with *C. gattii* R265, followed by those infected with *C. gattii* 5815 and *C. neoformans* H99. These findings suggest that *C. gattii* R265 may have the poorest potential to induce a recognition cascade in the immune system. Accordingly, the present histopathological examinations focusing on macrophage response with MGC formation can imply that *C. gattii* may possess some unknown mechanisms to escape the recognition by macrophages or other antigen-presenting cells.

In conclusion, the present study revealed that there is a different pathophysiology leading to death during *C. gattii* and *C. neoformans* infections. Our findings also suggest that there are two important characteristics of *C. gattii*: one is that *C. gattii* may possess some unknown mechanisms to escape recognition by macrophages and another is a high performance of structural alteration of the lung. Furthermore, these characteristics are presumably associated with the high virulence of *C. gattii* in infected mice. However, because only one strain of *C. neoformans* and two strains of *C. gattii* were investigated in the present study, further investigations are required to elucidate the detailed virulence factors of *C. gattii*.

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Review Article

How Histopathology Can Contribute to an Understanding of Defense Mechanisms against Cryptococci

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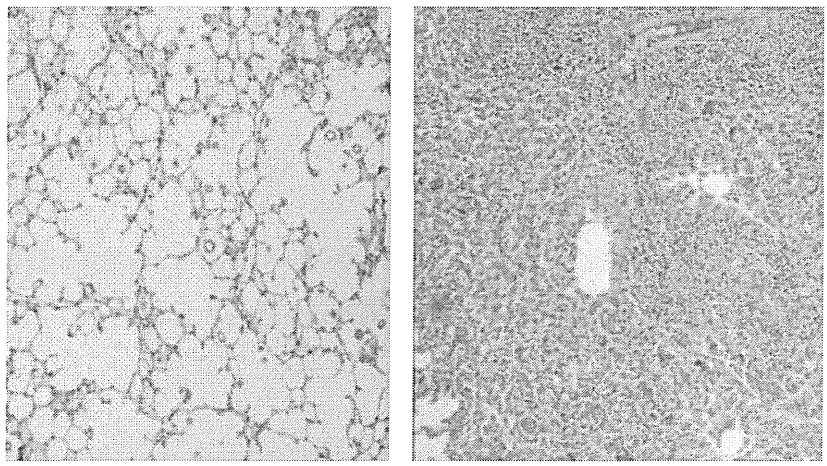
Invasive fungal infections, particularly those considered opportunistic, have become a common and significant complication of procedures performed in advanced contemporary medicine. Among such infections, cryptococcosis, which is usually caused by infection with *Cryptococcus neoformans* and *Cryptococcus gattii*, is particularly problematic because this fungal infection occurs in immunocompromised and apparently immunocompetent individuals. It has been largely accepted that *Cryptococcus* species are recognized by cellular receptors and that Th1-type immune responses play an important role in defense mechanisms against the yeast. However, the interaction between the yeast and host tissue varies depending on the characteristics of the yeast and the immune status of the host. To gain a better understanding of the pathophysiology of cryptococcosis, we wish to emphasize the usefulness of histopathological examinations, because it allowed more detailed information of an extremely complex interaction between the causative yeasts and tissue response. In the present review, we describe the pathophysiology of cryptococcosis as largely revealed in our previous histopathological investigations of the experimental infection.

1. Introduction

The increasing use of invasive monitoring and aggressive therapeutic technologies in developed countries has resulted in improved survival of individuals with life-threatening diseases [1–3]. However, it has also resulted in an increased number of patients at risk of invasive fungal infections, including cryptococcosis. Cryptococcosis is a localized or systemic fungal infection mainly caused by *Cryptococcus neoformans* (*C. neoformans*) and *Cryptococcus gattii* (*C. gattii*). *C. neoformans* is widely distributed throughout the world [4], whereas *C. gattii* has been limited in tropical and subtropical regions [5]. However, there was an outbreak of *C. gattii* infection in a temperate region of British Columbia in 1999, which expanded towards US Pacific Northwest and Japan [5–10].

Recent molecular biological investigations have contributed significantly toward an elucidation of the pathogenesis of the yeast. It has been largely accepted that *Cryptococcus* species are recognized by cellular receptors and that Th1-type immune responses play an important role in defense mechanisms against the yeast [11]. However, this defense mechanism may vary among individuals, depending on the



(a) (b)

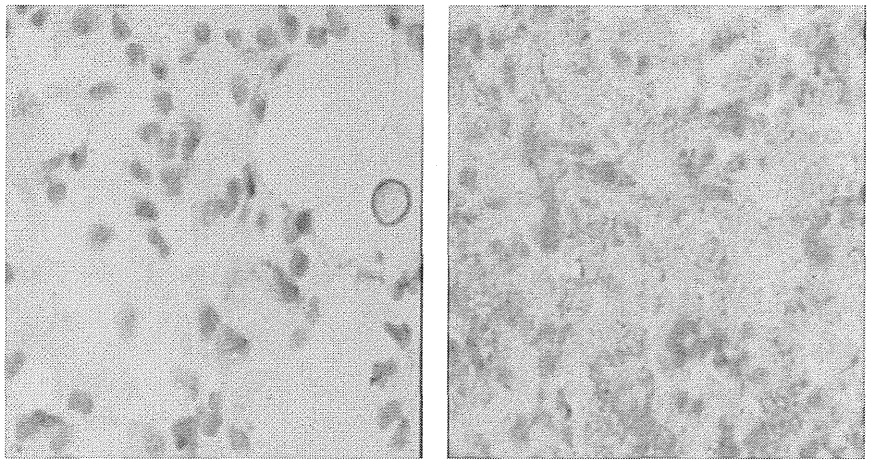
FIGURE 1: (a) The lung of mice infected with *C. neoformans* TIMM 0362 showed yeast cell proliferation in the alveoli but showed a very limited response of macrophage (13 days after infection, hematoxylin and eosin (HE) double stain, and original magnification ×200). (b) The lung of mice infected with *C. neoformans* TIMM 0372 showed numerous multinucleated giant cells (MGCs) due to the prominent macrophage response (13 days after infection, HE double stain, and original magnification ×200).

agents, tissues involved, and immune status of the host [12, 13]. Therefore, experimental animal models remain essential for an elucidation of the pathophysiology of cryptococcosis. In particular, histopathological examination of lesions at the site of infection and altered structures generally reveals an extremely complex interaction between the causative microbes and tissue response [14]. In the present review, we describe the pathophysiology of cryptococcosis as largely revealed in our previous histopathological investigations using an experimental animal model of cryptococcosis.

2. Elucidation of Virulence Factors of *Cryptococcus neoformans*

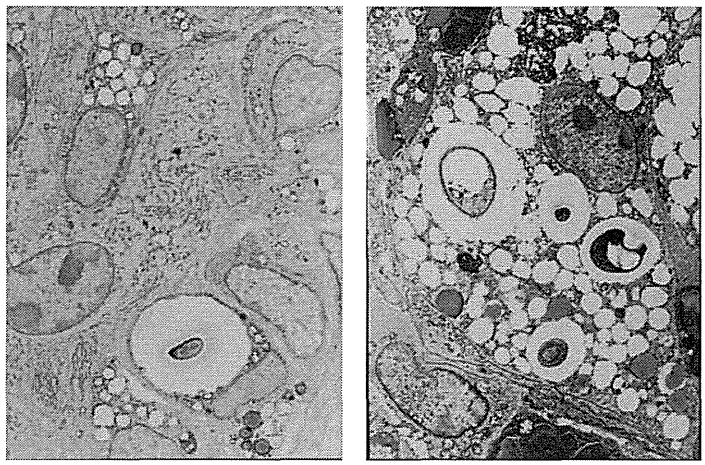
Primary foci of a cryptococcal infection tend to be established in the lungs before disseminating to the central nervous system [15]. To determine the pathogenesis of pulmonary cryptococcosis, we conducted histopathological investigations using mouse models that had been infected with either *C. neoformans* TIMM 0362, a relatively high-virulence and thus high-mortality strain, or TIMM 0372, a relatively low-virulence and thus low-mortality strain, via the intranasal route of infection [16].

Histopathological examination of the lungs of mice infected with *C. neoformans* TIMM 0362 showed yeast cell proliferation in the alveoli and a lesser macrophage response (Figure 1(a)). Time-dependent histopathological examination revealed decreased macrophage response but increased yeast-cell proliferation and alveolar space expansion with disease progression. Ultimately, fatal infection was established that involved not only the lungs but also the brain and several other organs. In contrast, the lungs of mice infected with *C. neoformans* TIMM 0372 showed numerous yeast-containing multinucleated giant cells (MGCs) due to the prominent macrophage response (Figure 1(b)). Time-dependent histopathological examination revealed a decrease in the number of yeast cells and in the macrophage response at the late phase of infection, but little or no alveolar expansion throughout the observation period. The most significant histopathological findings in the lungs of mice infected with *C. neoformans* TIMM 0362 were poor macrophage response against invasion of yeast cells, subsequent structural alteration of the lungs, and induction of cerebral cystic lesions by fatal disseminated infection. To elucidate the poor macrophage response following infection by *C. neoformans* TIMM 0362, we focused on the cell-mediated immune response against the yeast. The interaction of major histocompatibility complex (MHC) class II molecules on the surface of the antigen-presenting cells (APCs) and CD4-positive T lymphocytes plays a central role in regulating the cell-mediated immune response against infection with the *Cryptococcus* species [11, 17]. We therefore investigated the expression of MHC class II IAd molecule on the macrophage in the lungs of mice infected with *C. neoformans* TIMM 0362 and TIMM 0372 by immunohistochemical examination. As a result, the former showed no expression of MHC class II IAd molecule, and the later expressed this molecule (Figure 2). In addition, an ultrastructural examination of the macrophage in the lung of mice infected with *C. neoformans* TIMM 0362 showed a small number of phagocytized yeasts and well-preserved organelles, including rough endoplasmic reticulum and Golgi (Figure 3(a)). In contrast, that with *C. neoformans* TIMM 0372 showed a large number of phagocytized yeasts, lipid droplets, and vacuolization (Figure 3(b)). These findings suggested that poor macrophage response confirmed by histopathological examinations implies the impaired Th1 dominant cell-to-cell interaction triggered by the recognition of APCs. In fact, similar phenomenon is confirmed in the nude mice. Namely, pulmonary cryptococcosis in homozygous (nu/nu) nude mice could not induce



(a) (b)

FIGURE 2: (a) Macrophages in the lung of mice infected *C. neoformans* TIMM 0362 showed no expression of MHC class II IAd molecule (immunohistochemistry, original magnification ×400). (b) Macrophages in the lung of mice infected with *C. neoformans* TIMM 0372 expressed the molecule MHC class II IAd molecule (immunohistochemistry, original magnification ×400).

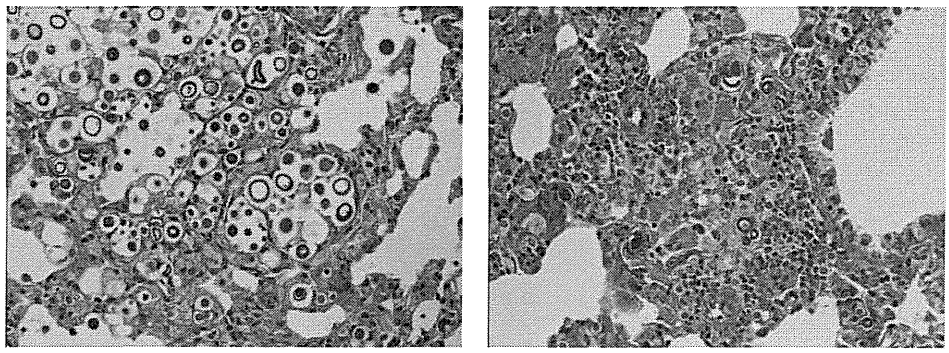


(a) (b)

FIGURE 3: (a) An ultrastructural examination of the macrophage in mice infected with *C. neoformans* TIMM 0362 showed that a large number of phagocytized lipid droplets and a small number of the yeasts, as well as lesser developed organelles. (b) An ultrastructural examination of the macrophage in mice infected with *C. neoformans* TIMM 0372 showed a large number of developed organelles, including rough endoplasmic reticulum and Golgi.

macrophage response (Figure 4(a)), whereas that in heterozygous (nu/−) nude mice could induce macrophage response with MGC formation (Figure 4(b)). Furthermore, similar histopathological findings were also observed in the lung of patients with acquired immunodeficiency syndrome (AIDS) [12, 18]. It has been well known that development of AIDS following infection with the human immunodeficiency virus (HIV) is associated with impaired cell-mediated immune response due to a reduction in CD4-positive T lymphocytes [19–21]. Histopathological examinations indicated that the absence of CD4-positive T lymphocytes the decreasing function of antigen-presenting activity in macrophages of patients with AIDS [12], and a large number of yeasts in the septal capillaries were observed (Figure 5).

The overall findings indicate that a Th1 dominant cell-to-cell interaction triggered by the macrophage recognition is one of the most important defense mechanisms against cryptococci. Our previous investigation revealed that inhaled *C. neoformans* TIMM 0362 can easily proliferate in alveoli due to the much lesser recognition by macrophages or other APCs via the MHC class II molecule, thereby causing greater structural alteration of the lungs and, ultimately, development of disseminated cryptococcosis. Consequently, poor cryptococcal yeast-cell recognition via macrophage or



(a) (b)

FIGURE 4: (a) Numerous and irregular-sized yeasts were diffusely observed in the pulmonary sections of cryptococcosis in homozygous (nu/nu) nude mice, but no multinucleated giant cell (MGC) was observed (periodic acid Schiff reaction, original magnification ×200). (b) MGCs with a well-developed and dense eosinophilic cytoplasm containing the yeasts were observed in the pulmonary sections of cryptococcosis in heterozygous (nu/−) nude mice (periodic acid Schiff reaction, original magnification ×200).

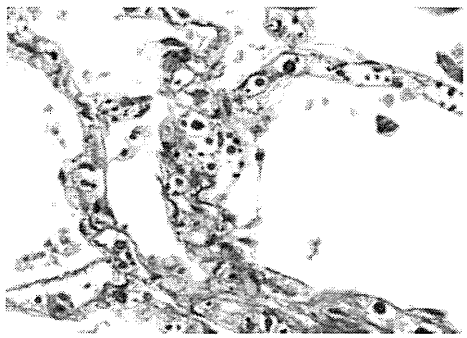


FIGURE 5: In patient with acquired immunodeficiency syndrome, a large number of yeast were observed in the pulmonary septal capillaries (periodic acid Schiff reaction, original magnification ×400).

other APCs may be closely associated with the virulence of *C. neoformans*.

3. Differences in Pathophysiology between *C. neoformans* and *C. gattii* Infection

C. gattii, which was responsible for the cryptococcosis outbreak on Vancouver Island, Canada, causes life-threatening infections in immunocompetent individuals, whereas *C. neoformans* typically causes fatal diseases only in immunocompromised individuals [22]. To elucidate the differences between *C. gattii* and *C. neoformans* infection, we previously investigated and compared the biological characteristics of the pathogens and the pathophysiology of mice infected with *C. neoformans* H99, *C. gattii* 5815, and *C. gattii* R265 [23] which is causing an outbreak on Vancouver Island, Canada [6]. The mortality rate in mice infected with *C. neoformans* H99 and *C. gattii* R265 was significantly higher than that of mice infected with *C. gattii* 5815. However, there was no significant difference in mortality rate between mice infected with *C. gattii* R265 and *C. neoformans* H99. We examined certain biological characteristics. The results showed that only the growth rate under physiological conditions suggested a correlation with the mortality rate of infected mice.

We also conducted a detailed histopathological examination. We found a significant difference in histopathological findings of the lung between mice infected with *C. gattii* R265 and *C. neoformans* H99. In particular, the former infection showed a very limited macrophage response, but eccentric enlargement of the alveolar space was prominent due to extensive proliferation of the yeast (Figures 6(a) and 6(b)), while the latter infection showed an extensive macrophage response with giant cell formation (Figures 6(c) and 6(d)). These results may suggest that extensive proliferation of yeast in alveoli and subsequent alveolar expansion during *C. gattii* R265 infection and extensive macrophage response to intruding *C. neoformans* H99 result in a significant decrease of the pulmonary gas-exchange function of alveolar septa. This highlights the fact that the pathophysiology leading to death in *C. gattii* infection differs significantly from that of *C. neoformans* infection, whereas mice infected with both strains exhibited the same mortality rate.

We now wish to describe the difference between the lungs of mice infected with *C. gattii* and *C. neoformans*. We found that sections of lungs from mice infected with both strains of *C. gattii* showed alveolar expansion, and the periphery of enlarged alveoli was usually encompassed by collapsed septa lying on top of each other. In contrast, sections of lungs from mice infected with *C. neoformans* H99 showed no alveolar expansion. This unique difference was also confirmed by our cross-point interval analysis, which is regarded as an indicator of structural alteration of the lung [24]. The results suggest that alveoli intruded by inhaled cryptococci were expanded due to extensive yeast proliferation, and that *C. gattii* can cause a greater structural alteration of the lung than *C. neoformans*.

Our study revealed that the poorest macrophage response was found in mice infected with *C. gattii* R265. It suggest that *C. gattii* R265 may have the poorest potential to induce

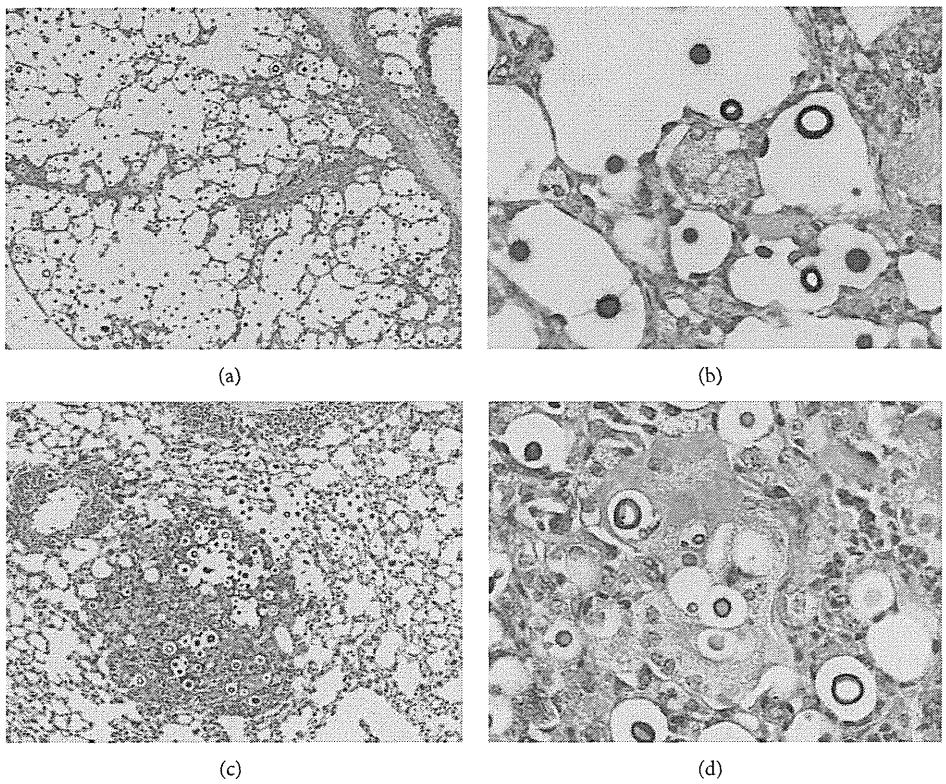


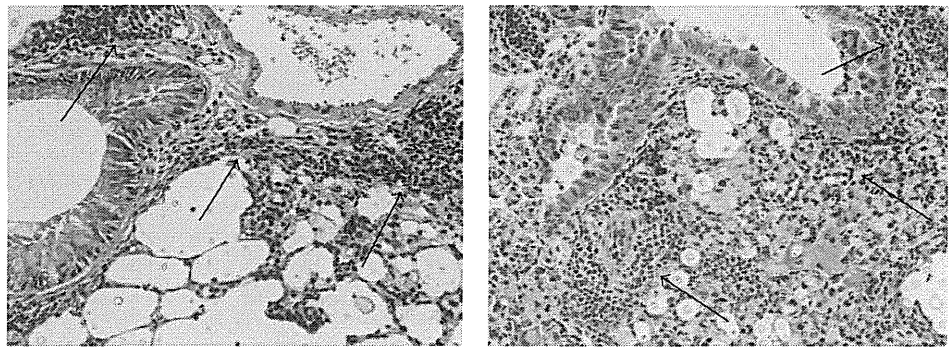
FIGURE 6: (a) Pulmonary sections of mice infected with *C. gattii* R265 exhibited eccentric pulmonary enlargement (periodic acid Schiff (PAS) reaction, ×200). (b) Pulmonary sections of mice infected with *C. gattii* R265 showed multinucleated giant cells with a foamy cytoplasm, and a small number of nuclei that were loosely aggregated (PAS reaction, ×1000). (c) Pulmonary sections of mice infected with *C. neoformans* H99 showed multiple well-demarcated nodular lesions, but no alveolar expansion was observed (PAS reaction, ×200). (d) Pulmonary sections of mice infected with *C. neoformans* H99 showed multinucleated giant cells with a well-developed and dense eosinophilic cytoplasm and a large number of nuclei (PAS reaction, ×1000).

a recognition cascade in the immune system which is consistent with a previous report [25]. We therefore conducted morphometric analyses of MGCs to quantitatively determine the strength of the macrophage response. We found that the findings of histopathological examinations may imply that *C. gattii* possesses some unknown mechanisms that allow it to avoid or escape recognition by macrophages or other antigen-presenting cells.

We now wish to provide more detailed discussion on the pathophysiology of cryptococcosis by comparing the previous investigation. Cheng et al. reported the histopathological differences between the mice infected with *C. gattii* R265 and *C. neoformans* H99 [26]. In their report, no significant difference was found for mortality rate between mice infected with *C. gattii* R265 and *C. neoformans* H99, as well as our present study. In addition, they suggested that *C. neoformans* H99 induces extensive neutrophil recruitment around the bronchovascular structures, but the ability was poor in *C. gattii* R265. Certainly, the same finding was also confirmed in our experimental murine model of cryptococcosis (Figures 7(a) and 7(b)). Since it has been reported that the migration of neutrophils into lung tissue is important in the early protection of mice against progressive cryptococcosis [27], the degree of neutrophil recruitment may be related to difference of pathophysiology between *C. gattii* and *C. neoformans* infections.

On the other hand, Cheng et al. reported that no significant difference was found in number of macrophages between *C. gattii* R265 and *C. neoformans* H99 infected mice, whereas our study revealed that macrophage response of the mice infected with *C. gattii* R265 was poor, but that infected with *C. neoformans* H99 was extensive. The contradictory result may attribute to the difference of the experimental design and analysis methods. There were two important differences: one is routes of pulmonary infection (intranasal infection versus intratracheal infection), and another is term of infection (7 days versus 14 days). Since we were able to observe histopathological findings of the lesion just before death of the infected mice, macrophage response could be amplified in our study. In addition, Cheng et al. employed flow-cytometric analysis to count the number of macrophages that is superior to manual counting of the cell, but it cannot count the number of macrophages within MGC, accurately. Since macrophage fused together to form a MGC, the flow-cytometric analysis was unable to accurately assess the macrophage response.

Consequently, there is an extensively different pathophysiology leading to death between *C. gattii* and *C. neoformans*



(a) (b)

FIGURE 7: (a) Pulmonary sections of mice infected with *C. gattii* R265 exhibited poor neutrophil recruitment around the bronchovascular structures (arrowheads, hematoxylin and eosin (HE) double stain, and ×400). (b) Pulmonary sections of mice infected with *C. neoformans* H99 showed extensive neutrophil recruitment around the bronchovascular structures (arrowheads, HE double stain, and ×400).

infections. Namely, both extensive proliferation of the yeast in alveoli during *C. gattii* R265 infection and macrophage response to intruding *C. neoformans* H99 would cause respiratory failure of the mice. Despite the same mortality rates between mice infected with *C. gattii* R265 and *C. neoformans* H99 was found, the pathophysiology leading to death between *C. gattii* and *C. neoformans* infection was different, which was only revealed by histopathological examination. We therefore wish to emphasize the usefulness of the yield of the histopathological examination for experimental animal model.

4. Exploration to Know Specific Virulence Factors of *C. gattii*

We found that *C. gattii* is characterized by its ability to induce high pulmonary structural alteration [23]. To gain an understanding of the cause of this unique feature, we investigated the characteristics of seven strains of *C. gattii* (TIMM 4097 and TIMM 4901 to 4906) [28, 29]. *C. gattii* TIMM 4097, TIMM 4901 to 4906 were isolated from Japanese zoo-bred koalas. Serotypes of the strain were confirmed using slide agglutination tests according to the manufacturer's instructions (Cryptocheck Iatron RM 304-K kit; Mitsubishi Kagaku Iatron, Inc., Tokyo, Japan). All animal studies were performed in accordance with the guidelines and permission of the animal experiment care committee of the Teikyo University. On day 100 after infection, the cumulative mortality rates in mice infected with *C. gattii* TIMM 4097 was 10/10 (100.0%), whereas the rates in mice infected with *C. gattii* TIMM 4901 to 4906 were 0/10 (0.0%). According to Kaplan-Meier survival analysis with a log-rank significance test, the mortality rate in mice infected with *C. gattii* TIMM 4097 was significantly higher than that of mice infected with *C. gattii* TIMM 4901 to 4906.

To elucidate factors affecting virulence of the yeast, we examined biological characteristics of seven strains of *C. gattii*, including growth rate, capsule thickness, melanin production, and hydrolase (urease, proteinase, and phospholipase) activity. Despite the significant differences in mortality rate among mice infected with the different strains, no significant differences were found among the strains regarding any of the characteristics examined, except for growth rate. The results showed that only the growth rate suggested a correlation with the mortality rate of infected mice.

TABLE 1: The molecular type of seven strains of *Cryptococcus gattii* was investigated using multilocus sequence typing (MLST) analysis. Although the mortality rate of mice infected with *C. gattii* TIMM 4907 was significantly higher than that of mice infected with *C. gattii* TIMM 4901 to 4906, MLST analysis revealed that the molecular type of all seven strains was VG I.

Molecular types of *Cryptococcus gattii*	
Strain	Molecular type
C. gattii TIMM 4097	VG I
C. gattii TIMM 4901	VG I
C. gattii TIMM 4902	VG I
C. gattii TIMM 4903	VG I
C. gattii TIMM 4904	VG I
C. gattii TIMM 4905	VG I
C. gattii TIMM 4906	VG I

Since previous investigations have reported that the molecular type of a *C. gattii* strain plays a strong role in its virulence [22], we investigated the molecular type of seven strains of *C. gattii* using multilocus sequence typing (MLST) analysis in accordance with the consensus *C. gattii* typing scheme established by the Cryptococcal Working Group I of the International Society for Human and Animal Mycology (ISHAM) [30], which includes the following seven unlinked genetic loci: *CAP59, GPD1, LAC1, PLB1, SOD1, URA5,* and *IGS1*. Although the mortality rate of mice infected with *C. gattii* TIMM 4907 was significantly higher than that of mice infected with *C. gattii* TIMM 4901 to 4906, MLST analysis revealed that the molecular type of all seven strains was VG I (Table 1), indicating that the virulence of these *C. gattii* strains may not be regulated by their molecular type.

Whereas there were no differences in biological characteristics among the seven *C. gattii* strains of VG I isolated

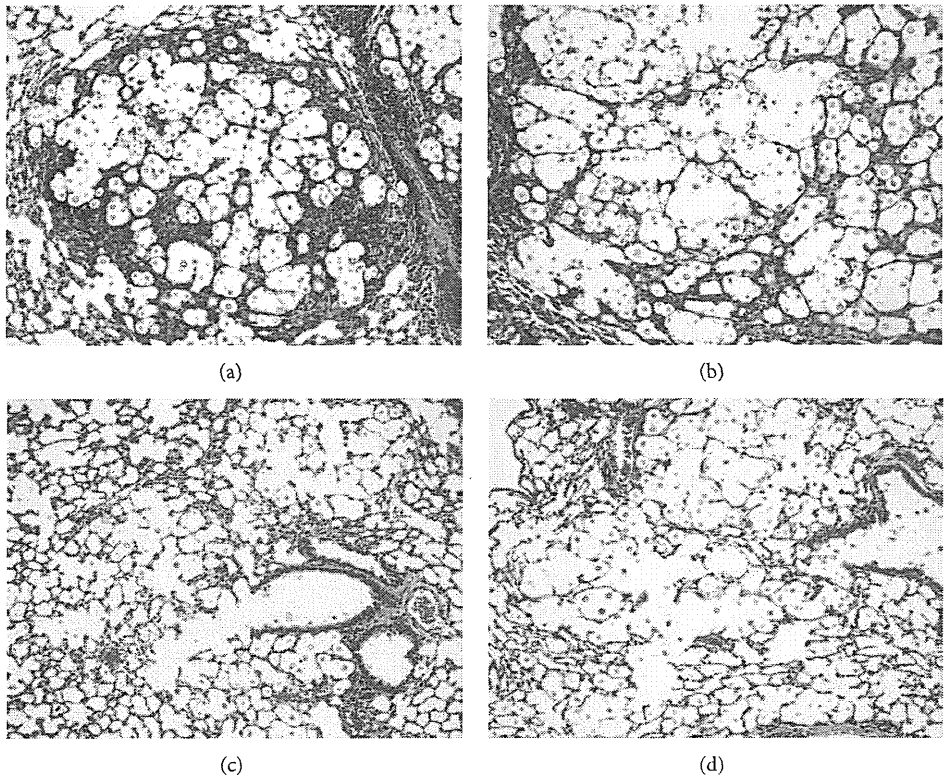


FIGURE 8: (a, b) On days 15 and 30 after infection, pulmonary sections of mice infected with *C. gattii* TIMM 4097 showed eccentric enlargement of alveoli containing prominent proliferation of yeast cells with disease progression (periodic acid Schiff (PAS) reaction, ×100, resp.). (c, d) On days 15 and 30 after infection, pulmonary sections of mice infected with *C. gattii* TIMM 4903 showed lesser degree of yeast cell proliferation and alveolar expansion than these with *C. gattii* TIMM 4097 (PAS reaction, ×100, resp.).

from Japanese zoo-bred koalas, the mortality rate of mice infected with *C. gattii* TIMM 4097 was significantly higher than that of mice infected with other six *C. gattii* strains. Therefore, we conducted detailed histopathological examination to gain an insight into the pathophysiology underlying the virulence. As the *C. gattii* TIMM 4901 to 4906 strains were found to have the same phenotypic expression, molecular type, and mortality rate in infected mice, *C. gattii* TIMM 4903 was randomly selected as a representative type for further examination and comparison with *C. gattii* TIMM 4097. Time-dependent histopathological examination revealed that pulmonary sections obtained from mice infected with *C. gattii* TIMM 4097 showed eccentric enlargement of alveoli containing prominent proliferation of yeast cells with disease progression (Figures 8(a) and 8(b)). In contrast, pulmonary sections of mice infected with *C. gattii* TIMM 4903 showed little or no alveolar expansion and contained a smaller number of yeast cells than sections of mice infected with *C. gattii* TIMM 4097 (Figures 8(c) and 8(d)), and quite interestingly, several yeast cells in the bronchi.

To gain a detailed understanding of the structural alteration of the lungs, the cross-point interval was measured. Analyses of the mean value and variance of the cross-point interval revealed significant differences between the mean value and variance of mice infected with *C. gattii* TIMM 4097 and 4903 on days 15 and 30 after infection (Figure 9).

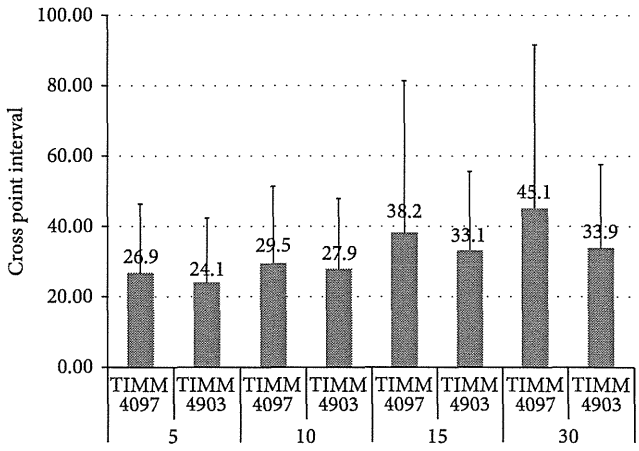


FIGURE 9: The mean ± standard deviation (SD) values of the cross-point interval of the lungs of the mice infected with *C. gattii* TIMM 4097 on days 5, 10, 15, and 30 after infection were 26.86 ± 19.48, 29.46 ± 21.89, 38.21 ± 43.08, and 45.13 ± 46.48 μm, respectively. In contrast, that of mice infected with *C. gattii* TIMM 4097 were 24.06 ± 18.30, 27.90 ± 20.00, 33.07 ± 22.49, and 33.92 ± 23.70 μm, respectively. Analyses of the mean value and variance of the cross-point interval revealed significant differences between the mean value and variance of mice infected with *C. gattii* TIMM 4097 and 4903 on days 15 and 30 after infection.

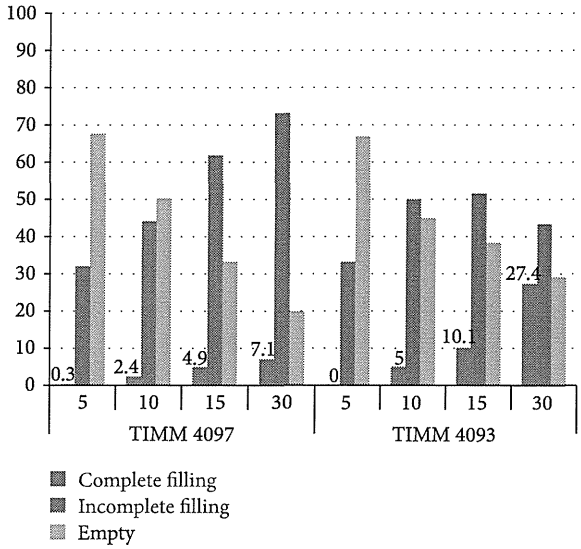


FIGURE 10: The percentage of bronchi that was "completely full" in mice infected with *C. gattii* TIMM 4097 on days 10, 15, and 30 after infection was 2.4, 4.9, and 7.1%, respectively, while that of mice infected with *C. gattii* 4903 was 5.0, 10.1, and 27.4%, respectively. Results revealed that the ratio of "completely full" bronchi in mice infected with *C. gattii* TIMM 4903 was significantly higher than that of mice infected with *C. gattii* TIMM 4097 throughout the observation period.

These results suggest that although alveoli invaded by inhaled cryptococci of any strain expand due to extensive yeast proliferation, inhalation of *C. gattii* TIMM 4097 may cause greater structural alteration of the lungs than *C. gattii* TIMM 4903 at a late phase of infection, which may be due to the greater ability of *C. gattii* TIMM 4097 to reside in the alveolar lumen compared to *C. gattii* TIMM 4903.

The results of the histopathological examination and morphometric analysis of the lungs of infected mice indicated that infection with *C. gattii* TIMM 4097 (a high-virulence strain) tend to induce greater alteration of lung structure than infection with *C. gattii* TIMM 4903 (a low-virulence strain). To investigate the hypothesis that *C. gattii* TIMM 4097 tends to reside in the alveoli, the extent to which the yeast cells filled the bronchi was determined by semiquantitative assessment. All bronchi of the pulmonary PAS-stained sections were observed, and the bronchi were classified as "empty," "partially full," or "completely full" based on the degree to which the bronchi were filled with yeast cells. Results revealed that the ratio of "completely full" bronchi in mice infected with *C. gattii* TIMM 4903 was significantly higher than that of mice infected with *C. gattii* TIMM 4097 throughout the observation period (Figure 10). This assessment of the bronchi supported our hypothesis that *C. gattii* TIMM 4097 can rigidly reside in the alveoli. To test this hypothesis, the number of viable cells in the lungs and trachea of mice infected with each strain was also counted. Statistical analysis revealed that the mean $\log_{10}$ CFU count in the lungs of mice infected with *C. gattii* TIMM 4097 was significantly higher than that of mice infected with *C. gattii* TIMM 4903

TABLE 2: The probability of the value of each data point was indicated as a "flag," such as "present" ($P \leq 0$ to < 0.04), "marginal" ($P \leq 0.04$ to < 0.06), or "absent" ($P \leq 0.06$ to < 0.5). Gene expression that contained the "absent" flag (expression of 39,181 genes) was excluded, and the expression of 5,920 genes was extracted.

	Absent	Marginal	Present
Control	22562	1016	21523
C. gattii TIMM 4097	26505	1068	17528
C. gattii TIMM 4903	37445	937	6719

in each evaluation period except for day 5 after infection (Figure 11). Nevertheless, and quite interestingly, the mean $\log_{10}$ CFU counts in the tracheae of mice infected with *C. gattii* TIMM 4903 on days 5, 10, and 15 after infection were significantly higher than those of mice infected with *C. gattii* 4097 (Figure 11). These results support the hypothesis that *C. gattii* TIMM 4097 tends to reside in the alveoli, whereas *C. gattii* TIMM 4903 tends to be washed out from the alveoli and move into the central side of the respiratory system.

A microarray assay with gene ontology analysis was also performed with reference to the previous investigation [31]. Gene expression in the lungs of mice infected with *C. gattii* TIMM 4097 and 4903 was measured and compared using microarray analysis (Affymetrix GeneChip microarrays). All the microarray data are deposited in GEO (accession number GSE48595). The microarray analysis detected the expression of 45,101 genes (Figure 12) and the probability of the value of each data point was indicated as a "flag," such as "present" ($P \leq 0$ to <0.04), "marginal" ($P \leq 0.04$ to <0.06), or "absent" ($P \leq 0.06$ to <0.5). Gene expression that contained the "absent" flag (expression of 39,181 genes) was excluded, and the expression of 5,920 genes was extracted (Table 2). As referenced in previous investigations, a second selection was then performed using a cut-off value indicating at least a ±1.5-fold change (log 2 ratio). The results revealed that although 219 genes were upregulated in the lungs of mice infected with *C. gattii* TIMM 4097, only 175 genes were upregulated in the lungs of mice infected with C. *gattii* TIMM 4903. Thus, 35 genes were upregulated in the lungs of mice infected with either *C. gattii* TIMM 4097 or 4903, while 184 genes were upregulated only in the lungs of mice infected with *C. gattii* TIMM 4097 (Figure 13). To examine the hypothesis that upregulation of genes in mice infected with *C. gattii* TIMM 4097 is related to the high virulence of the strain, Gene Ontology (GO) analysis of the stored genes was conducted using the Biological Networks Gene Ontology tool (BINGO, http://www.psb.ugent.be/cbd/papers/BiNGO/) to identify statistically overrepresented GO categories among the biological data. For the 184 genes upregulated only in the lungs of mice infected with *C. gattii* TIMM 4097, 163 terms related to molecular functioning were detected, and GO terms related to binding accounted for about 75% of all terms (Figure 14).

Our previous investigations suggested that *C. gattii* TIMM 4097 rigidly resides in the alveolar space and that this may cause alveolar expansion, whereas *C. gattii* TIMM 4903 is likely to be washed out from the alveoli and move

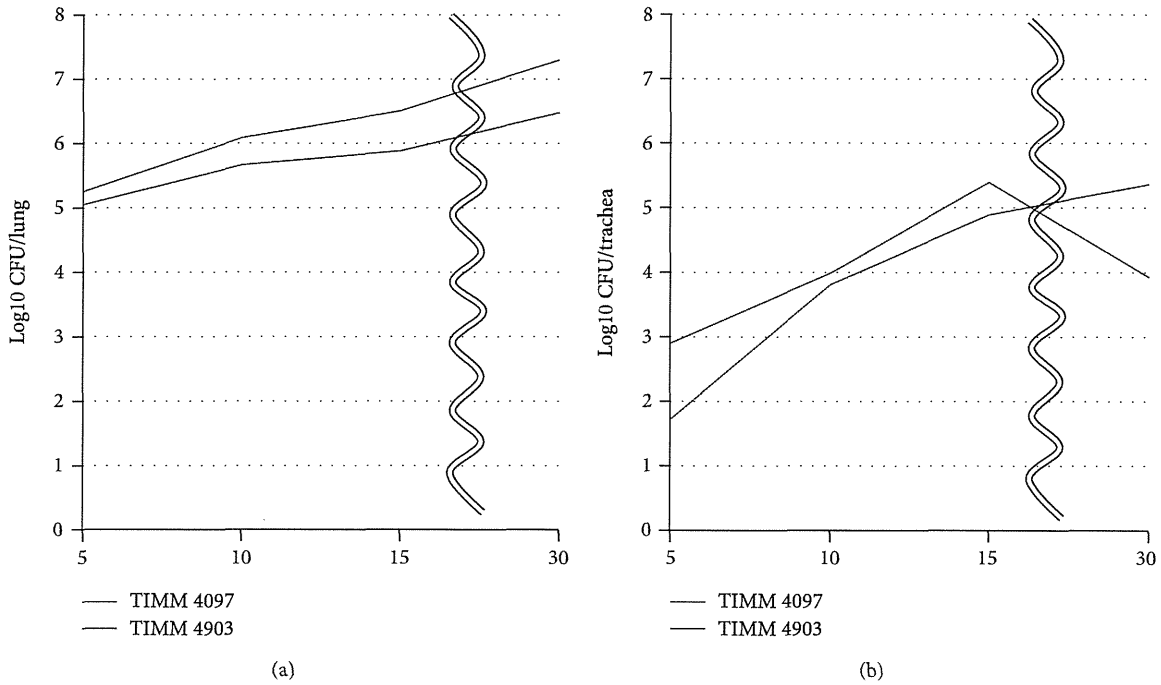


FIGURE 11: The mean ± SD $\log_{10}$ CFU/mL counts in the lungs of mice infected with *C. gattii* TIMM 4097 on days 5, 10, 15, and 30 after infection were 5.25 ± 0.16, 6.09 ± 0.05, 6.51 ± 0.12, and 7.30 ± 0.08, respectively, similar to those of mice infected with *C. gattii* TIMM 4903, which were 5.05 ± 0.19, 5.67 ± 0.14, 5.89 ± 0.12, and 6.48 ± 0.24, respectively. In contrast, the mean ± SD $\log_{10}$ CFU/mL counts in the tracheae of mice ys 5, 10, 15, and 30 after infection were 1.72 ± 0.54, 3.81 ± 0.11, 4.89 ± 0.60, and 5.37 ± 0.18, respectively, similar to those of mice infected with *C. gattii* TIMM 4903, which were 2.90 ± 0.14, 3.99 ± 0.04, 5.40 ± 0.10, and 3.93 ± 0.52, respectively. Statistical analysis revealed that the mean $\log_{10}$ CFU count in the lungs of mice infected with *C. gattii* TIMM 4097 was significantly higher than that of mice infected with *C. gattii* TIMM 4903 in each evaluation period except for day 5 after infection. Nevertheless, the mean $\log_{10}$ CFU counts in the tracheae of mice infected with *C. gattii* TIMM 4903 on days 5, 10, and 15 after infection were significantly higher than those of mice infected with *C. gattii* 4097.

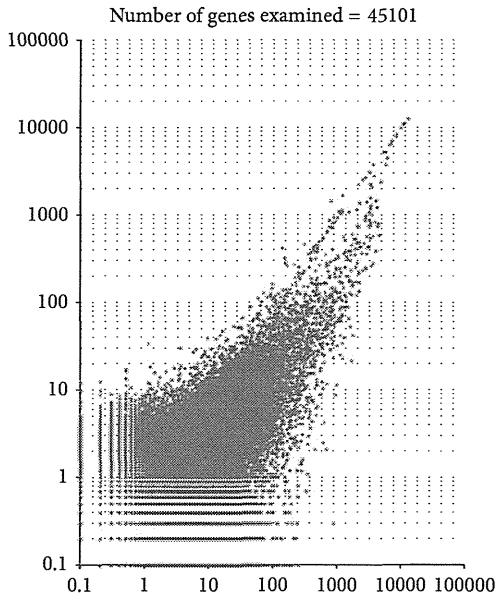


FIGURE 12: Gene expression in the lungs of mice infected with *C. gattii* TIMM 4097 and 4903 was measured and compared using microarray analysis. The microarray analysis detected the expression of 45,101 genes.

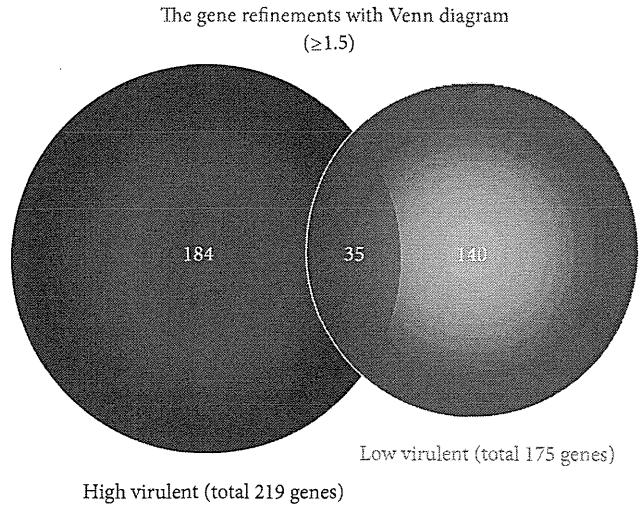


FIGURE 13: Whereas 219 genes were upregulated in the lungs of mice infected with *C. gattii* TIMM 4097 (a high-virulence strain), only 175 genes were upregulated in the lungs of mice infected with *C. gattii* TIMM 4903 (a low-virulence strain). Thus, 35 genes were upregulated in the lungs of mice infected with either *C. gattii* TIMM 4097 or 4903, while 184 genes were upregulated only in the lungs of mice infected with *C. gattii* TIMM 4097.

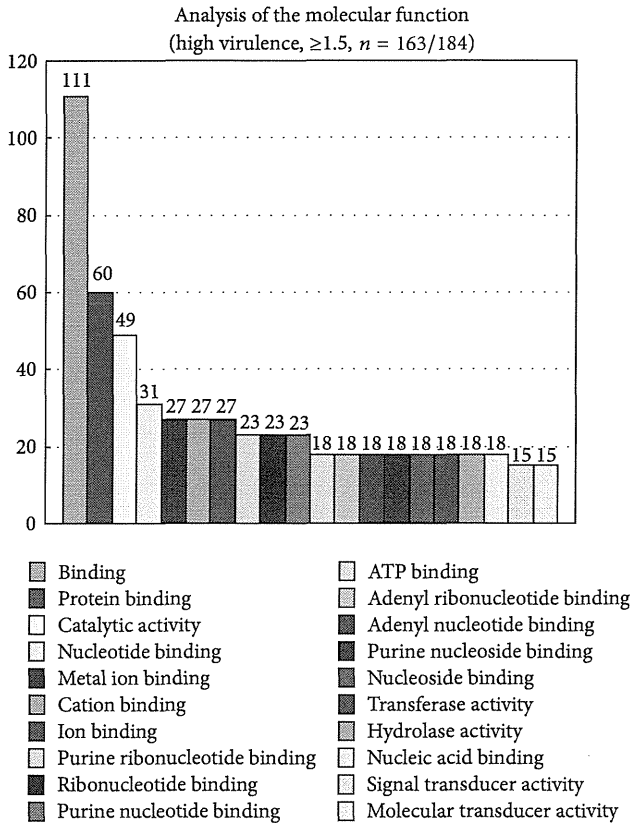


FIGURE 14: Analysis of the molecular function of stored genes was conducted using the Biological Networks Gene Ontology tool (BINGO, http://www.psb.ugent.be/cbd/papers/BiNGO/) to identify statistically overrepresented GO categories among the biological data. For the 184 genes upregulated only in the lungs of mice infected with *C. gattii* TIMM 4097, 163 terms related to molecular functioning were detected, and GO terms related to binding accounted for about 75% of all terms.

into the central side of the respiratory system, as indicated by observation of little change in lung structure of mice infected with this strain. These differences may have arisen from the different capacity of the two strains to adhere onto the alveolar epithelium and is partly confirmed by our previous study.

5. Conclusion

The defense mechanism against cryptococci may vary among individuals, depending on the agents, tissues involved, and immune status of the host. Given these considerations, we wish to emphasize the usefulness of histopathological examinations, because it allowed more detailed information of an extremely complex interaction between the causative microbes and tissue response.

Authors' Contributions

All authors contributed towards the conceptualization, writing, reading, and approval of the final version of the paper. In particular, Yoichiro Okubo and Naobumi Tochigi conceptualized this study, integrated the data, wrote the paper, and contributed equally to this work.

Conflict of Interests

Dr. Shibuya reports receiving research grants from Pfizer Inc., Janssen Pharmaceutical K. K., Dainippon Sumitomo Pharma Co., Astellas Pharma Inc., Taiho Pharmaceutical Co., and POLA-Pharma Inc. Other authors declare that they have no competing interests.

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Multilocus sequence typing of *Cryptococcus neoformans* in non-HIV associated cryptococcosis in Nagasaki, Japan

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Cryptococcosis is primarily caused by two *Cryptococcus* species, i.e., *Cryptococcus neoformans* and *C. gattii*. Both include several genetically diverse subgroups that can be differentiated using various molecular strain typing methods. Since little is known about the molecular epidemiology of the *C. neoformans/C. gattii* species complex in Japan, we conducted a molecular epidemiological analysis of 35 *C. neoformans* isolates from non-HIV patients in Nagasaki, Japan and 10 environmental isolates from Thailand. All were analyzed using *URA5*-restriction fragment length polymorphism (RFLP) and multilocus sequence typing (MLST). Combined sequence data for all isolates were evaluated with the neighbor-joining method. All were found to be serotype A and mating type MATα. Thirty-two of the 35 clinical isolates molecular type VNI, while the three remaining isolates were VNII as determined through the *URA5*-RFLP method. Thirty-one of the VNI isolates were identified as MLST sequence type (ST) 5, the remaining one was ST 32 and the three VNII isolates were found to be ST 43. All the environmental isolates were identified as molecular type VNI (four MLST ST 5 and six ST 4). Our study shows that *C. neoformans* isolates in Nagasaki are genetically homogeneous, with most of the isolates being ST 5.

Keywords MLST, *C. neoformans*, Japan, ST 5, VNII

Introduction

It had been previously proposed that *Cryptococcus neoformans* contained two varieties, i.e., *C. neoformans* var. *neoformans*, the opportunistic agent of cryptococcosis in immunosuppressed hosts, and *C. neoformans* var. *gattii*, a probable causative agent of cryptococcosis in immunocompetent hosts [1]. Recently, *C. neoformans* var. *gattii* was proposed and accepted as a separate species, *Cryptococcus gattii*, due to its divergent ecological, biochemical, and molecular characteristics [2]. Today, the *C. neoformans/C. gattii* species complex comprises *C. neoformans* var. *neoformans* (serotype D), the hybrid isolates (serotype AD), *C. neoformans* var. *grubii* (serotype A) [3], and *C. gattii* (serotypes B and C) [4]. Several molecular typing methods are widely used for epidemiological molecular analysis of this species complex [5–8]. For example, polymerase chain

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reaction (PCR) fingerprint patterns based on M13 microsatellite DNA has been employed to identify eight major molecular types [8]. The molecular types VNI and VNII are associated with serotype A (*C. neoformans* var. *grubii*), while VNIII is linked with serotype AD (hybrid) and VNIV with serotype D (*C. neoformans* var. *neoformans*). Although the majority of isolates of molecular types VGI and VGII belong to serotype B (*C. gattii*), some molecular types VGI, VGII isolates, as well as VGIII, and VGIV types do not correlate with members of this specie [9].

Numerous studies on strain typing have been reported worldwide [8,10–16] with no geographical differences being found in the distribution of the types, including investigation in Far East Asian countries such as Korea and China [17,18]. However, many of these studies employed clinical isolates recovered from HIV-positive patients, whereas studies in Korea and China dealt with those obtained from non-HIV patients. Whether predominant strains with unique genotypes exist in patients with different backgrounds is not clear. Because the prevalence of HIV infection is lower in Japan (less than 0.1% approximately) compared to other countries, the epidemiological features of genotypes are worth investigating in order to compare the distribution of molecular types found in Asia as compared with other countries from different continents [19]. To investigate the molecular epidemiology of the *C. neoformans/C. gattii* species complex in Japan, we performed *URA5*-restriction fragment length polymorphism (RFLP) and MLST with 35 *C. neoformans* isolates from HIV-negative patients.

Materials and methods

Isolates

A total of 10 environmental *Cryptococcus* isolates obtained in Thailand and 35 clinical isolates recovered in Nagasaki, Japan, between 1996 and 2010 were analyzed. Species identification was initially determined by standard mycological methods [20] and confirmed with a commercial identification system (RapID Yeast Plus System) [21]. All isolates were stored in 25% glycerol at − 80°C until use and maintained on Sabouraud dextrose agar at 25°C during the study. Reference strains of the known major molecular types of the *C. neoformans* and *C. gattii* species complex were included, i.e., WM148 (serotype A, VNI), WM626 (serotype A, VNII), WM628 (serotype AD, VNIII), WM629 (serotype D, VNIV), WM179 (serotype B, VGI), WM178 (serotype B, VGII), WM175 (serotype B, VGIII), and WM779 (serotype C, VGIV) [8]. These were kindly provided by Dr June Kwon-Chung (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA).

Determination of serotype and mating type by PCR

Genomic DNA was extracted from each isolate and purified using a MasterPure yeast DNA purification kit (Epicentre Biotechnologies, Madison, WI, USA) following the manufacturer's protocol. The mating type of each of the isolates was established through the use of four different PCR amplification reactions. Primers specific to the MATα or MATa allele of the *STE20* locus for either serotype A or D isolates were used, that is, primers JOHE7270 and JOHE7272 (aA), JOHE7273/JOHE7275 (aD), JOHE7264/JOHE7265 (αA), and JOHE7267/JOHE7268 (αD), as described previously [22–24]. The PCR products were electrophoresed on 1% or 1.5% agarose gels in 1 × Tris-acetate-EDTA buffer at 100 V for 30 min and then visualized under ultraviolet light.

Genotype determination by URA5-RFLP

URA5-RFLP analysis with the enzymes *Hha*I and *Sau*96I was performed initially to verify the molecular type of each studied isolate as previously described [8].

MLST analysis

All isolates were analyzed by MLST, using the ISHAM consensus scheme of seven genetic loci (*CAP59*, *GPD1*, *LAC1*, *IGS1*, *PLB1*, *SOD1*, and *URA5*). Each locus was amplified using the primers and amplification parameters described by the ISHAM Cryptococcal Working Group [25] and analyzed as reported previously [7,17,18]. Sequences of additional strains of the *C. neoformans/C. gattii* species complex were retrieved from previous studies [7,8,17,26,27] (Table 1). Combined sequence data for all isolates were analyzed with the neighbor-joining method. Alleles and sequence types were designated in accord with previous publications [7,28] and based on the *Cryptococcus neoformans* MLST database maintained at the Molecular Mycology Research Laboratory at the University of Sydney (mlst.mycologylab.org).

Clinical records

Information on the 26 clinical isolates recovered in Nagasaki University Hospital between 1996 and 2010 was acquired from medical records. We investigated patients' sex, age, underlying diseases, steroid use, specimen used for identification, complication of encephalomeningitis, and case outcomes. This retrospective study including the analysis and release of

Table 1 List of additional isolates used in MLST analysis.

Name	Country	Source	Molecular type	Genotype*	References
c8	United States	Clinical	VNI	M5	[7]
jp1088	Japan	Clinical	VNI	M5	[7]
CHC186	China	Clinical	VNIc	M5	[17]
bt134	Botswana	Clinical	VNI	M5	[7]
it743	Italy	Unknown	VNI	M5	[7]
c48	United States	Clinical	VNI	M5	[7]
K1	Korea	Clinical	VNIc	M5	[18]
ug2463	Uganda	Clinical	VNI	M10	[7]
K30	Korea	Clinical	VNI	M10	[18]
A3 1-1	United States	Environmental	VNI	M3	[7]
H99	United States	Clinical	VNI	M1	[27]
WM148	Australia	Clinical	VNI	M1	[8]
K37	Korea	Clinical	VNI	M3	[18]
br795	Brazil	Unknown	VNI	M4	[7]
ug2471	Uganda	Clinical	VNI	M4	[7]
K54	Korea	Clinical	VNI	M4	[18]
K53	Korea	Clinical	VNI	M4	[18]
bt88	Botswana	Clinical	VNI/VNB	NA	[7]
bt85	Botswana	Clinical	VNI/VNB	NA	[7]
bt131	Botswana	Clinical	VNI/VNB	NA	[7]
WM626	Australia	Clinical	VNII	NA	[8]
WM629	Australia	Clinical	VNIV	NA	[8]

*Genotype determined previously by 12 loci MLST [7].

clinical data was approved by the ethical committee of Nagasaki University Hospital.

Results

Serotypes and mating types

All of the 35 clinical and 10 environmental isolates corresponded to *C. neoformans*, serotype A and mating type MATα.

Genotyping

Thirty-two of the 35 clinical isolates proved to be VNI and the remaining 3 were VNII. All 10 environmental isolates were identified as molecular type VNI by *URA5*-RFLP.

MLST analysis

Of the 35 clinical isolates, 32 and 3 were identified as molecular type VNI and VNII, respectively. Thirty-one VNI isolates were identified as MLST sequence type (ST) 5, which possess alleles 1, 3, 5, 2, 1, 1 and 1 at loci *CAP59*, *GPD1*, *LAC1*, *PLB1*, *SOD1*, *URA5*, and *IGS1*, respectively. The other VNI isolate was identified as ST 32, whereas the 3 VNII type isolates were identified as ST 43. All environmental isolates were identified as molecular type VNI (four ST 5 and six ST 4). Table 2 gives a summary of the genotypes and molecular types of all studied isolates. Thirty-one of the clinical isolates, which were ST 5, correlated with the VNIc/M5 genotype clade, as identified by 12 loci MLST typing [7], while one ST 32 isolate (ngs23) was identified as belonging to the VNI-M4 clade by neighbor-joining analysis [7] (Fig. 1). ST 4 isolates belong to VNI-M10 (Fig. 1). The ST 5 isolates were identical with the VNIc/M5 type, which represents the major molecular type of the Korean and Chinese isolates identified by the M13 fingerprinting method.

Clinical features

The 35 patients with cryptococcosis (19 males and 16 females) were Japanese and confirmed as HIV-negative. Detailed information on 26 of the clinical isolates was available from the medical records, but detailed records, except HIV infection status, of the other nine clinical isolates could not be obtained. Table 3 gives a summary of the patients' clinical features.

Patient ages ranged from 21–86 years (mean 62.3 years) and none had a history of foreign travel. The majority of isolates were recovered from bronchoalveolar lavage fluid ($n = 19$), followed by sputum ($n = 7$), cerebrospinal fluid ($n = 2$), of unknown origin ($n = 3$), autopsy specimen ($n = 2$), blood specimen ($n = 1$), and lymph node specimen ($n = 1$).

Nine of the 26 patients were apparently healthy and 17 had underlying diseases and conditions, including cancer ($n = 4$), diabetic mellitus ($n = 3$), collagen disease

Table 2 The allelic profiles of the 35 clinical isolates from Nagasaki and the 10 environmental isolates from Thailand typed by MLST in this study.

Name	*CAP59*	*GPD1*	*IGS1*	*LAC1*	*PLB1*	*SOD1*	*URA5*	Serotype	Mating type	Sequence type	Molecular type	Year of isolation
ngs1	1	3	1	5	2	1	1	A	α	5	VNI	2007
ngs2	1	3	1	5	2	1	1	A	α	5	VNI	2008
ngs3	1	3	1	5	2	1	1	A	α	5	VNI	2008
ngs4	1	3	1	5	2	1	1	A	α	5	VNI	2008
ngs6	1	3	1	5	2	1	1	A	α	5	VNI	2008
ngs9	1	3	1	5	2	1	1	A	α	5	VNI	2009
ngs10	1	3	1	5	2	1	1	A	α	5	VNI	2009
ngs11	1	3	1	5	2	1	1	A	α	5	VNI	2003
ngs12	1	3	1	5	2	1	1	A	α	5	VNI	2004
ngs14	1	3	1	5	2	1	1	A	α	5	VNI	2004
ngs16	1	3	1	5	2	1	1	A	α	5	VNI	2005
ngs17	1	3	1	5	2	1	1	A	α	5	VNI	2005
ngs18	1	3	1	5	2	1	1	A	α	5	VNI	2005
ngs20	1	3	1	5	2	1	1	A	α	5	VNI	2006
ngs21	1	3	1	5	2	1	1	A	α	5	VNI	1997
ngs24	1	3	1	5	2	1	1	A	α	5	VNI	1998
ngs29	1	3	1	5	2	1	1	A	α	5	VNI	1998
ngs30	1	3	1	5	2	1	1	A	α	5	VNI	1999
ngs32	1	3	1	5	2	1	1	A	α	5	VNI	2000
ngs33	1	3	1	5	2	1	1	A	α	5	VNI	2000
ngs42	1	3	1	5	2	1	1	A	α	5	VNI	2001
ngs43	1	3	1	5	2	1	1	A	α	5	VNI	2001
ngs45	1	3	1	5	2	1	1	A	α	5	VNI	2001
ngs47	1	3	1	5	2	1	1	A	α	5	VNI	2001
ngs50	1	3	1	5	2	1	1	A	α	5	VNI	2001
ngs51	1	3	1	5	2	1	1	A	α	5	VNI	2001
ngs53	1	3	1	5	2	1	1	A	α	5	VNI	2002
ngs54	1	3	1	5	2	1	1	A	α	5	VNI	2002
ngs55	1	3	1	5	2	1	1	A	α	5	VNI	2003
ngs57	1	3	1	5	2	1	1	A	α	5	VNI	2003
ngs72	1	3	1	5	2	1	1	A	α	5	VNI	2003
ngs23	1	1	10	3	4	1	1	A	α	32	VNI	1997
ngs28	2	9	14	8	11	11	4	A	α	43	VNII	1998
ngs19	2	9	14	8	11	11	4	A	α	43	VNII	2006
ngs35	2	9	14	8	11	11	4	A	α	43	VNII	2000
ngsth59	1	3	1	5	2	1	1	A	α	5	VNI	2001
ngsth60	1	3	1	5	2	1	1	A	α	5	VNI	2001
ngsth62	1	3	1	5	2	1	1	A	α	5	VNI	2001
ngsth66	1	3	1	5	2	1	1	A	α	5	VNI	2001
ngsth61	1	1	1	4	2	1	5	A	α	4	VNI	2001
ngsth64	1	1	1	4	2	1	5	A	α	4	VNI	2001
ngsth65	1	1	1	4	2	1	5	A	α	4	VNI	2001
ngsth67	1	1	1	4	2	1	5	A	α	4	VNI	2001
ngsth68	1	1	1	4	2	1	5	A	α	4	VNI	2001
ngsth70	1	1	1	4	2	1	5	A	α	4	VNI	2001

ngs, clinical strain in Nagasaki; ngsth, environmental strain in Thailand.
Sequence type is the type defined using 10-locus MLST. Molecular type is the type defined using the URA5-RFLP method.

($n = 3$), rheumatoid arthritis ($n = 2$), malignant lymphoma ($n = 1$), adult T-cell leukemia ($n = 1$), acute interstitial pneumonia ($n = 1$), end-stage kidney disease ($n = 2$), neurofibromatosis type 1 ($n = 1$), and pregnancy ($n = 1$). A total of 24 patients received lumbar puncture to rule out central nervous system infection, and 4 patients had encephalomeningitis.

All of the patients with isolates of ST 5 responded to treatment with fluconazole, voriconazole, and itraconazole except one who died of systemic cryptococcosis after steroid pulse treatment for acute interstitial pneumonia. Only one ST 32 was isolated from the sputum of a 72-year-old man with pulmonary cryptococcosis. The patient was human adult T cell leukemia virus-I carrier with pharyngeal

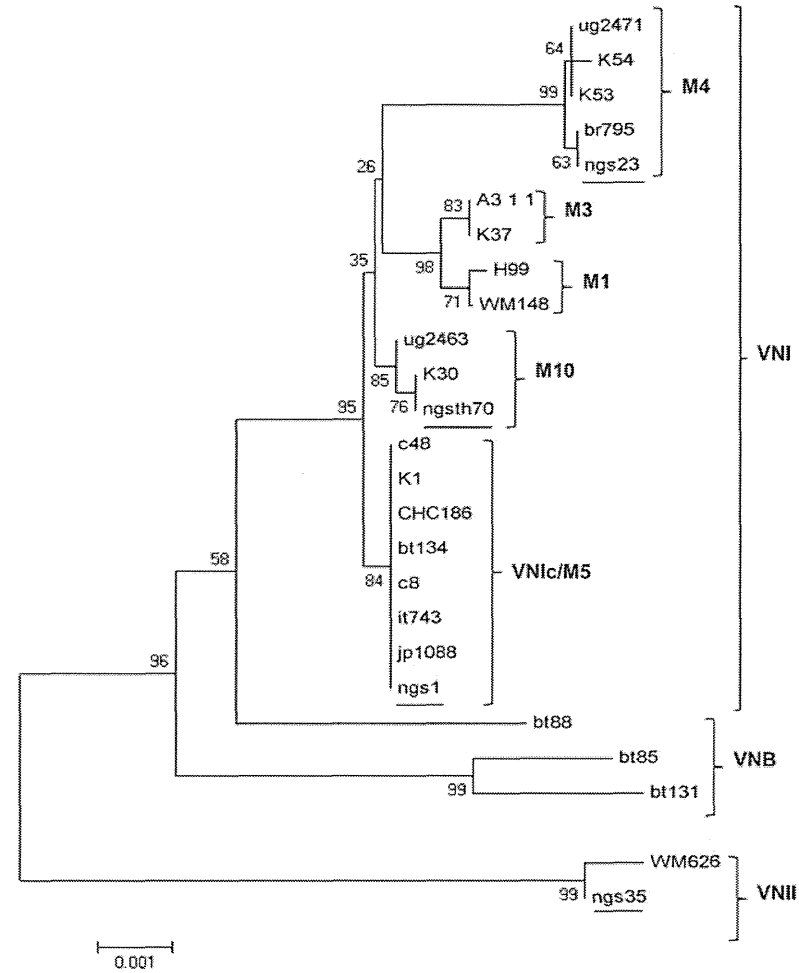

Fig. 1 Dendrogram of the neighbor-joining tree based on the concatenated seven multilocus sequence typing of selected Nagasaki strains (underlined) and other *Cryptococcus neoformans* strains isolated globally from previous studies. M typing (M1, M3, M4, M5 and M10) was designated according to a previous 12-locus MLST study [7]. The ST 5 isolates (ngs 1 and other 30 clinical isolates) were identical with the VNIc/M5 type, which represents the major molecular type of the Korean and Chinese isolates identified by the M13 fingerprinting method. One ST 32 isolate (ngs23) was identified to belong to the VNI-M4 clade. ST 4 isolates belong to VNI-M10 (ngsth70). Three isolates were classified as VNII (ngs 35 and other two isolates).

cancer (Case, ngs23 in Table 3). The outcome of *Cryptococcus* infection of this patient was unknown.

VNII isolates were recovered from three patients, one of whom, a 72-year-old woman, had pulmonary cryptococcosis after postoperative chemotherapy for lung cancer. She recovered within 6 months of administration of fluconazole. In another case, an 83-year-old woman with rheumatoid arthritis, who was on long-term steroids and immunosuppressive agents, had miliary tuberculosis, heart failure, nephrosis, and disseminated intravascular coagulation as complications. She died of exacerbation of heart failure without responding to cryptococcosis treatment and *C. neoformans* was detected in the right lung tissue on autopsy. In a third case, a 56-year-old male had multiple nodules in both upper lung fields, with *Mycobacterium tuberculosis* and *C. neoformans* detected in the sputum. His underlying disease was hepatocellular carcinoma and he had received antituberculous drugs and hepatic artery embolization. However, he died of hepatic rupture before the administration of antifungal drugs for cryptococcosis.

Discussion

In the last decade, a number of interesting reports have been published worldwide concerning molecular typing of yeasts belonging to the *C. neoformans/C. gattii* species complex [10–12,15,29–31]. Although there are no geographical differences in the distribution of *C. neoformans* and *C. gattii*, there is a predominant molecular type for each species (VNI and VGI, respectively).

Table 3 List of clinical features of *Cryptococcus neoformans* isolates from Nagasaki, Japan used in the study.

Name	Sex	Age	Source	Underlying condition	meningitis	Steroid usage	outcome
ngs1	M	39	BALF	-	-	-	cured
ngs2	F	unknown	CSF	unknown	+	unknown	unknown
ngs3	F	85	LN	ATL	-	-	unknown
ngs4	M	39	BALF	-	-	-	cured
ngs6	F	74	BALF	RA, DM	unknown	+	cured
ngs9	F	75	BALF	gastric cancer	-	-	cured
ngs10	F	unknown	BALF	unknown	-	unknown	unknown
ngs11	M	45	BALF	-	-	-	cured
ngs12	M	86	CSF	end stage kidney disease	+	unknown	unknown
ngs14	F	29	Sputum	pregnancy	-	-	cured
ngs16	M	32	Sputum	Neurofibromatosis type 1	-	-	cured
ngs17	F	78	BALF	Lung cancer	-	-	cured
ngs18	M	70	BALF	-	-	-	cured
ngs20	F	unknown	BALF	Lymphoma	-	unknown	unknown
ngs21	M	21	BALF	-	-	-	cured
ngs24	M	unknown	BALF	unknown	unknown	unknown	unknown
ngs29	M	unknown	unknown	unknown	unknown	unknown	unknown
ngs30	F	79	BALF	-	-	-	cured
ngs32	M	74	BALF	DM	-	-	cured
ngs33	F	65	BALF	-	-	-	cured
ngs42	M	74	Blood	Wegener's granulomatosis	+	unknown	unknown
ngs43	M	54	BALF	-	-	-	cured
ngs45	M	unknown	BALF	unknown	unknown	unknown	unknown
ngs47	F	unknown	Sputum	unknown	unknown	unknown	unknown
ngs50	F	77	unknown	Systemic Scleroderma	unknown	+	unknown
ngs51	M	43	BALF	-	-	-	cured
ngs53	F	unknown	BALF	RA, Secondary amyloidosis	+	unknown	unknown
ngs54	M	unknown	Sputum	unknown	unknown	unknown	unknown
ngs55	M	67	Lung (autopsy)	Acute interstitial pneumonia	unknown	+	dead (cryptococcosis)
ngs57	F	unknown	unknown	unknown	unknown	unknown	unknown
ngs72	M	71	Sputum	unknown	unknown	unknown	unknown
ngs23	M	72	Sputum	cancer	-	-	unknown
ngs28	F	82	Lung (autopsy)	RA, DM, miliary TB	unknown	+	dead (heart failure)
ngs19	F	72	BALF	Cancer, chemotherapy	-	-	cured
ngs35	M	56	Sputum	DM, HCC	-	-	dead (HCC)

BALF, bronchoalveloar lavage fluid; CSF, cerebrospinal &fi;uid; LN, lymph node; DM, diabetes mellitus; RA, Rheumatoid arthritis; TB, tuberculosis; HCC, hepatocellular carcinoma; unknown, missing data.

Our data indicated 31 of the 35 clinical isolates from Nagasaki showed the same genotyping result (ST 5), and only one isolate was identified as ST 32. Epidemiological studies from Korea and China indicated that the majority of *C. neoformans* strains isolated in these two countries were classified as VNIc/M5 by M13 fingerprinting method [8]. The nucleotide sequences of VNIc/M5 were identical to those of ST 5, and the majority of the studied strains gave the same sequences. These results indicate that *C. neoformans* isolates from patients in Nagasaki, Japan, are genetically homogenous with the VNIc/M5 type strains isolated in Korea and China. Although several ST 32 strains were isolated from Africa, USA and Brazil, it has never been, until this report, isolated in Asia to date [7]. Since clinical data of the patient with ST 32 is too limited, we were not able to state the relation between clinical feature and this unique isolate. Environmental strains from Thailand were classified as ST 4 and 5. Previous population genetic analyses indicated that Thailand isolates from 11 provinces were highly homogenous, consisting of the same genetic background (VNI) and exhibiting only ten nearly identical sequence types (STs), with three (STs 44, 4 and 5) dominating samples [28].

Although the number of isolates investigated in Korea (78 strains) and China (129 strains) was higher than that in the current study, no single VNII strain was found in these two countries as was the case in our investigation [17,18]. This may indicate a certain level of genetic diversity of *C. neoformans* across different Asian countries.

Our study showed that the majority of *C. neoformans* isolates (74.3%) were recovered from bronchoalveolar lavage fluid and sputum, and only two were isolated from

cerebral fluid. On the other hand, the majority of *C. neoformans* isolates were obtained from cerebral spinal fluid in studies from China (89.1%) and Korea (65.3%) [17,18]. Although there are only a few epidemiological reports of affected organs in *C. neoformans* infection cases in Japan, a retrospective study of the visceral mycoses in autopsied cases indicated that the most common sites of cryptococcal infection were the lungs (53.8%), followed by liver (14.9%), brain and meninges (10.6%), and spleen (10.6%) [32]. Additionally, we previously reported that only 11 cryptococcosis cases were complicated with encephalomeningitis of the 126 patients [33]. One possible reason of this difference may be that we have a periodic mass-screening check-up system of chest radiographs in local residents in Japan. It may provide a means to detect pulmonary cryptococcosis cases with small nodule or nodules but these cases usually do not develop central nervous system infections. Another possibility is that susceptibility to pulmonary cryptococcosis without encephalomeningitis is peculiar to Japanese. Further studies are warranted to investigate these unique findings in large scale Japanese epidemiological study in the future. However, our data indicated ST 5 is still dominant in Japan, Korea and China, even though the isolation site of *C. neoformans* is different.

The first epidemiological study regarding genotyping was reported in Japan in 1983 [34]. In this investigation, 31 of 32 clinical strains were characterized as serotype A and mating type MATα, one was identified as serotype D, and serotype AD isolates were recovered from the environment. In Kohno *et al.*'s study in 1994, a total of 51 *C. neoformans* serotype A isolaters were divided into three subtypes by genomic DNA probe UT-4p methods and four were determined to be AD serotype [35]. These data indicate that VNIII and VNIV type strains have actually existed for more than 20 years in Japan. However, we have found only serotype A isolates in the current study. The possible reason for this is that we have investigated a limited number of clinically isolates and no environmental ones. A few cases involving *C. gattii* have been previously reported in Japan, one of which involved a Japanese patient without a history of traveling aboard with the isolate found to be VGIIa, known as endemic to North America [36]. Additionally, a single *C. gattii* isolate was investigated by a molecular method in 1995, although no clinical information was available [37]. The current prevalence of *C. gattii* infection in Japan has not been extensively evaluated, though it is expected to be as low. However, it is possible that a few *C. gattii* infection cases without strain identification may exist in the country.

Similar to the Chinese and Korean isolates, most of the Japanese isolates were of the genotype VNIc/M5 [17,18]. However, in China, VNIc/M5 is the predominant genotype in the non-HIV patients without any apparent risk factors which contributed to the investigation of the virulence of these isolates. The results showed that these VNIc/M5 strains were all far less virulent than strain H99, the type culture of *C. neoformans* var. *grubii*. Hence, the infection may be correlated with the host factors rather than with the virulence of the VNIc/M5 strains. The assumption may also be applicable to Japan where non-HIV cryptococcosis is mainly caused by the same genotype. However, as in Korea, most of the HIV negative patients in Japan had a variety of underlying diseases. Thus, the difference in virulence of Japanese and Chinese isolates, including *in vivo* virulence studies, are warranted.

Although much less common than VNI strains, VNII strains have been isolated from many continents such as North America, South America, and Africa [7,8,38], as well as from Asian countries. A total of 1%, 4.5%, and 15.6% of *C. neoformans* isolates were identified as VNII in Taiwan, Malaysia, and China, respectively [11,14,30]. In this study, two of three patients with infections caused by VNII isolates died of underlying diseases other than cryptococcosis. Therefore, it is difficult to determine if VNII is more pathogenic than other genotypes or if infection caused by these two isolate may have been involved in the death of the patients. In fact, although a comprehensive study comparing the pathogenicity of VNI and VNII has never been done, by using murine inhalation model, a virulence study of a few VNII isolates suggested their virulence was lower than that of VNI strains [39]. This suggested that Far East Asians might have different susceptibilities to cryptococcosis than patients in other parts of the world. Again, further experiments need to be done before drawing final conclusions.

In conclusion, we demonstrated that the *C. neoformans* clinical isolates from Nagasaki, Japan, were genetically homogeneous, with most of the isolates belonging to the same genotype (ST 5), which is also the major type found in Korea and China [17,18]. Since the number of tested isolates in this study is still limited, studies using additional clinical isolates from other parts of Japan, from HIV patients in Japan, and environmental isolates to determine phenotypic differences are warranted. Furthermore, examinations of host factors including genetic factors in clinical prospective studies among non-HIV patients in Asia are also warranted.

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