Final Report

Molecular epidemiology of the non-HIV associated cryptococcal genotype, VNIc/M5/ST5, in Thailand

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December 2016

Contract No. TRG5780088

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Molecular epidemiology of the non-HIV associated cryptococcal genotype, VNIc/M5/ST5, in Thailand

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This project granted by the Thailand Research Fund

Abstract

Project Code: TRG5780088

Project Title: Molecular epidemiology of the non-HIV associated cryptococcal genotype, VNIc/M5/ST5, in Thailand

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Project Period: 2nd June 2014 - 1st December 2016

Abstract:

Despite a strong association between *C. neoformans* and HIV status of the patients globally, most of cryptococcosis in Far East Asia occurs in non-HIV individuals. Further molecular epidemiological studies, using MLST, revealed more than 95% of the cryptococcal strains belonged to a specific sub-type of the molecular type VNIc/ST5 genotype. Such association of this genotype has never been specifically explored in other part of Asia. Therefore, this study aims to investigate the VNIc/ST5 genotype distribution among cryptococcosis patients in Thailand, a South East Asian country.

Fifty-one of *C. neoformans* isolates were collected from clinical samples in Siriraj Hospital, Bangkok, Thailand. The strains were mostly isolated from HIV positive (88.57%) and all were molecular type VNI MATα. Multilocus Sequence Typing (MLST) analysis identified five sequence types (ST) in Siriraj Hospital. ST4 (45.10%) and ST6 (35.29%) were found to be the two most common. The other less common were ST5 (15.69%), ST32 (1.96%) and ST93 (1.96). The ST5 was, unlike what reported in the Far East Asia, mostly (83.3%) found in HIV patient ($p = 0.657$) and there was no significant change in ST5 prevalence over the past 10 years ($p = 0.548$). Further analysis of co-morbidities showed higher morbidity and delays in cryptococcal diagnosis in a group with TB co-infection or with non-HIV status.

Our study suggested that, though Thai is genetically closely-related to the Far East Asian, ST5 in Thailand is not associated with non-HIV status. Thus, such association might not relate to the host genetic. However, the definite mechanism remains a mystery.

Keywords: Cryptococcus, HIV, AIDS, molecular epidemiology

Executive summary

C. neoformans is found worldwide and can be classified by molecular techniques showing VNI is the most common molecular type in Thailand and worldwide. 51 cryptococcal isolates were obtained from clinical samples at Siriraj Hospital. Cryptococcus occurred more frequently in HIV positive (88.6%) and HIV negative (11.4%). 57% of the isolates were from male and 43% were from female patients. An average age of the patients was 40.2 years. All strains in this study is VNI and *MAT*α. This research found five STs; ST4 (45%), ST5 (15.3%), ST6 (35.7%), ST32 (2%) and ST93 (2%). In conclusion, the non-HIV specific ST5 was still uncommon compared to other STs. Phylogenetic analysis showed five STs were highly homogenous. However, the relationship between the sequence type and HIV status were unclear. Further collection of were clinical strains are needed to ensure such correlation.

Objective

1. To explore molecular epidemiology of cryptococcosis in Thailand
2. To identify association of the ST5 and non-HIV status in Thailand

Research methodology

Clinical isolates

Cryptococcal isolates, from 2012-2014 were obtained from clinical samples in the Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. All clinical isolates were identified using RapID™ Yeast Plus System (Thermo Fisher Scientific, MA, USA). L-canavanine-glycine-bromothymol blue (CGB) agar was used to differentiate *C. neoformans* from *C. gattii*.

Reference strains

A set of laboratory standard reference strains representing each of the eight major molecular type were used for molecular type identification: WM148 VNI), WM626 (VNII), WM 628 (VNIII), WM 629 (VNIV), WM 179 (VGI), WM 178 (VGII), WM 175 (VGIII), and WM 779 (VGIV) (1). KN99α and KN99a were used as a reference strains for mating type analysis (2).

Genotype and mating type analysis

DNA extraction was performed using the phenol–chloroform–isoamyl alcohol (25:24:1, v:v:v) method (3). Genotypes were determined by *URA5*- RFLP analysis with the enzymes *HhaI* and *Sau96I* (Thermo Fisher Scientific, MA USA) (1). Mating types were identified by PCR using two specific primer pairs STE20Aα and STE20Aa (4).

Multilocus sequence typing (MLST)

Analysis of MLST was performed on *C. neoformans* isolates, using the ISHAM consensus scheme of seven unlinked loci (*CAP59, GPD1, IGS1, LAC1, PLB1, SOD1* and *URA5*). Each locus was amplified using the primers and amplification parameters described previously (5). Allele types (AT) and sequence types (ST) were defined according to ISHAM MLST database for *C. neoformans* (http://mlst.mycologylab.org) (2).

Statistical analysis

Statistical analysis was performed using the Fisher exact test. Unknown data were regarded as lost data and not included in the calculations. Fisher Exact test was done by using online program SISA (http://www.quantitativeskills.com/sisa/statistics/fisher.htm). Statistical significance was defined when p value $\leq$ 0.05.

Result

Distribution of major molecular types and mating types

A total of 165 *C. neoformans* isolates were collected from 51 patients. Only one isolate per patient were selected for subsequent analysis. Cerebrospinal fluid (29 strains, 56.86%) was the most common site of isolation and followed by blood (21 strains, 41.18%) and bone marrow (1 strain, 1.96%). All isolates were identified as molecular type VNI and mating type MATα.

MLST analysis

All isolates were identified as molecular type VNI (table 1). MLST analysis divided the 51 *C. neoformans* isolates into five STs, including ST4 (45.10%, n = 23), ST6 (35.29%, n = 18), ST5 (15.69%, n = 8), ST93 (1.96%, n = 1) and ST32 (1.96%, n = 1).

Table 1. The alleles and sequence types of cryptococcal isolates in this study.

Strain	Molecular type	Mating type	Allelic profiles							Sequence type
			CAP59	*GPD1*	*IGS1*	*LAC1*	*PLB1*	*SOD1*	*URA5*	
SICN_001	VNI	α	1	3	1	5	2	1	1	5
SICN_002	VNI	α	1	1	1	4	2	1	5	4
SICN_003	VNI	α	1	1	1	3	2	1	5	6
SICN_004	VNI	α	1	1	1	4	2	1	5	4
SICN_005	VNI	α	1	1	1	4	2	1	5	4
SICN_006	VNI	α	1	1	1	4	2	1	5	4
SICN_008	VNI	α	1	1	1	3	2	1	5	6
SICN_009	VNI	α	1	1	1	3	2	1	5	6
SICN_011	VNI	α	1	1	1	4	2	1	5	4
SICN_012	VNI	α	1	3	1	5	2	1	1	5
SICN_013	VNI	α	1	1	1	4	2	1	5	4
SICN_014	VNI	α	1	1	1	4	2	1	5	4
SICN_015	VNI	α	1	1	1	3	2	1	5	6
SICN_017	VNI	α	1	1	1	4	2	1	5	4
SICN_018	VNI	α	1	23	10	3	4	1	1	93

SICN_019	VNI	α	1	3	1	5	2	1	1	5
SICN_021	VNI	α	1	3	1	5	2	1	1	5
SICN_022	VNI	α	1	1	1	3	2	1	5	6
SICN_024	VNI	α	1	1	1	3	2	1	5	6
SICN_025	VNI	α	1	1	1	3	2	1	5	6
SICN_026	VNI	α	1	1	1	3	2	1	5	6
SICN_027	VNI	α	1	1	1	3	2	1	5	6
SICN_028	VNI	α	1	3	1	5	2	1	1	5
SICN_029	VNI	α	1	1	1	3	2	1	5	6
SICN_030	VNI	α	1	3	1	5	2	1	1	5
SICN_031	VNI	α	1	1	1	3	2	1	5	6
SICN_032	VNI	α	1	1	1	4	2	1	5	4
SICN_033	VNI	α	1	1	1	3	2	1	5	6
SICN_034	VNI	α	1	1	1	3	2	1	5	6
SICN_035	VNI	α	1	1	1	4	2	1	5	4
SICN_036	VNI	α	1	1	1	4	2	1	5	4
SICN_038	VNI	α	1	1	1	4	2	1	5	4
SICN_039	VNI	α	1	1	1	4	2	1	5	4
SICN_040	VNI	α	1	1	1	4	2	1	5	4
SICN_041	VNI	α	1	1	10	3	4	1	1	32
SICN_060	VNI	α	1	1	1	4	2	1	5	4
SICN_061	VNI	α	1	1	1	3	2	1	5	6
SICN_062	VNI	α	1	1	1	4	2	1	5	4
SICN_063	VNI	α	1	1	1	3	2	1	5	6
SICN_064	VNI	α	1	1	1	4	2	1	5	4
SICN_065	VNI	α	1	1	1	4	2	1	5	4
SICN_066	VNI	α	1	1	1	4	2	1	5	4
SICN_067	VNI	α	1	1	1	4	2	1	5	4
SICN_068	VNI	α	1	1	1	4	2	1	5	4
SICN_069	VNI	α	1	1	1	4	2	1	5	4
SICN_070	VNI	α	1	3	1	5	2	1	1	5
SICN_071	VNI	α	1	1	1	3	2	1	5	6
SICN_072	VNI	α	1	1	1	4	2	1	5	4
SICN_073	VNI	α	1	1	1	3	2	1	5	6

SICN_074	VNI	α	1	3	1	5	2	1	1	5
SICN_075	VNI	α	1	1	1	3	2	1	5	6

Demographic data

Medical record of thirty-five patient with cryptococcosis were available. Patient ages were ranged from 24-82 years (mean 40.2 years) and most were males (65.71%). Thirty-one patients (88.57%) were HIV positive.

ST5 is not associated with non-HIV strains in Thailand

Recent data showed significant association between ST5 and non-HIV patients. Thus, we expected similar phenomenon among Thai isolates. However, our result showed ST5 were found mainly in HIV positive patient (83.3%) and no significant difference in prevalence of HIV infections ($p = 0.657$) were found between ST5 and non-ST5 group (table 2).

No change in ST distributions in Thailand during the last 10 years

After a national campaign of highly active antiretroviral therapy (HAART), prevalence of cryptococcosis dropped significantly. According to our records, 90% decrease in cryptococcosis cases was evident in the past 10 years (data not shown). Thus, we speculated changes in ST distributions might occurred. However, data showed such change were not evident significantly ($p = 0.548$) as showed in table 2.

Table 2. Association of sequence types with HIV status and time periods

Data		Sequence type		P-value
		ST5	Non-ST-5	
HIV status[#]	Positive (n=31)	5 (16.13%)	26 (83.87%)	0.657
	Negative (n=4)	1 (25.00%)	3 (75.00%)	
Years	2003-2005 (n=231)	29 (12.55%)	201 (87.45%)	0.548
	2012-2014 (n=51)	8 (15.70%)	43 (84.30%)	

[#]only data available in medical records were considered.

High mortality was associated with tuberculosis and non-HIV strains

As other comorbidities may affect treatment efficiency in cryptococcosis, we investigated effect of tuberculosis (TB) and HIV infection on treatment outcome. As expected, patients with active TB had higher morbidity (75% VS 25%; p = 0.031). However, patients without HIV infection surprisingly had higher morbidity (66.67% VS 33.33%; p = 0.041) (table 3).

Table 3. Association of comorbidities (HIV or TB) with treatment outcome[#]

Disease		Treatment outcome		P-value
		Cure	Dead	
TB	Active (n=4)	1 (25.00%)	3 (75.00%)	0.031
	Non active (n=27)	18 (78.26%)	5 (21.74%)	
HIV	Positive (n=22)	17 (77.27%)	5 (22.73%)	0.041
	Negative (n=6)	2 (33.33%)	4 (66.67%)	

[#]only data available in medical records were considered.

As comorbidities may complicate investigations of cryptococcosis, we tested whether TB or HIV infection delayed the diagnosis. The data showed having TB co-infection or being non-HIV patients delayed diagnosis of cryptococcosis (took 1.9 and 2.3 times longer, respectively) (table 4) with the significant difference in the TB co-infection group (p = 0.024).

Table 4. Association of comorbidities with time from onset of symptom to the disease diagnosis[#]

Disease	Mean of time from onset of symptom to the disease diagnosis (days)		P-value
	Active/ positive status	Non-active/ negative status	
TB	42.50 (n = 4)	22.35 (n = 31)	0.401
HIV	18.74 (n = 27)	44.63 (n = 8)	0.024

[#]only data available in medical records were considered.

Conclusion and Discussion

Cryptococcosis is mostly considered as on opportunistic infection with more than 90% association in AIDs-patients. A patient typically gets infected by inhaling the spores of *Cryptococcus* and, primarily, causes disease in the lungs. Subsequently, they can disseminate via bloodstream to other organs, especially, central nervous system. Similarly, this study found that the most common source of cryptococcal isolates were from cerebrospinal fluid (CSF) (56.86 %). Moreover, 41.18% of the isolates were from blood and 1.96% was from bone marrow which is known to be involved in pathogenesis of this organism. Our result was in line with a previous study that more than 80% of specimens came from cerebrospinal fluid (CSF) (5).

C. neoformans molecular type VNI is the most commonly found in Thailand and around the world (5, 6) and mostly infected immunocompromised persons. Surprisingly, in 2008, it is reported that the majority (91.5%) of *C. neoformans* VNIc/ST5 from China occurred in immunocompetent patients (7) and also reported in other area of Far East Asia (5, 8-10). Therefore, an epidemiology study of the non-HIV associated genotype, ST5, in Thailand where is closely related to the Far East Asia was initiated. Our result is consistent with all the previous worldwide results that more than 90% of the isolates were VNI (5, 11). MLST analysis divided *C. neoformans* strains from Siriraj Hospital into five STs of the molecular types VNI, including ST4 (45.10%, n = 23), ST5 (15.67%, n = 8), ST6 (35.29%, n = 18), ST93 (1.96%, n = 1) and ST32 (1.96%, n = 1). The high prevalence of ST4 and ST6 genotypes is in line with the previous study in Thailand (5). ST93 was dominant in India and Indonesia (8). A recent report showed genotype ST32 was also rarely (3.2%) found in Japan. (9) The data showed a clear genetic variation among different Asian regions (5, 8-10). A phylogram, constructed by neighbor-joining analysis based on a concatenated data from the seven loci, showed that the five STs (ST4, 5, 6, 32 and 93) in this study were genetically homogenous. In fact, such clonality of *C. neoformans* was also emphasized by the fact that all strains were belonged to the MATα.

The demographic data showed that most strains were isolated from HIV positive patients (88.6%) which is in accordance with a previous study (5). The male predominance (65.71%) was in line with previous data in Thailand that more than 60 % of cryptococcosis patients were male (5). A slight increase in the mean age from 30.5 years in 1997 to 32.4 years in 2004 was reported (12). A later study in 2013 reported a further increase average age in 37.97 years (5). This trend was shown again in our study where average age was increased to 40.2 years. One reason might be that HARRT therapy improved quality of patient lives and, therefore, increased their life spans (13).

Unlike cryptococcosis in Japan (9), China (14), and Korea (15), association between ST5 and non-HIV status in Siriraj Hospital was not evident in this study. Only 14.29% of the clinical cases were ST5 and 2.86% of ST5 was isolated from a HIV negative patient. In fact, it is reported that ST5 could be found both in patients who had HIV positive and negative status (8). Our data emphasizes that the association between ST5 and non-HIV status is unique to the Far East Asia. Moreover, despite the fact that such association might be due to specific genetic susceptibility of the Far East Asians (8), our data suggested otherwise. With a report of close genetic background between the Fast East Asians and the South East Asians (16), the specific genetic susceptibility theory would not explain such difference in ST5-HIV relationship between the two Asian populations. However, since number of samples is limited, a further sampling is needed.

Co-infection of cryptococcosis and tuberculosis (TB) are relatively difficult to be treated. Our result also confirmed this finding that co-infection with tuberculosis decreased treatment efficiency. There was an evidence that both *Mycobacterium tuberculosis* and *Cryptococcus* synergistically suppressed immune system and, subsequently, reduced sign and symptom of patients resulting in delays in diagnosis and treatment (17). Moreover, the failure of treatment also occurred more frequently in HIV negative patients in this study. A similar suggestion was proposed in one report where a delay in diagnosis of cryptococcosis in non-HIV patients due to negative or low antigen titer in samples (18). These proposals were finally supported by our finding that having a co-infection with TB or being a non-HIV patient caused delayed on diagnosis of cryptococcosis. Therefore, timely diagnosis of the diseases, especially in endemic areas of TB, is mandatory (19, 20).

In conclusion, the non-HIV specific ST5 was still uncommon compared to other STs in Thailand. Phylogenetic analysis showed five STs were highly homogenous. However, the relationship between the sequence type and HIV status were unclear. Further collection of more clinical strains are needed to ensure such correlation.

Appendix

Patients data of cryptococcosis in Siriraj Hospital

Patients data in Siriraj Hospital in 2012-2014

SICN_Code	Sex	Age	Underlying disease	Address	Occupation	HIV status	Medication	Treatment outcome
SICN_001	F	34		Bangkok	Sales	+	[D]Ampho	Cure
SICN_002	F	82	ESRD, IHD	Bangkok	Retired	-	[D]Ampho	[a]None
SICN_003	F	33		Laos	None	+	[D]Ampho	Cure
SICN_004	M	50	Chronic HCV	Nontaburi	Employee	+	[D]Ampho	Cure
SICN_005	M	29		Bangkok	None	+	[D]Ampho	Cure
SICN_006	M	37		Laos	None	+	[D]Ampho	Dead
SICN_008	F	80	Pauci Immune	Surin	Retired	-	[a]None	Dead
SICN_009	M	61		Petchaburi	Retired	-	[D]Ampho	Cure
SICN_011	M	28		Bangkok	Sales	+	[a]None	[a]None
SICN_012	M	24		Bangkok	Employee	+	[D]Ampho	Cure
SICN_013	M	33		Prajuab	None	+	[D]Ampho	[a]None
SICN_014	M	27		Nakornsri	Employee	+	[D]Ampho	Cure
SICN_015	M	38		Roied	None	+	[D]Ampho	Cure
SICN_017	M	39		Bangkok	Employee	+	[D]Ampho	Dead
SICN_018	F	39		Krabi	Sales	+	[D]Ampho	Cure
SICN_019	M	41	Tribal	Chiangmai	Constructer	+	[b]IRZE/Ampho	Dead
SICN_021	F	42	Evan syndrome	Buririum	Employee	-	[D]Ampho	Dead
SICN_024	F	33	Epilepsy	Bangkok	Employee	+	[D]Ampho	[a]None

Patients data of cryptococcosis in Siriraj Hospital (cont.)

Patient data in Siriraj Hospital in 2012-2014

SICN_Code	Sex	Age	Underlying disease	Address	Occupation	HIV status	Medication	Treatment outcome
SICN_025	M	64	Ischemic stroke	Bangkok	Retired	-	Ampho	Cure
SICN_026	F	39		Bangkok	Employee	+	Ampho	Cure
SICN_027	M	46		Bangkok	Employee	+	Ampho	Cure
SICN_028	M	42		Bangkok	Employee	+	Ampho	[a]None
SICN_029	F	41	SLE	Bangkok	Government	-	Ampho	Dead
SICN_030	M	48		Uthaitani	Constructer	+	Ampho	[a]None
SICN_031	M	50	Lacuna stroke	Bangkok	Government	+	Ampho	Cure
SICN_032	M	38		Bangkok	Employee	+	[b]IRZE/Ampho	Dead
SICN_033	M	36		Bangkok	Programmer	+	Fluconazole	Cure
SICN_034	M	67	Conjuctival	Yasothorn	Farmer	-	Ampho	Dead
SICN_035	M	25		Bangkok	Singer/Host	+	[b]IRZE/Ampho	Cure
SICN_036	M	32		Ayuthaya	Employee	+	Ampho	Cure
SICN_038	F	30		Bangkok	Employee	+	Ampho	Cure
SICN_039	M	55		Bangkok	Employee	-	Ampho	[a]None
SICN_040	F	30		Bangkok	Student	+	[c]MDR TB	Dead
SICN_041	F	66		Bangkok	Retired	+	Ampho	Cure
SICN_069	M	44		Bangkok	Police	+	Ampho	Cure

[a]None, unknown

[b]IRZE, isoniazid rifampicin pyrazinamide

[c]MDR TB, multi-drug-resistant tuberculosis

[D]Ampho, Amphotericin B

M, male

F, female

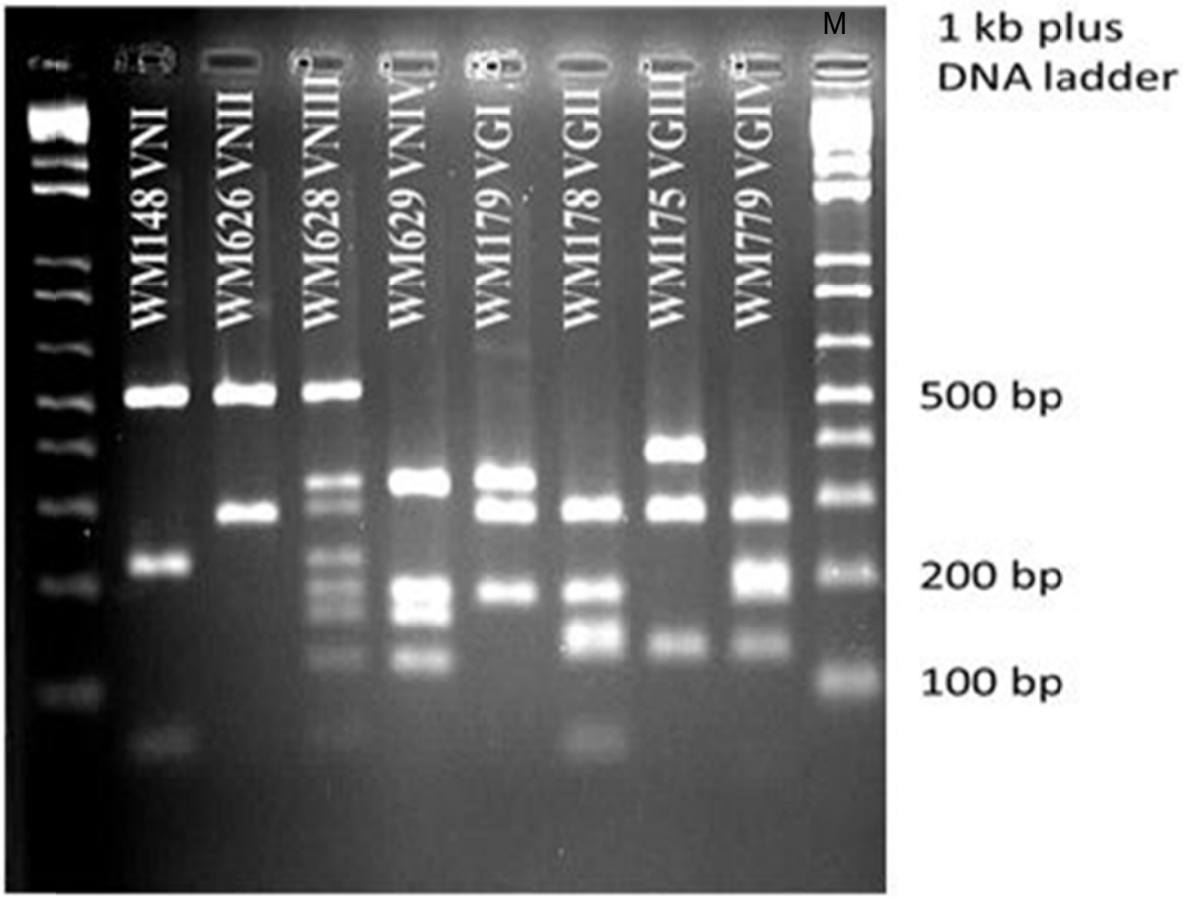
+, positive

-, negative

***URA5*-RFLP**

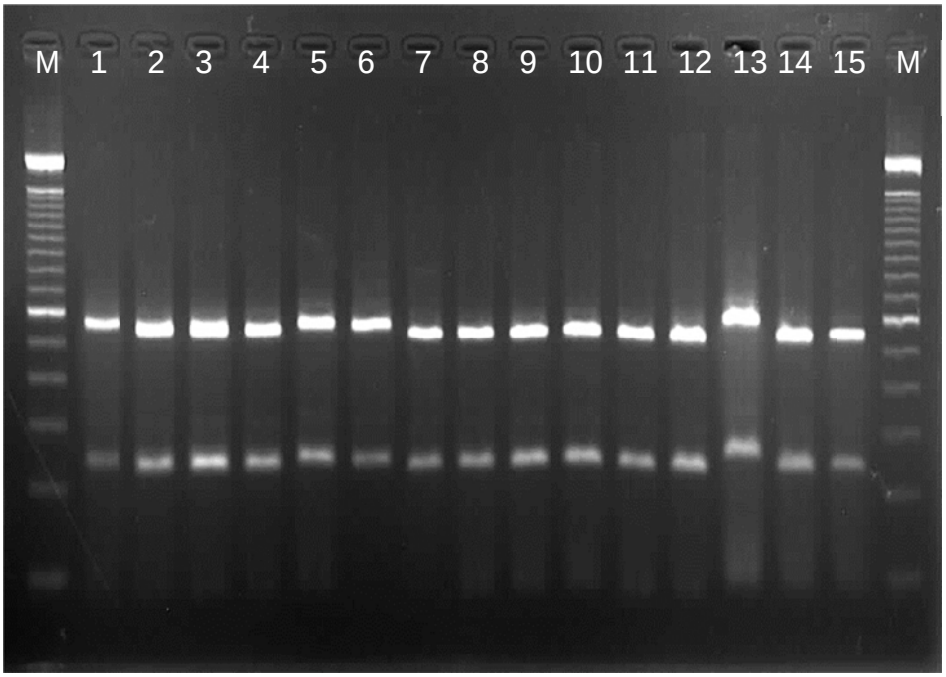
Reference strains

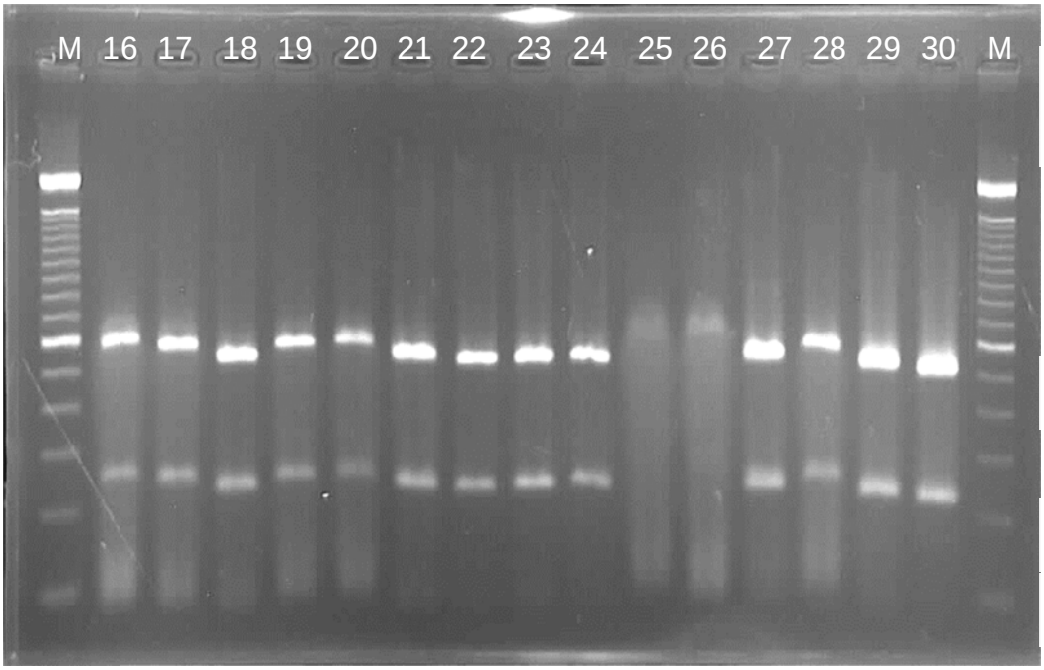
Major molecular types were determinated by *URA5*-RFLP. Lane 2-9 are VNI to VNIV and VGI to VGIV respectively. Lane 1 and 10 are 1 Kb plus DNA maker.

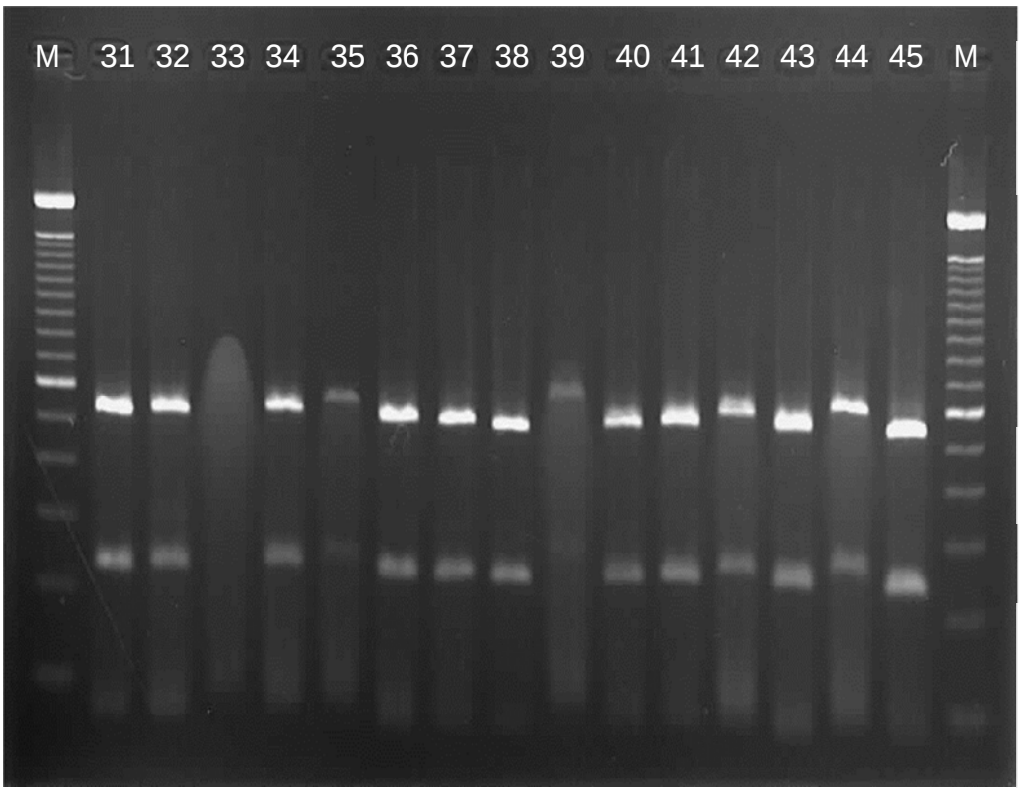


51 strains from Siriraj Hospital

51 strains showed only one *URA5*-RFLP pattern, consisting of two major bands. Top bands had size of 550 basepair and bottom bands had size of 250 basepair. The patterns were similar to the VNI pattern.



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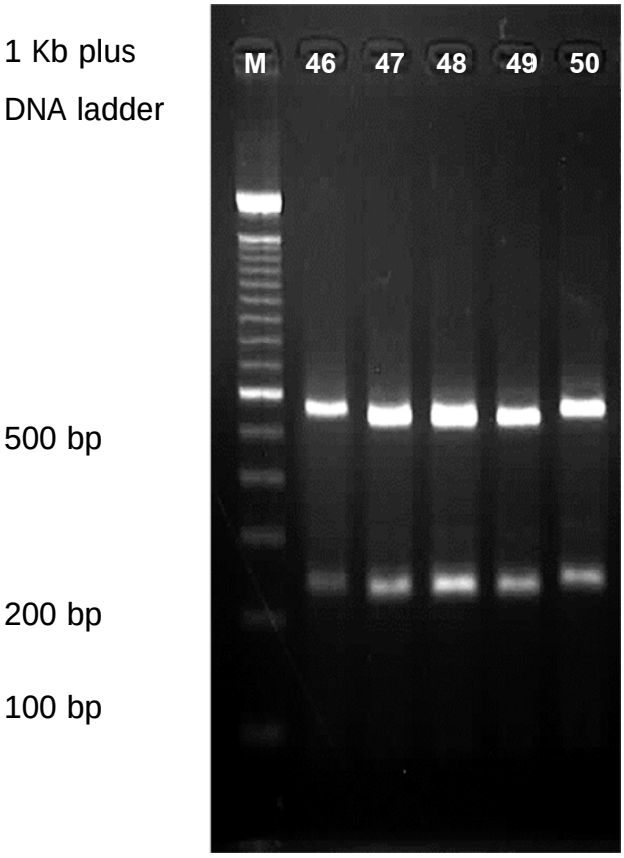
DNA ladder
500 bp
200 bp
100 bp

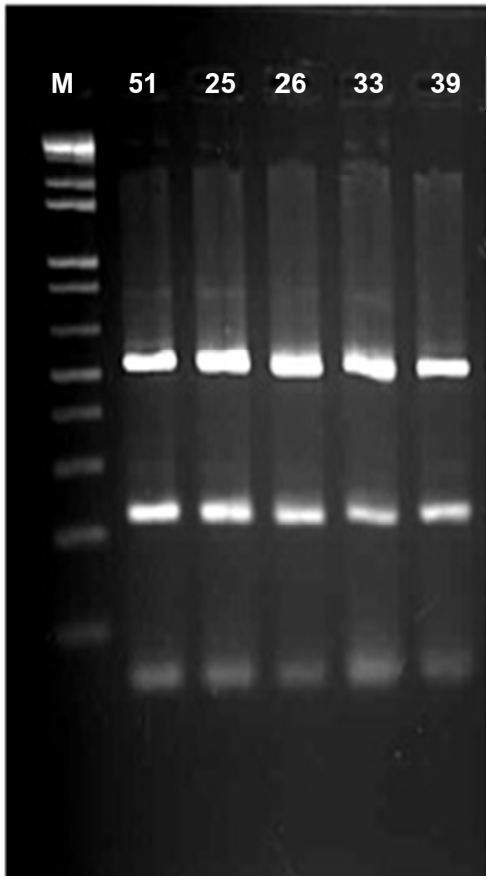

1 Kb plus
DNA ladder
M 46 47 48 49 50
500 bp
200 bp
100 bp

1 Kb plus DNA ladder

500 bp

200 bp

100 bp

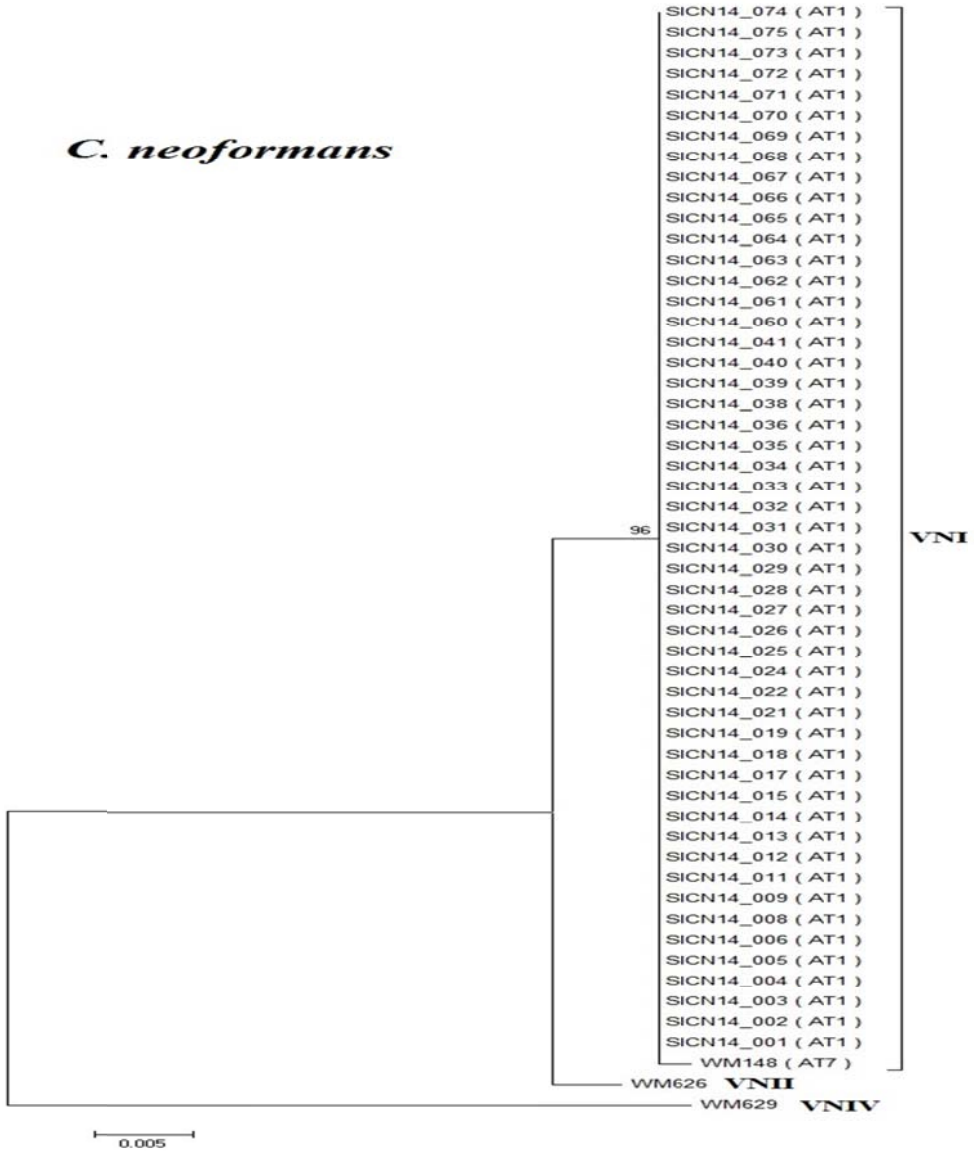


PHYLOGRAM CONSTRUCTION OF THE SEVEN LOCI

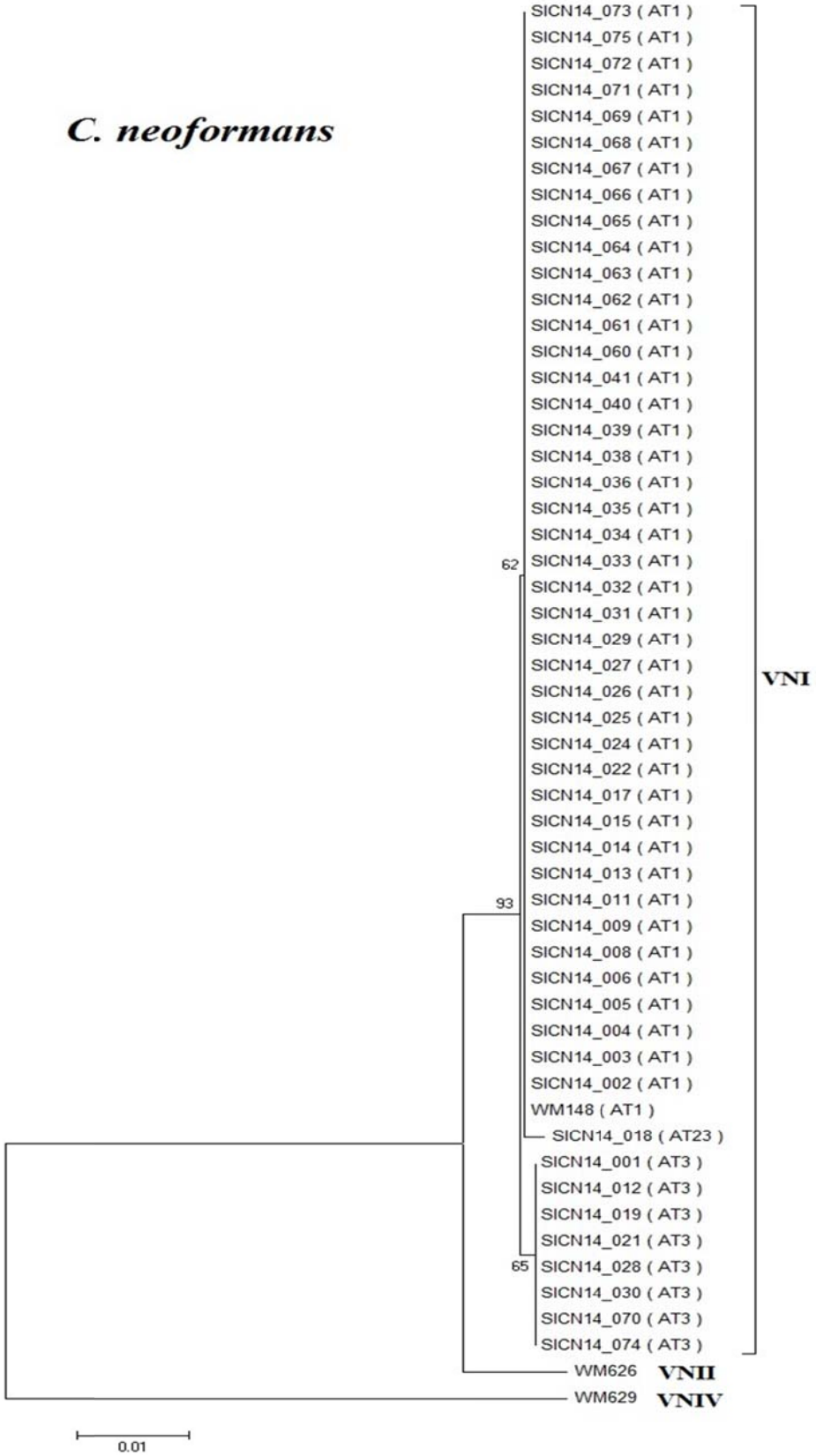
Phylogram construction

The sequence alignments of 51 strains, using the ISHAM consensus MLST typing scheme, seven set of loci, including conserved and variable regions of *CAP59*, *GPD*1, IGS1, *LAC*1, *PLB*1, *SOD*1 and *URA*5. Data were imported to the program MEGA 6.00 and analyzed by using the neighbor-joining method to construct phylogram. Bootstrap analysis using 1000 replicates was used to estimate support for clades of the concatenate dataset.

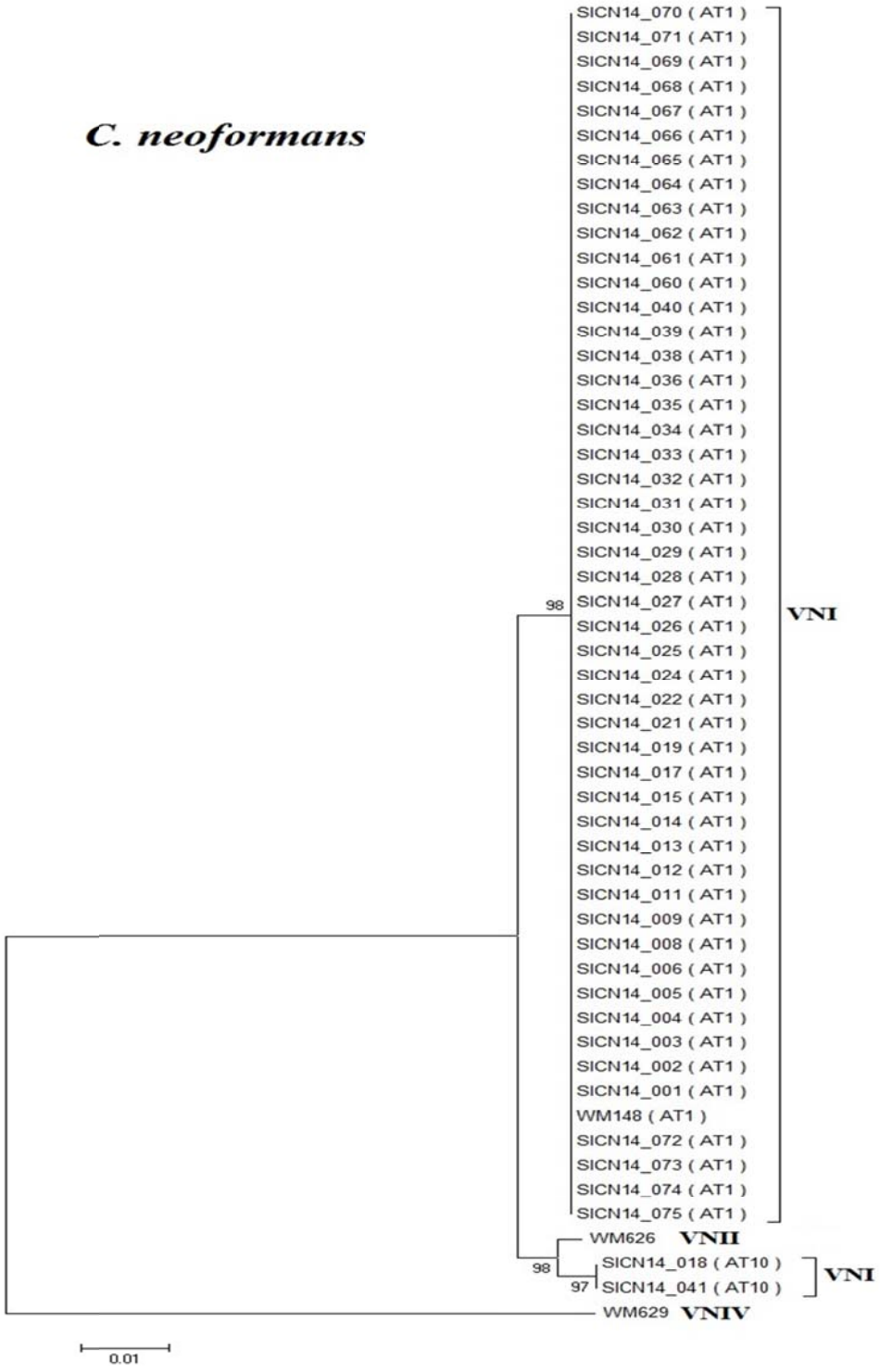
Phylogram of *CAP59* gene



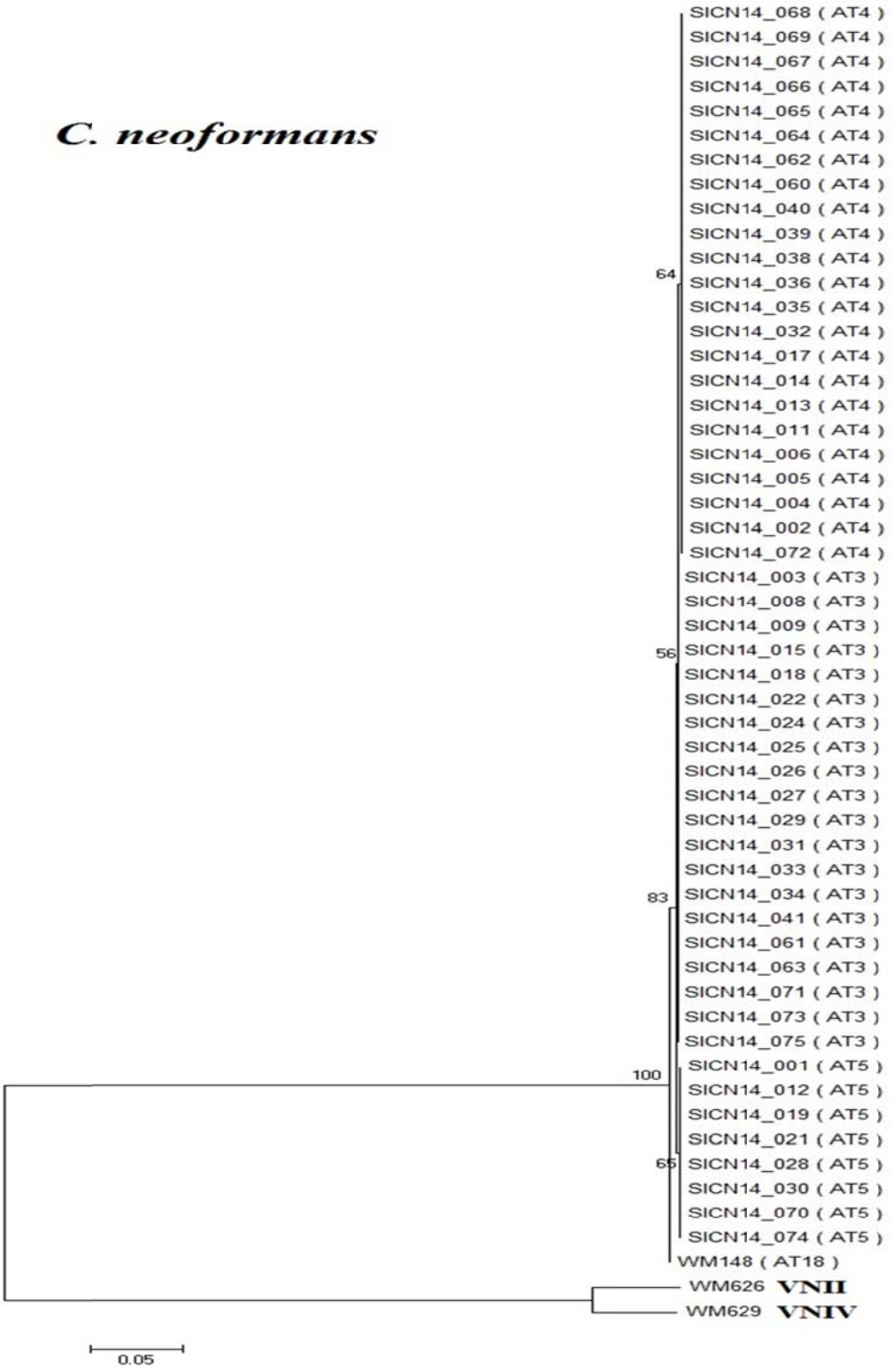
Phylogram of *GPD*1 gene



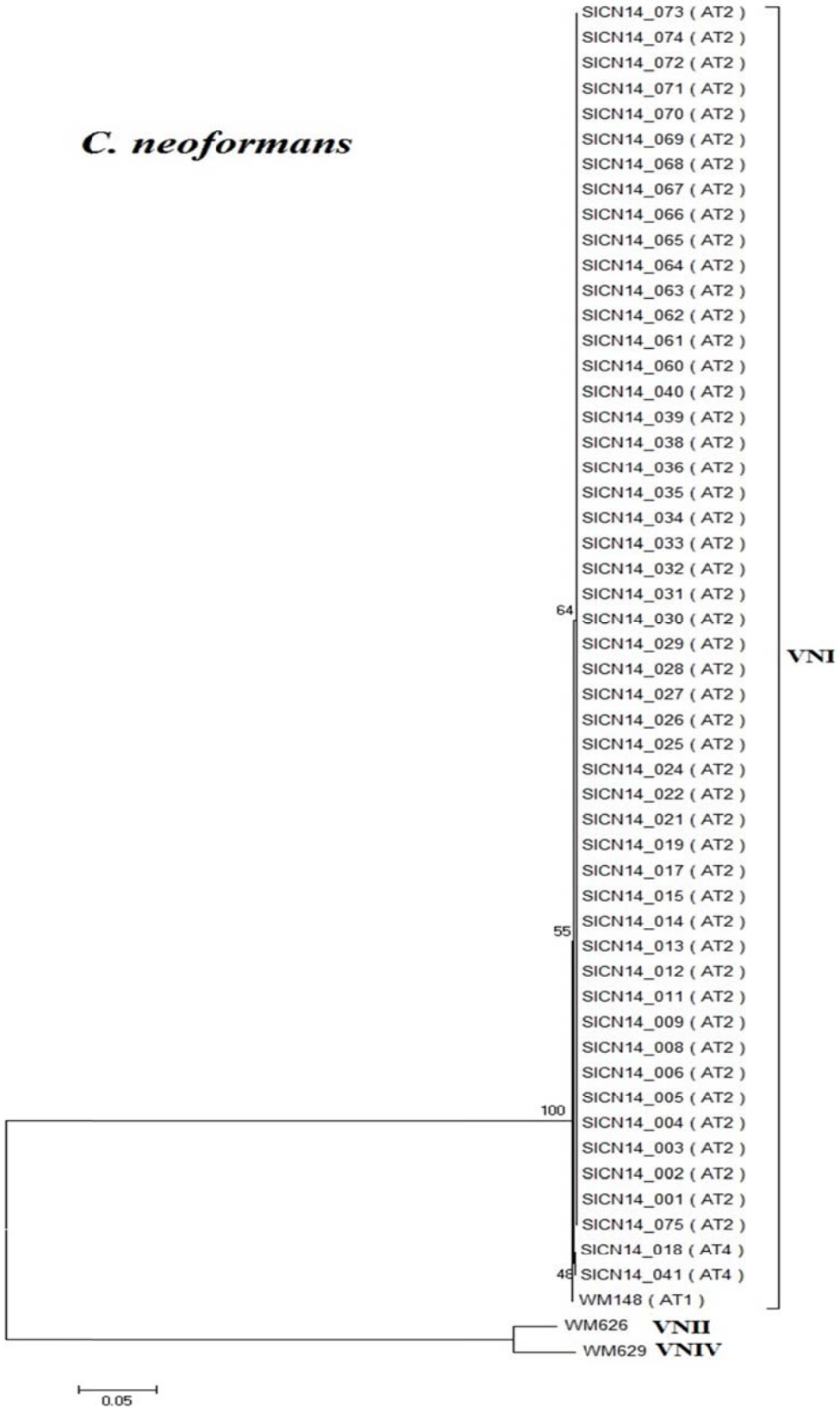
Phylogram of IGS1 gene



Phylogram of *LAC*1 gene



Phylogram of *PLB*1 gene



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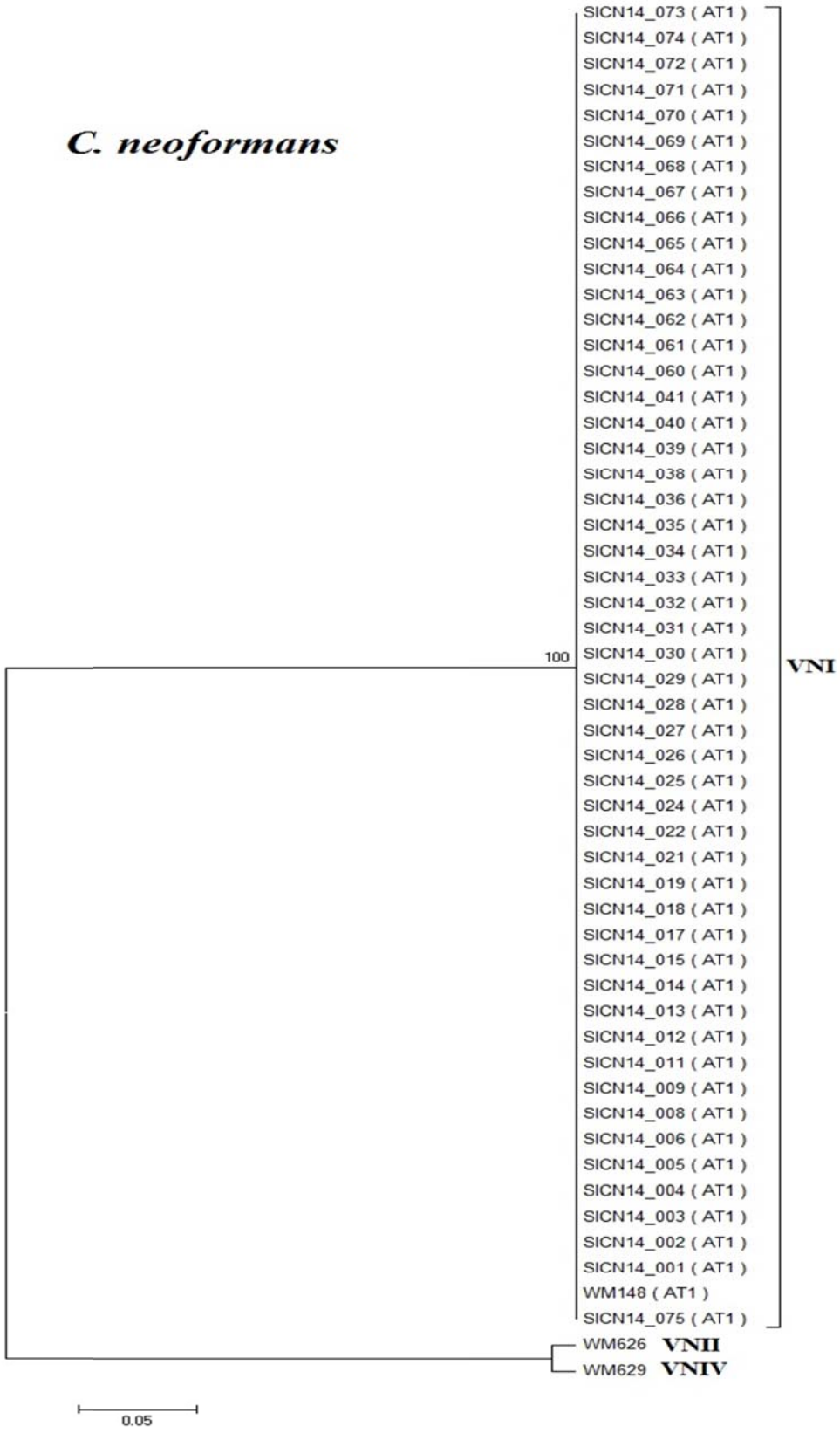
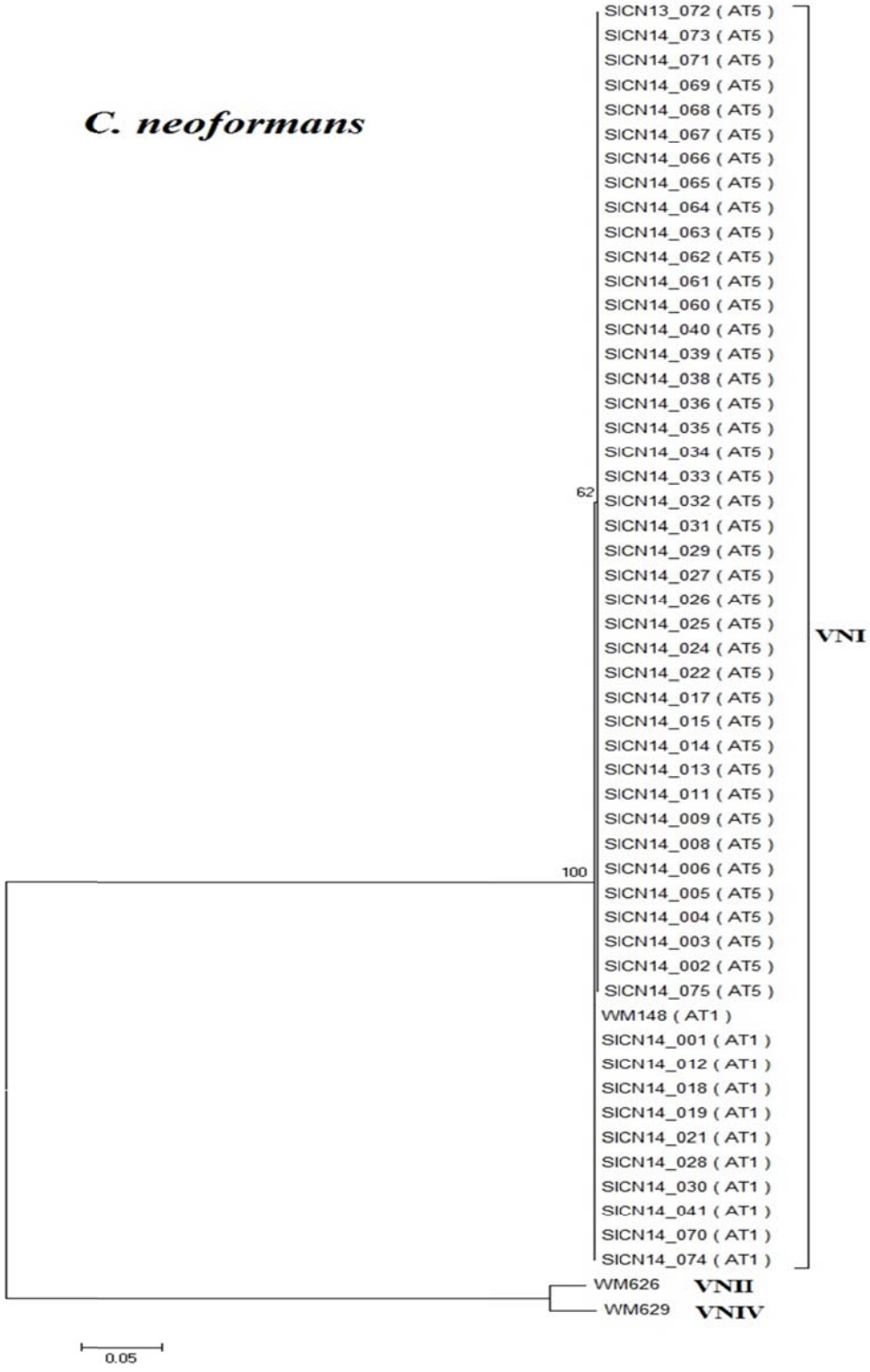


Phylogram of *URA*5 gene



Output

International Journal Publication

Molecular Epidemiology of Cryptococcal Genotype VNIc/ST5 in Siriraj Hospital, Thailand

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Abstract

Despite the strong association between *Cryptococcus neoformans* infection and the *Human immunodeficiency virus* (HIV) status of patients globally, most cryptococcosis in Far East Asia occurs in non-HIV individuals. Molecular epidemiological studies, using multilocus sequence typing (MLST), have shown that more than 95% of cryptococcal strains belong to a specific subtype of the VNIc/ST5 genotype. However, this association has never been specifically examined in other parts of Asia. Therefore, in this study, we investigated the VNIc/ST5 genotype distribution among cryptococcosis patients in Thailand, Southeast Asia.

Fifty-one *C. neoformans* isolates were collected from clinical samples in Siriraj Hospital, Bangkok, Thailand. The strains were predominantly isolated from HIV-positive patients (88.57%) and all were molecular type VNI MATα. An MLST analysis identified five sequence types (ST) in Siriraj Hospital, of which ST4 (45.10%) and ST6 (35.29%) were most common, and ST5 (15.69%), ST32 (1.96%), and ST93 (1.96) were less common. Contrary to reports from Far East Asia, ST5 was predominantly (83.3%) found in HIV patients (P = 0.657), and there was no significant change in the prevalence of ST5 over the past 10 years (P = 0.548). A further analysis of comorbidities showed higher morbidity and delays in the cryptococcal diagnosis in patients with tuberculosis coinfection or without HIV.

Our study suggests that although the Thai population is genetically closely related to the Far East Asian population, ST5 is not associated with non-HIV status in Thailand. Therefore, this association may not be related to the host's genetic background. However, its mechanism remains unclear.

Introduction

Members of the *Cryptococcus* species complex include two major species, *C. neoformans* and *C. gattii*. *Cryptococcus* is an important opportunistic human pathogen causing life-threatening meningitis, with significant morbidity and mortality. *Cryptococcus neoformans* and *C. gattii* are the major causative agents of human and animal cryptococcosis. *Cryptococcus neoformans* is known to infect mainly immunocompromised hosts, whereas immunocompetent hosts are usually infected by *C. gattii* (1, 2). *Cryptococcus* infection occurs after the inhalation of infectious propagules (basidiospores or blastoconidia), which primarily colonize the lung and subsequently invade the central nervous system (CNS) (3). The yeast can survive in humans by expressing virulence factors such as the capsule protein, melanin, because it grow at 37 °C, and because it has a unique mating system.

PCR fingerprinting has been the major typing technique used to classify *C. neoformans* and *C. gattii* into eight major molecular types: VNI (var. *grubii*, serotype A), VNII (var. *grubii*, serotype A), VNIII (serotype AD), VNIV (var. *neoformans*, serotype D), VGI, VGII, VGIII, and VGIV (*C. gattii*, serotypes B and C). VNI is the most common molecular type among the strains collected from cases of clinical infection and is the leading causes of mortality among HIV patients worldwide, including in Thailand (4, 5). The *Cryptococcus* species complex was believed to cause only opportunistic infections, but cryptococcosis in immunocompetent patients is increasingly reported. For example, the ongoing outbreaks of cryptococcosis on Vancouver Island, Canada, and in the Pacific Northwest area of the USA have seen *C. gattii* emerge as a primary pathogen (6). Furthermore, although VNI-type *C. neoformans* was previously believed to

predominantly infect immunocompromised patients, is also reported to cause disease in healthy people (7). Cryptococcosis caused by *C. neoformans* in immunocompetent hosts has been increasingly reported in Far East Asia regions, including China and South Korea, where 77.4%–100% of cryptococcosis infections occur in non-HIV patients. Multilocus sequence typing (MLST) has shown that more than 90% of these cases are infected by a specific subtype of molecular type VNI, the ST5 genotype. Although the overall association between ST5 infection and non-HIV status was reported again in a recent Asian study (8), this association has never been evaluated in other parts of Asia. Therefore, in this study, we investigated the association between HIV status and ST5 infection in Thailand, a Southeast Asian country.

Materials and Methods

Clinical isolates

Cryptococcal isolates from 2012–2014 were obtained from clinical samples in the Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. All the isolates were identified with the RapID™ Yeast Plus System (Thermo Fisher Scientific, MA, USA). L-Canavanine–glycine–bromothymol blue (CGB) agar was used to differentiate *C. neoformans* from *C. gattii*.

Reference strains

A set of standard laboratory reference strains representing each of the eight major molecular types were used for molecular typing: WM148 (VNI), WM626 (VNII), WM 628 (VNIII), WM 629 (VNIV), WM 179 (VGI), WM 178 (VGII), WM 175 (VGIII), and WM 779 (VGIV) (3). KN99α and KN99a were used as the reference strains for the mating-type analysis (9).

Genotype and mating-type analyses

DNA was extracted with the phenol–chloroform–isoamyl alcohol (25:24:1, v:v:v) method (10). Genotypes were determined with a restriction fragment length polymorphism (RFLP) analysis of the *URA5* gene with enzymes *Hha*I and *Sau*96I

98 (Thermo Fisher Scientific, MA USA) (3). Mating
99 types were identified with PCR using two specific primers, STE20Aα and STE20Aa (11).
100

101 MLST

102

103 An MLST analysis of the *C. neoformans* isolates was performed, using the
104 International Society for Human and Animal Mycology (ISHAM) consensus scheme of
105 seven unlinked loci (*CAP59*, *GPD1*, *IGS1*, *LAC1*, *PLB1*, *SOD1*, and *URA5*). Each locus
106 was amplified with previously described primers and amplification parameters (3, 4). The
107 allele types and sequence types (STs) were defined according to the ISHAM MLST
108 database for *C. neoformans* (http://mlst.mycologylab.org) (2).

109

110 Statistical analysis

111

112 The statistical analysis was performed with Fisher's exact test using the online
113 program SISA (http://www.quantitativeskills.com/sisa/statistics/fisher.htm). Unknown
114 data were regarded as lost and were not included in the calculations. Statistical
115 significance was defined as $P \leq 0.05$.

Results

Distribution of major molecular types and mating types

A total of 165 *C. neoformans* isolates were collected from 51 patients. Only one isolate per patient was selected for analysis. Cerebrospinal fluid (29 strains, 56.86%) was the most common site of isolation, followed by blood (21 strains, 41.18%) and bone marrow (one strain, 1.96%). All the isolates were identified as molecular type VNI and mating type MATα.

MLST analysis

All isolates were identified as molecular type VNI (Table 1). The MLST analysis divided the 51 *C. neoformans* isolates into five STs: ST4 (45.10%, n = 23), ST6 (35.29%, n = 18), ST5 (15.69%, n = 8), ST93 (1.96%, n = 1), and ST32 (1.96%, n = 1).

131 **Table 1. Alleles and sequence types of the cryptococcal isolates in this study.**

Strain	Molecular type	Mating type	Allelic profiles							Sequence type
			CAP59	*GPD1*	*IGS1*	*LAC1*	*PLB1*	*SOD1*	*URA5*	
SICN_001	VNI	α	1	3	1	5	2	1	1	5
SICN_002	VNI	α	1	1	1	4	2	1	5	4
SICN_003	VNI	α	1	1	1	3	2	1	5	6
SICN_004	VNI	α	1	1	1	4	2	1	5	4
SICN_005	VNI	α	1	1	1	4	2	1	5	4
SICN_006	VNI	α	1	1	1	4	2	1	5	4
SICN_008	VNI	α	1	1	1	3	2	1	5	6
SICN_009	VNI	α	1	1	1	3	2	1	5	6
SICN_011	VNI	α	1	1	1	4	2	1	5	4
SICN_012	VNI	α	1	3	1	5	2	1	1	5
SICN_013	VNI	α	1	1	1	4	2	1	5	4
SICN_014	VNI	α	1	1	1	4	2	1	5	4
SICN_015	VNI	α	1	1	1	3	2	1	5	6
SICN_017	VNI	α	1	1	1	4	2	1	5	4
SICN_018	VNI	α	1	23	10	3	4	1	1	93
SICN_019	VNI	α	1	3	1	5	2	1	1	5
SICN_021	VNI	α	1	3	1	5	2	1	1	5
SICN_022	VNI	α	1	1	1	3	2	1	5	6
SICN_024	VNI	α	1	1	1	3	2	1	5	6
SICN_025	VNI	α	1	1	1	3	2	1	5	6
SICN_026	VNI	α	1	1	1	3	2	1	5	6
SICN_027	VNI	α	1	1	1	3	2	1	5	6
SICN_028	VNI	α	1	3	1	5	2	1	1	5
SICN_029	VNI	α	1	1	1	3	2	1	5	6
SICN_030	VNI	α	1	3	1	5	2	1	1	5
SICN_031	VNI	α	1	1	1	3	2	1	5	6
SICN_032	VNI	α	1	1	1	4	2	1	5	4
SICN_033	VNI	α	1	1	1	3	2	1	5	6
SICN_034	VNI	α	1	1	1	3	2	1	5	6
SICN_035	VNI	α	1	1	1	4	2	1	5	4
SICN_036	VNI	α	1	1	1	4	2	1	5	4

SICN_038	VNI	α	1	1	1	4	2	1	5	4
SICN_039	VNI	α	1	1	1	4	2	1	5	4
SICN_040	VNI	α	1	1	1	4	2	1	5	4
SICN_041	VNI	α	1	1	10	3	4	1	1	32
SICN_060	VNI	α	1	1	1	4	2	1	5	4
SICN_061	VNI	α	1	1	1	3	2	1	5	6
SICN_062	VNI	α	1	1	1	4	2	1	5	4
SICN_063	VNI	α	1	1	1	3	2	1	5	6
SICN_064	VNI	α	1	1	1	4	2	1	5	4
SICN_065	VNI	α	1	1	1	4	2	1	5	4
SICN_066	VNI	α	1	1	1	4	2	1	5	4
SICN_067	VNI	α	1	1	1	4	2	1	5	4
SICN_068	VNI	α	1	1	1	4	2	1	5	4
SICN_069	VNI	α	1	1	1	4	2	1	5	4
SICN_070	VNI	α	1	3	1	5	2	1	1	5
SICN_071	VNI	α	1	1	1	3	2	1	5	6
SICN_072	VNI	α	1	1	1	4	2	1	5	4
SICN_073	VNI	α	1	1	1	3	2	1	5	6
SICN_074	VNI	α	1	3	1	5	2	1	1	5
SICN_075	VNI	α	1	1	1	3	2	1	5	6

Demographic data

The medical records of 35 patients with cryptococcosis were available. The patients' ages ranged from 24 to 82 years (mean, 40.2 years) and most were males (65.71%). Thirty-one patients (88.57%) were HIV positive.

ST5 not associated with non-HIV patients in Thailand

Recent data have shown a significant association between ST5 and non-HIV patients. Therefore, we expected a similar phenomenon among the Thai isolates. However, our results show that ST5 was mainly detected in HIV-positive patients (83.3%), and there was no significant difference in the prevalence of HIV infection inST5- and non-ST5-infected patients (P = 0.657) (Table 2).

No change in ST distributions in Thailand in the last 10 years

After a national campaign of highly active antiretroviral therapy (HAART), the prevalence of cryptococcosis dropped significantly. According to our records, a 90% reduction in cases of cryptococcosis occurred in the past 10 years (data not shown). Therefore, we speculated that changes in the ST distributions may have occurred. However, our data showed no such change (P = 0.548), as shown in Table 2.

165 **Table 2. Association between sequence types, HIV status, and time.**

Data		Sequence type		P value
		ST5	Non-ST-5	
HIV status[#]	Positive (n=31)	5 (16.13%)	26 (83.87%)	0.657
	Negative (n=4)	1 (25.00%)	3 (75.00%)	
Years	2003-2005 (n=231)	29 (12.55%)	201 (87.45%)	0.548
	2012-2014 (n=51)	8 (15.70%)	43 (84.30%)	

166 [#]only data available in medical records were considered

167

168 ## High mortality was associated with tuberculosis and non-HIV

169 ## strains

170

171 Because comorbidities may affect the efficacy of treatment for cryptococcosis, we
172 investigated the effects of tuberculosis (TB) and HIV infection on treatment outcomes.
173 As expected, patients with active TB had higher morbidity than those without TB (75%
174 vs 25%, respectively; P = 0.031). However, surprisingly, patients without HIV infection
175 had higher morbidity than those without HIV (66.67% vs 33.33%, respectively; P =
176 0.041) (Table 3).

177

178

179

180 **Table 3. Association between comorbidity (HIV or TB) and treatment outcome[#].**

Disease		Treatment outcome		P value
		Cure	Death	
TB	Active (n = 4)	1 (25.00%)	3 (75.00%)	0.031
	Non active (n = 27)	18 (78.26%)	5 (21.74%)	
HIV	Positive (n = 22)	17 (77.27%)	5 (22.73%)	0.041
	Negative (n = 6)	2 (33.33%)	4 (66.67%)	

181 [#]only data available in medical records were considered.

182

183 Because comorbidities can complicate the investigation of cryptococcosis, we
184 tested whether TB or HIV infections delayed the diagnosis of cryptococcosis. Our data
185 showed that coinfection with TB or being HIV-negative delayed the diagnosis of
186 cryptococcosis (1.9 and 2.3 times longer, respectively) (Table 4), and the difference
187 between the TB-coinfected and -uninfected patients was significant (P = 0.024).

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196 **Table 4. Association between comorbidity (HIV or TB) and time from symptom**

197 **onset to disease diagnosis[#].**

Disease	Mean of time from symptom onset to disease diagnosis (days)		P value
	Active/positive status	Non-active/negative status	
TB	42.50 (n = 4)	22.35 (n = 31)	0.401
HIV	18.74 (n = 27)	44.63 (n = 8)	0.024

198 [#]only data available in medical records were considered.

199

Discussion

Cryptococcosis is usually considered an opportunistic infection, and is associated with acquired immune deficiency syndrome (AIDS) in 90% of patients. A patient typically becomes infected by inhaling the spores of *Cryptococcus*, which primarily cause a lung disease. The spores can then disseminate to other organs via the bloodstream, especially to the CNS. In this study, we found that the commonest source of cryptococcal isolates was the cerebrospinal fluid (56.86%), whereas 41.18% of the isolates were from blood and 1.96% from bone marrow, which is known to be involved in the pathogenesis of this organism. Our results are consistent with a previous study, in which more than 80% of specimens were isolated from cerebrospinal fluid (4).

Cryptococcus neoformans VNI is the molecular type most commonly found in Thailand and around the world (4, 7), and usually infects immunocompromised individuals. Surprisingly, in 2008, it was reported that the majority (91.5%) of *C. neoformans* VNIc/ST5 isolates detected in China were from immunocompetent patients (12). This phenomenon has also been reported in other areas of Far East Asia (4, 8, 13, 14). Therefore, we undertook an epidemiological study of the putatively non-HIV-associated genotype, ST5, in Thailand, where the population is closely genetically related to that in Far East Asia. Our results are consistent with those of all previous studies throughout the world, in that more than 90% of the isolates were VNI (4, 15). Our MLST analysis divided the *C. neoformans* strains from Siriraj Hospital into five STs of molecular type VNI: ST4 (45.10%, n = 23), ST6 (35.29%, n = 18), ST5 (15.67%, n = 8), ST93 (1.96%, n = 1), and ST32 (1.96%, n = 1). The high prevalence of the ST4 and ST6 genotypes is consistent with a previous study in Thailand (4). ST93 is the dominant

224 sequence type in India and Indonesia (8). A recent report showed that genotype ST32 is
225 rarely (3.2%) found in Japan. (12). These data demonstrate clear genetic variations
226 among different Asian regions (4, 8, 13, 14). A phylogram, constructed with a neighbor-
227 joining analysis based on concatenated data from seven loci, showed that the five STs
228 (STs 4, 5, 6, 32, and 93) detected in this study were genetically homogeneous. This
229 clonality of *C. neoformans* was also confirmed by the fact that all the strains belonged to
230 MATα.

231 Most strains were isolated from HIV-positive patients (88.6%), which is
232 consistent with a previous study (4). The male predominance (65.71%) is consistent with
233 previous data collected in Thailand, which showed that more than 60% of cryptococcosis
234 patients were male (4). A slight increase in the mean age, from 30.5 years in 1997 to 32.4
235 years in 2004, has been reported (16). A later study in 2013 reported a further increase in
236 the average age to 37.97 years (4). This trend was evident again in our study, where the
237 average age had increased to 40.2 years. One explanation might be that HARRT
238 improves the quality of patients' lives and therefore increases their life spans (17).

239 Unlike cryptococcosis in Japan (13), China (18), and Korea (19), no association
240 was evident between ST5 and non-HIV status in patients at Siriraj Hospital in this study.
241 Only 15.69% of the clinical isolates were ST5 and 12.50% of the ST5 isolates were from
242 an HIV-negative patient. It has been reported that ST5 is found in both HIV-positive and
243 -negative patients (8). Our data confirms that the association between ST5 and non-HIV
244 status is unique to Far East Asia. Although this association has been tentatively attributed
245 to the specific genetic susceptibility of the Far East Asian population (8), our data suggest
246 otherwise. Because the genetic backgrounds of the Fast East Asian and South East Asian
247 populations are reportedly close (20), the specific genetic susceptibility theory is
248 inconsistent with this difference in the ST5–HIV relationship in these two Asian

249 populations. However, because the number of samples analyzed in the present study was
250 limited, a larger study is required.
251 Coinfection with *Cryptococcus* and *Mycobacterium tuberculosis* is relatively difficult to
252 treat. Our results confirm that comorbidity with tuberculosis reduces the treatment
253 efficacy for cryptococcosis. There is evidence that *M. tuberculosis* and *Cryptococcus*
254 synergistically suppress the immune system, and consequently reduce the signs and
255 symptoms of patients, delaying their diagnosis and treatment (21). Treatment also failed
256 more frequently in HIV-negative patients in this study. A similar suggestion was
257 proposed in another report, in which a delay in the diagnosis of cryptococcosis in non-
258 HIV patients was attributed to the negative or low antigen titers in their samples (22).
259 These proposals are also supported by our finding that coinfection with TB or being a no
260 HIV infection delayed the diagnosis of cryptococcosis. Therefore, the timely diagnosis of
261 the disease, especially in TB endemic areas, is essential (23, 24).

262 In conclusion, the non-HIV-specific ST5 is still less common than other STs in
263 Thailand. A phylogenetic analysis showed that the five STs detected were highly
264 homogeneous. However, the relationship between the sequence type and the host's HIV
265 status is still unclear. The collection of more clinical strains is required to clarify this
266 relationship.
267

268 # Acknowledgments

269

270 This work was supported by a Thailand Research Fund Grant for New
271 Researchers to P.N. (TRG5780088).
272

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353

Application

Review article

ReviewArticle

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: *Cryptococcoccus*, genotype, molecular type, epidemiology, HIV

Siriraj Med J 2015;67:301-305
E-journal: http://www.sirirajmedj.com

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

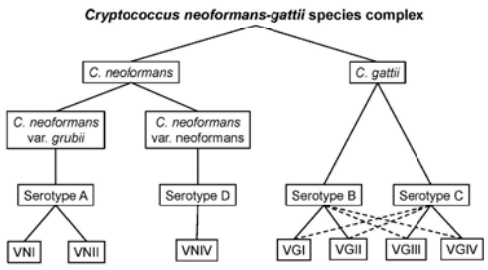

Fig 1. A correlation between different classifications of *C. neoformans-gattii* species complex. Note that minority of serotype B can be classified to VGIII or VGIV and Minority of serotype C can be classified to VGI or VGII (represented in dashed lines)

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-gattii* 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

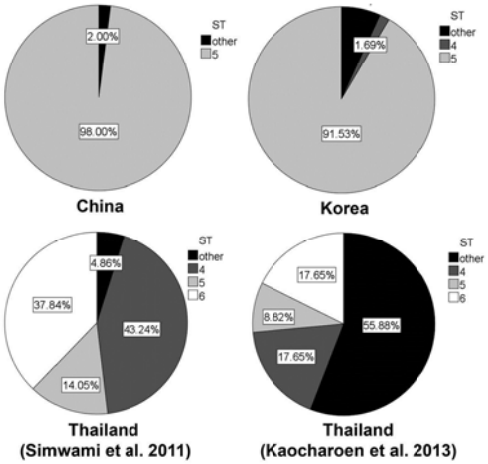

Fig 2. Prevalence of each major sequence type (ST) in each study.

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.

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International conference presentation

1. **A chair session and invited speaker** “Genotyping and the taxonomy of the *C. neoformans*/*C. gattii* species complex” and an invited speaker “Molecular Epidemiology of the *Cryptococcus species* complex in Asia” at the 19th International Society for Human and Animal Mycology Congress on 3rd-7th May 2015 at the Melbourne Exhibition Centre, Melbourne, Victoria, Australia

National conference presentation

1. **A poster presentation** “Prevalence of a non-HIV specific genotype ST5 of Cryptococcus neoformans-gattii species complex in Thailand” at the TRF-OHEC Annual Congress 2016 on 6th-8th January 2016 at Reagent Cha-am Beach Resort, Cha-am, Petchaburi, Thailand

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