A Non-HIV Specific ST5 Genotype of *Cryptococcus neoformans-gattii* Species Complex

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ABSTRACT

Cryptococcosis is a basidiomycetous yeast infection caused by *Cryptococcus neoformans-gattii* species complex which comprises of two sibling species, *Cryptococcus neoformans* and *Cryptococcus gattii*. Since the beginning of the acquired immune deficiency syndrome (AIDS) pandemic in 1980s, the prevalence of cryptococcosis has increased dramatically. More than 95% of cryptococcosis was AIDS-associated thus cryptococcosis was considered as an opportunistic infection. However, over the years, this paradigm has been challenged by several epidemiological studies reporting non-AIDS-associated cryptococcosis. Firstly, in 2008, Chang et al. reported that most (91.5%) of 129 cryptococcosis cases from China occurred in immunocompetent patients. Secondly, in 2010, an epidemiological survey of cryptococcosis in Korea revealed 77.4% of the 62 cases were non-HIV patients. Further molecular epidemiological study revealed the ST5 genotype is responsible for most cases (91-98%) of non-HIV cryptococcosis. Thus, genetic susceptibility to cryptococcosis by the Far East Asian bloodline was suspected. As close siblings of the Far East Asian bloodline, molecular epidemiological surveys of cryptococcosis were conducted. However, two molecular epidemiological studies in Thailand revealed 98% of cryptococcal cases occurred in HIV infected patients and, as expected, only 8-14% belonged to the non-HIV specific ST5 genotype.

Keywords: *Cryptococococcus*, genotype, molecular type, epidemiology, HIV

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The *Cryptococcus neoformnans-gattii* species complex is composed of two closely related species, *C. neoformans* and *C. gattii*. Although the species was described over a century ago, it was only reported as sporadic infections in humans before the 1980s. However, after the event of AIDS pandemic, the prevalence of cryptococcosis, an infection caused by the fungal species, has dramatically increased.[1] Since then, this pathogenic yeast has been the leading cause of fungal meningoencephalitis resulting in morbidity and mortality worldwide especially in immunocompromised patients. It is estimated that the species kills at least half of the estimated one million global new cases of cryptococcal meningitis occurring each year.[2]

The taxonomic classification within the *C. neoformans-gattii* species complex is constantly changing. In the 1950s, after several times of renaming, one of the current species name, *Cryptococcus neoformans*, was finally proposed by Benham.[3,4] The first strain typing method based on the antigenic properties of the extracellular polysaccharide was established in 1949 and four serotypes, A, B, C and D, were recognized in the species complex.[5,6] In 1978, Kwon-Chung raised

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serotype B and C to a species status as *Cryptococcus bacillisporus* due to a sufficient morphological difference in a perfect (sexual) state from the serotypes A and D.[7] However, in 1982, further studies showed high homology in DNA-DNA association study, high similarity of biological properties and ability to produce viable spores in inter-species mating between *C. neoformans* and *C. bacillisporus,* so Kwon-Chung reclassified *C. bacillisporus* to a variety level as *C. neoformans* var. *gattii* (serotype B and C). *C. neoformans* were given a varietal name as *C. neoformans* var. *neoformans* (serotype A and D).[8] In 1999, a new variety of *C. neoformans* was proposed for the serotype A by Franzot *et al.* as *C. neoformans* var. *grubii*, based upon detection of significant genotypic differences between serotype A and D.[9] Finally, in 2002, *Cryptococcus neoformans* variety *gattii* was again raised to the species level by Kwon-Chung according to a sufficient difference in an analysis of DNA sequences and lack of genetic recombination between the *C. neoformans* var. *grubii/neoformans* and *C. neoformans* var. *gattii*.[10] Therefore, at the present time, two species, two varieties and four serotypes are recognized within *C. neoformans-gattii* species complex, namely *C. neoformans var. grubii* (serotype A), *C. neoformans* var. *neoformans* (serotype D) and *C. gattii* (serotype B and C) (Fig 1).

As a cosmopolitan pathogenic yeast, numerous studies of molecular epidemiology have been reported over the past 20 years. Several molecular typing methods have been used to study the genetic diversity of *C. neoformans-gattii* species

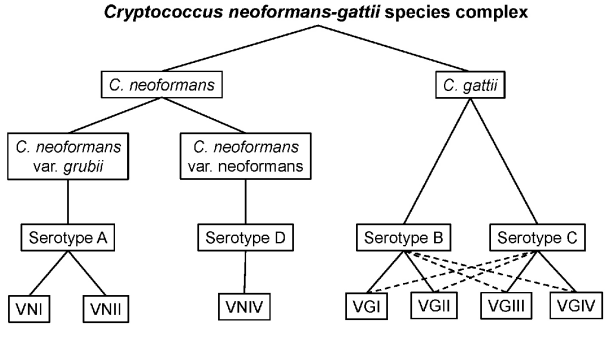

Fig 1. A correlation between different classifications of *C. neoformans-gattii* species complex. Note that minority of serotype B can be classified to VGIII or VGIV and Minority of serotype C can be classified to VGI or VGII (represented in dashed lines)

complex[11] such as M13 fingerprinting,[12] *URA5*[13] or *PLB1*[14] Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP),[15] Multi Locus Sequence Typing (MLST),[16] Multi-Locus Microsatellite Typing (MLMT)[17] or Matrix-Assisted Laser Desorption Ionization-Time-of-Flight Mass Spectrometry (MALDI-TOF MS).[18] All methods consistently identified seven haploid molecular types among thousands of isolates of *C. neoformans-gattii* species complex which have been recognized globally as standard molecular types of the yeast, namely VNI and VNII (*C. neoformans* variety *grubii*, serotype A); VNIV (*C. neoformans* variety *neoformans*, serotype D); VGI, VGII, VGIII and VGIV (*C. gattii*, serotype B and C). (Fig 1)

Of all the molecular typing methods, the *URA5*-RFLP, M13 fingerprinting and MLST are the most widely used.[13,16] Mostly, the *URA5*-RFLP method is used as the first step to designate the major molecular type of cryptococcal strains.[13] Despite its straightforwardness and unequivocal interpretation, *URA5*-RFLP can only differentiate genetic diversity of the cryptococcal strains to the level of the standard molecular types. Thus, their subtypes are subsequently determined by using a more discriminatory method, e.g. M13 PCR-fingerprinting.[12] However, the results of the M13 PCR-fingerprinting can vary depending on several factors, such as type of DNA polymerase, buffer, amount of primers/DNA template and water quality. Therefore, the reproducibility between different laboratories cannot be easily achieved and requires standard controls and optimization of the PCR conditions. Recently, the Cryptococcal Working Group on *C. neoformans-gatti*i species complex genotyping of the International Society for Human and Animal Mycology (ISHAM) proposed a MLST consensus typing scheme as a standard method for epidemiological studies of the *C. neoformans-gattii* species complex.[16] Based on 500-700 base pairs of DNA sequences from each of the seven unlinked genetic loci, including *CAP59, GPD1, LAC1, PLB1, SOD1, URA5* and the IGS1 region, the allele types (AT) and sequence types (ST) are being designated to each strain and deposited in the database which is published online at http://mlst.mycologylab.org/.

This MLST scheme allows flawless inter-laboratory comparison and is presently recognized as the most robust method for global epidemiological studies of cryptococcosis. Until now, approximately 600 STs were designated by the MLST method among *C. neoformans-gattii* species complex.

The global epidemiology according to the major molecular types has been reported by Meyer *et al.* based on the integrated analysis of 2755 cryptococcal isolates from several studies.[11] The molecular type VNI is the most common molecular type among both clinical (63%) and environmental (41%) isolates. The VGI and VGII are the second and third most common molecular types with comparable percentages. This paradigm is applicable to most parts of the world including Thailand.[19-21] However, the molecular type VNIV is more frequently found in Europe. The molecular types VGIII and VGIV of *C. gattii* are more common in South America.[11]

A correlation between the cryptococcal species and immunological status of the patients has long been reported. *C. neoformans* is known to majorly cause cryptococcosis in immunocompromised patients especially with HIV infection. On the other hand, *C. gattii* is more likely associated with immunocompetent patients (Table 1). Based on a number of international molecular epidemiological studies, the molecular type VNI of *C. neoformans* is the most common among cryptococcosis in immunocompromised patients while VGII of *C. gattii* is the most common among immunocompetent patients.[11] However, this model was recently challenged by studies in Far East Asian countries, China, Japan and Korea which reported the majority of immunocompetent patients were infected with *C. neoformans* (Table 1). Further studies by MLST revealed the strains belonged to the immunocompetent-specific, ST5 genotype. This special genotype lies within the molecular type VNI which was thought to cause disease only in HIV patients.[22,23] In 2008, a study of 129 clinical cryptococcal isolates in China revealed 91.5% were from non-HIV patients and 98% of *C. neoformans* isolates from the patients belonged to the ST5 genotype (Table 1, Fig 2).[22] In 2010, a subsequent study in Korea also revealed most (77.4%) of the 62 cryptococcosis cases were from non-HIV patients and 96.8% were identified as *C. neoformans*. A further MLST study revealed 91.53% of *C. neoformans* isolates belonged to the ST5 genotype (Table 1, Fig 2).[23] Finally, in 2012, an molecular epidemiological study of non-HIV cryptococcosis from Japan revealed a similar fact that all patients were infected with *C. neoformans* (Table 1) and 88.57% of these isolates belonged to the ST5 genotype.[24] These results suggested that either a subset of isolates in the VNI molecular type evolved to be a hypervirulent genotype, the ST5, or a Far East genetic background of the human host is more susceptible to cryptococcal infections which has contributed to this finding as suggested in the Chinese study.[22]

Though several epidemiological surveys of cryptococcosis in Thailand were done,[25-28] only two molecular epidemiological studies used the standard molecular typing systems.[20,21] Comparing to the countries in Far East Asia, cryptococcosis in Thailand occurred mainly in HIV patients and

TABLE 1. Associations between HIV status and cryptococcal species in different region.

Country (Total cases, *%C. neoformans/%C. gattii*)	**% HIV *(%C. neoformans/%C. gattii)***	**% non-HIV *(%C. neoformans/%C. gattii)***
Global[11] (1121, 81.9/18.1)	68.9 (97.4/2.6)	31.1 (47.7/52.3)
China[22] (129, 93/7)	8.5 (81.8/18.2)	91.5 (94.1/5.9)
Korea[23] (62, 96.8/3.2)	22.6 (100/0)	77.4 (95.8/4.2)
Japan[24] (35, N/A)	N/A (N/A)	N/A (100/0)
Thailand[20,21] (209, 96.2/3.8)	95.7 (98.5/1.5)	4.3 (44.4/55.6)

N/A; not applicable as only non-HIV cryptococcosis was included in the study; only strains with HIV status information were included in this analysis

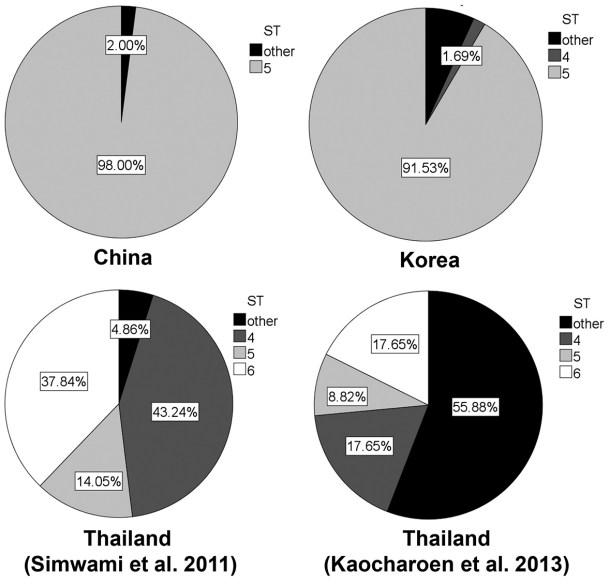

Fig 2. Prevalence of each major sequence type (ST) in each study.

was mostly caused by *C. neoformans* similar to the global data (Table 1). The first study was reported in 2011 in which 183 cryptococcal isolates collected mainly from the North and Northeastern parts of the country belonged to the molecular type VNI and only 14.1% were of the ST5 genotype based on MLST analysis.[21] A subsequent study in 2013, based on M13 PCR-fingerprinting and MLST analysis, showed that 498 *C. neoformans* and *C. gattii* isolates, mainly collected from the Middle and Western part of Thailand, revealed 94.8% belonged to the molecular type VNI. A further study with MLST showed that only 8.8% of the isolates were the ST5 genotype (Fig 2).[20] Interestingly, one of the ST5 isolates, E38, was isolated from the environment which suggested that the ST5 genotype existed in nature and could potentially pose a threat to people in Thailand.[20] The marked difference in ST genotype distribution and HIV status of the cryptococcosis cases between Thailand and the Far East Asain countries is still unexplainable. Thus, further epidemiological studies of the cryptococcal genotypes in Thailand are indispensable.

REFERENCES

1. Perfect JR, Casadevall A. Cryptococcosis. Infect Dis Clin North Am. 2002 Dec;16(4):837-74, v-vi.
2. Heitman J, Kozel TR, Kwon-Chung J, Perfect JR, Casadevall A, editors. *Cryptococcus*: from human pathogen to model yeast. Washington DC: ASM press; 2011.
3. Benham RW. Cryptococcosis and blastomycosis. Ann N Y Acad Sci. 1950 Sep;50(10):1299-314.
4. Benham RW. The genus *Cryptococcus*. Bacteriol Rev. 1956 Sep;20(3):189-201.
5. Evans EE. The antigenic composition of *Cryptococcus neoformans*. I. A serologic classification by means of the capsular and agglutination reactions. J Immunol. 1950 May; 64(5):423-30.
6. Wilson DE, Bennett JE, Bailey JW. Serologic grouping of *Cryptococcus neoformans*. Proc Soc Exp Biol Med. 1968 Mar;127(3):820-3.
7. Kwon-Chung KJ, Bennett JE, Theodore TS. *Cryptococcus bacillisporus* sp. nov.: serotype B-C of *Cryptococcus neoformans*. Int J Syst Bacteriol. 1978;28:616-20.
8. Kwon-Chung KJ, Bennett JE, Rhodes JC. Taxonomic studies on *Filobasidiella species* and their anamorphs. Antonie Van Leeuwenhoek. 1982;48(1):25-38.
9. Franzot SP, Salkin IF, Casadevall A. *Cryptococcus neoformans* var. *grubii*: separate varietal status for *Cryptococcus neoformans* serotype A isolates. J Clin Microbiol. 1999 Mar;37(3):838-40.
10. Kwon-Chung KJ, Boekhout T, Fell JW, Diaz M. (1557) Proposal to conserve the name *Cryptococcus gattii* against *C.hondurianus* and *C.basillisporus* (Basidiomycota, Hymenomycetes, Tremellomycetidae). Taxon. 2002 Nov; 51:804-6.
11. Meyer W, Gilgado F, Ngamskulrungroj P, Trilles L, Castañeda E, Boekhout T. Molecular typing of the *Cryptococcus neoformans*/*C. gattii* species complex. In: Heitman J, Kozel TR, Kwon-Chung J, Perfect JR, Casadevall A, editors. *Cryptococcus*: From Human Pathogen to Model Yeast. Washington DC: ASM press; 2011. p. 327-58.
12. Meyer W, Marszewska K, Amirmostofian M, Igreja RP, Hardtke C, Methling K, et al. Molecular typing of global isolates of *Cryptococcus neoformans* var. *neoformans* by polymerase chain reaction fingerprinting and randomly amplified polymorphic DNA-a pilot study to standardize techniques on which to base a detailed epidemiological survey. Electrophoresis. 1999 Jun;20(8):1790-9.
13. Meyer W, Castaneda A, Jackson S, Huynh M, Castaneda E. Molecular typing of IberoAmerican *Cryptococcus neoformans* isolates. Emerg Infect Dis. 2003 Feb;9(2):189-95.
14. Latouche GN, Huynh M, Sorrell TC, Meyer W. PCR-restriction fragment length polymorphism analysis of the phospholipase B (PLB1) gene for subtyping of *Cryptococcus neoformans* isolates. Appl Environ Microbiol. 2003 Apr;69(4):2080-6.
15. Boekhout T, Theelen B, Diaz M, Fell JW, Hop WC, Abeln EC, et al. Hybrid genotypes in the pathogenic yeast *Cryptococcus neoformans*. Microbiology. 2001 Apr;147 (Pt 4):891-907.
16. Meyer W, Aanensen DM, Boekhout T, Cogliati M, Diaz MR, Esposto MC, et al. Consensus multi-locus sequence typing scheme for *Cryptococcus neoformans* and *Cryptococcus gattii*. Med Mycol. 2009 Jun 12:1-14.
17. Hanafy A, Kaocharoen S, Jover-Botella A, Katsu M, Iida S, Kogure T, et al. Multilocus microsatellite typing for

Cryptococcus neoformans var. grubii. Med Mycol. 2008 May 16:1-12.
18. Firacative C, Trilles L, Meyer W. MALDI-TOF MS enables the rapid identification of the major molecular types within the *Cryptococcus neoformans/C. gattii* species complex. PLoS One. 2012;7(5):e37566.
19. Arjratanakul W, Ngamskulrungroj P. Isolation Prevalence of Mycobacterial and Fungal Infections in Thailand. Siriraj Med J. 2013;65 (Suppl):S24-S8.
20. Kaocharoen S, Ngamskulrungroj P, Firacative C, Trilles L, Piyabongkarn D, Banlunara W, et al. Molecular Epidemiology Reveals Genetic Diversity amongst Isolates of the *Cryptococcus neoformans/C. gattii* Species Complex in Thailand. PLoS Neglected Tropical Diseases. 2013 Jul;7 (7):e2297.
21. Simwami SP, Khayhan K, Henk DA, Aanensen DM, Boekhout T, Hagen F, et al. Low diversity *Cryptococcus neoformans* variety *grubii* multilocus sequence types from Thailand are consistent with an ancestral African origin. PLoS Pathog. 2011 Apr;7(4):e1001343.
22. Chen J, Varma A, Diaz MR, Litvintseva AP, Wollenberg KK, Kwon-Chung KJ. *Cryptococcus neoformans* strains and infection in apparently immunocompetent patients, China. Emerg Infect Dis. 2008 May;14(5):755-62. .
23. Choi YH, Ngamskulrungroj P, Varma A, Sionov E, Hwang SM, Carriconde F, et al. Prevalence of the VNIc genotype of *Cryptococcus neoformans* in non-HIV-associated cryptococcosis in the Republic of Korea. FEMS Yeast Res. 2010 Sep;10(6):769-78.
24. Mihara T, Izumikawa K, Kakeya H, Ngamskulrungroj P, Umeyama T, Takazono T, et al. Multilocus sequence typing of *Cryptococcus neoformans* in non-HIV associated cryptococcosis in Nagasaki, Japan. Med Mycol. 2012 Aug 17.
25. Sukroongreung S, Lim S, Tantimavanich S, Eampokalap B, Carter D, Nilakul C, et al. Phenotypic switching and genetic diversity of *Cryptococcus neoformans*. J Clin Microbiol. 2001 Jun;39(6):2060-4.
26. Sukroongreung S, Nilakul C, Ruangsomboon O, Chuakul W, Eampokalap B. Serotypes of *Cryptococcus neoformans* isolated from patients prior to and during the AIDS era in Thailand. Mycopathologia. 1996;135(2):75-8.
27. Imwidthaya P, Dithaprasop P, Egtasaeng C. Clinical and environmental isolates of *Cryptococcus neoformans* in Bangkok (Thailand). Mycopathologia. 1989 Oct;108(1): 65-7.
28. Imwidthaya P, Poungvarin N. Cryptococcosis in AIDS. Postgrad Med J. 2000 Feb;76(892):85-8.