Phenotype and Genotype of *Cryptococcus neoformans* in Khon Kaen Province, Thailand

ฟีโนไทป์และจีโนไทป์ของเชื้อ *Cryptococcus neoformans* ในจังหวัดขอนแก่น ประเทศไทย

Jeerapar Noiseeluang (จีรภา น้อยสีเหลือง)* Sukanya Srigulbutr (สุกัญญา ศรีกุลบุตร)**
Phuangphaka Sadee (พวงผกา สาดี)** Dr.Kunyaluk Chaikumpar (ดร.กัญญลักษณ์ ชัยคำภา)***
Dr.Arunee Sangka (ดร.อรุณนี สังกา)**** Dr.Maitree Pakarasang (ดร.ไมตรี ปะการะสังข์)****
Dr.Chularu Prariyachatigul (ดร.จุฬารัตน์ ปริยชาติกุล)*****

ABSTRACT

Cryptococcosis is an opportunistic disease in immunocompromised patients by *Cryptococcus neoformans*. The differentiation and classification of pathogenic *Cryptococcus* species provide useful data for molecular epidemiological studies. The objective is to study the molecular epidemiology of *URA5* gene in *C. neoformans* infections. Seventy isolate of *C. neoformans* from clinical samples were typing by PCR-RFLP technique. Sixty-nine isolates were identified as molecular type VNI (98.57%) and one isolate were VGII (1.43%). It was susceptible 100% with 3 antifungals as follow: fluconazole, voriconazole and posaconazole. The susceptibility of itraconazole, 5-flucytosine, amphotericin B were 97.22% (MIC_{90}= 0.25 µg/ml), 94.44% (MIC_{90}= 8 µg/ml) and 80.56% (MIC_{90}= 1 µg/ml) respectively. *C. neoformans* (VNI) is the most wide spread species throughout the world, as the same situation in Khon Kaen, Thailand.

บทคัดย่อ

โรคคริปโตคอคโคซีส เป็นโรคที่เกิดจากการติดเชื้อราฉวยโอกาสในผู้ป่วยที่มีภูมิคุ้มกันบกพร่องจากเชื้อราคริปโตคอคคัสนีโอฟอร์แมนส์ที่ซับซ้อน ความแตกต่างและการจำแนกสายพันธุ์จะให้ข้อมูลที่เป็นประโยชน์สำหรับการศึกษาระบาดวิทยาระดับโมเลกุล การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาระบาดวิทยาระดับโมเลกุลและลักษณะที่แสดงออกต่อความไวของยาชนิดต่างๆ ของเชื้อราคริปโตคอคคัสนีโอฟอร์แมนส์ การคัดกรองรูปแบบทางพันธุกรรมจำนวน 70 สายพันธุ์ที่แยกได้จากผู้ป่วยด้วยวิธี *URA5*-RFLP พบว่าเชื้อมีรูปแบบทางพันธุกรรมเป็น VNI ร้อยละ 98.57 (69/70) และเป็น VGII ร้อยละ 1.43 (1/70) การศึกษาความไวของยา 3 ชนิด คือ fluconazole voriconazole และ posaconazole ให้ผลความไวต่อยาร้อยละ 100 ในขณะที่ยา itraconazole 5-flucytosine และ amphotericin B ให้ผลความไวร้อยละ 97.22 ร้อยละ 94.44 และร้อยละ 80.56 ตามลำดับ ค่า MIC_{90} ของยาดังกล่าวนี้คือ 0.25 8 และ 1 ไมโครกรัมต่อมิลลิลิตรตามลำดับ ในการศึกษานี้ได้ข้อสรุปเชื้อราว่าคริปโตคอคคัสนีโอฟอร์แมนส์มีรูปแบบพันธุกรรมชนิด VNI เป็นสายพันธุ์ที่แพร่หลายมากที่สุดทั่วโลก รวมทั้งในประเทศไทยด้วยซึ่งสอดคล้องกับการศึกษาส่วนใหญ่

Keywords: *Cryptococcus neoformans*, Molecular Epidemiology, PCR-RFLP
คำสำคัญ: เชื้อราคริปโตคอคคัสนีโอฟอร์แมนส์ ระบาดวิทยาระดับโมเลกุล พีซีอาร์ อาเอฟแอลพี

* *Student, Master of Science Program in Medical Sciences, Faculty of Associated Medical Sciences, Khon Kaen University*
** *Supportive Staff, Division of Clinical Laboratory, Srinagarind Hospital, Faculty of Medicine, Khon Kaen University,*
*** *Assistant Professor, Department of Microbiology, Faculty of Medicine, Khon Kaen University*
**** *Assistant Professor, Department of Clinical Microbiology, Faculty of Associated Medical Sciences, Khon Kaen University*
***** *Associate Professor, Department of Clinical Microbiology, Faculty of Associated Medical Sciences, Khon Kaen University*

Introduction

Cryptococcus neoformans and *C. gattii* species complex cause cryptococcosis in humans and animals worldwide. Moreover, *C. neoformans* is an opportunistic pathogen that mostly infected in HIV infections or cancer, primarily affecting the central nervous system. The difference of varieties have been based on molecular typing by the polymerase chain reaction (PCR) and follow with fingerprinting, restriction fragment length polymorphism (RFLP) (Boekhout et al., 2001; Currie et al., 1994; Latouche et al., 2003; Meyer et al., 2003), amplified fragment length polymorphism (AFLP) and multi locus sequence typing (MLST) (Bovers et al., 2008; Meyer et al., 2009). To identification of concordant genotype in *C. neoformans* and *C. gattii* species complex, these technique could distinguish for each clearly species. Previous studies showed the outbreak strain in the worldwide as molecular type VNI, which are also the major case of infection with HIV-positive patients (Dromer et al., 1996). In Thailand, *C. gattii*, serotype B and C were reported the main cause of disease before the AIDs era (Sukroongreung et al., 1996). In 2013, Kaocharoen and coworker that were reported molecular type VNI more than 90% from clinical and environmental isolates in Thailand. In addition, they had reported molecular type VNII (2.45% in clinical isolates) and VNIV (0.2% from environmental isolates) and VGI (0.2% clinical isolate), VGII (2.4% clinical isolate) (Kaocharoen et al., 2013). Moreover, two species of *C. neoformans* and *C. gattii* has been numerous differences geographical distribution, clinical presentation and molecular characters. Treatment of the fungal pathogen is difficult, due to the many similarities between the cellular machinery and humans. In the current study amphotericin B is the main treatment of choice and often complement with 5-flucytosine. Azole, such as fluconazole and itraconazole are frequency used in the management of cryptococcosis. In addition, In Africa had reported of resistance to fluconazole that primarily used therapy in AID patients (Manosuthi et al., 2006). Nowadays, the focus of monitoring molecular epidemiology, drug resistant against *C. neoformans* species complex and *in vitro* each other drug, which are important to provide treatment.

Objective of the study

To study the epidemiology of *Cryptococcus neoformans* infection in patients in Khon Kaen, Thailand by phenotypic (antifungal susceptibilities) and genotypic (PCR-RFLP).

Materials and methods

Patients and ethics statement

The cryptococcosis patients were recruited from Khon Kaen Hospital and Srinagarind Hospital, Khon Kaen, Thailand. The study was reviewed and approved by the Office of The Khon Kaen University Ethics Committee in Human Research (HE561236). Written informed consent was provided by patients' legal guardians. The epidemiological profiles of the patients (age, sex, immunological status, ward, biological sample and residence) were obtained by analysis of the examination requisitions.

Sample size

The sample sizes were determined from Yamane (Yamane, 1967). This formula assumes a degree of variability of 0.05 and a confidence level of 95%. Population size is the number of *C. neoformans* infected patients from May 2012 to May 2013, in which 84 cases were reported from Khon Kaen

Hospital and Srinagarind Hospital. Therefore the sample size would consist of at least 70 isolates as substituted by the formula.

The formula from Yamane is:

$$n = \frac{N}{1 + N(e^2)} = \frac{84}{1 + 84(0.05^2)} = 69.42$$

Where: n = sample size

N = population size

e = the level of precision

This formula assumes a degree of variability (i.e. proportion) of 0.05 and a confidence level of 95%.

Isolation of *C. neoformans* from clinical specimens

C. neoformans isolates were isolated from clinical specimens of patients in Khon Kaen Hospital and Srinagarind Hospital during October 2013 to December 2014 which was identified by medical technologists. Forty-one isolates from Khon Kaen Hospital and 29 isolates from Srinagarind Hospital. The isolates from both hospitals were reconfirmed by conventional methods. The 48 h single colony of 35 °C from Sabouraud dextrose agar (SDA, HiMedia, India) were picked and stained with Nigrosin (Sigma, Singapore) and examined for capsules. The isolates were also confirmed by using urease activity on Christensen (BD, USA) urea agar medium.

References strains

The reference strains of eight molecular types of the *C. neoformans* and *C. gattiis* species complex were provide by Medical Doctor Popchai Ngamskulrungroj, Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand. The original reference strains were kindly permitted from the research of Dr. Wieland Meyer (Meyer et al., 2003). A set of Laboratory standard *C. neoformans* reference strains representing each molecular type were WM 148 (serotype A, VNI), WM 626 (serotype A, VNII), WM 628 (serotype AD, VNIII), WM 629 (serotype D, VNIV), WM 179 (serotype B, VGI), WM 178 (serotype B, VGII), WM 175 (serotype B, VGIII) , WM 779 (serotype C, VGIV).

DNA extraction and purification

Genomic DNA was extracted by glass bead-phenol:chloroform method (Yamada et al., 2002) slightly modified with CTAB (Appold, 2008; Ros-Chumillas et al., 2007).

***URA5* gene RFLP analysis**

All 70 isolates of *C. neoformans* were test for *URA5* gene RFLP analysis. The *URA5* gene was amplified with the two flanking primers: *URA5* (5'ATGTCCTCCCAAGCCCTCGACTCCG3') and *SJO1* (5'TTAAGACCTCTGAACACCGTACTC3'). PCR of the *URA5* gene was performed in a final volume of 50 µl containing 50 ng DNA. 1x PCR buffer (500mM KCl, 100mM Tris-HCl (pH 9.1 at 20 °C) and 0.1% Triton^{TM} X-100) were used. The buffer was optimized with 0.2mM of each dNTP (Vivantis, Molecular laboratory grade, Malaysia), 0.2mM each of dATP, dCTP, dGTP and dTTP (Vivantis, Malaysia), 0.3mM $MgCl_2$ and 1.5 U *taq* DNA polymerase (Vivantis, Malaysia). PCR of the *URA5* gene was performed for 35 cycles in a thermal cycler at initial denaturation at 94 °C, 2 min, denaturation at 94 °C, 45 s, annealing at 61 °C, 1 min and extension at 72 °C, 2 min and followed by a final extension cycle at 72 °C, 10 min. Amplification products were visualized by 1.5% agarose gel electrophoresis in 1x TBE buffer at 75 V for 1 h and strained with 0.3 µg/ml ethidium bromide. Then, each of the amplified products 15 µl were mixed with 3 ml buffer and were

digested with *Sau*96I (10 U/µl) and *Hha*I (20 U/µl) for overnight at 37 °C and separated by 3% agarose gel and run electrophoresis at 100 V for 3 h by using a 50 bp DNA ladder. The molecular types were determined with comparison with the profile from the standard strains of the eight principle genotype (VNI-VNIV and VGI-VGIV) of *C. neoformans* and *C. gattii* species complex (Meyer et al., 2003).

Antifungal susceptibility testing

The *In vitro* susceptibility profiles of 36 isolates of *C. neoformans* against antifungal agent were determined by the reference methods of broth microdilution in accordance with document M27-A3 of the CLSI (Park et al., 2008; Pfaller et al., 2007; Pfaller et al., 2014). Stock inoculum suspensions of yeast were obtained from 24 h cultures on SDA at 35 °C. Picked several well-isolate colonies of >1 mm diameter from pure culture of yeast isolate and added in sterile saline to turbidity equal to 0.5 McFarland standard (Oxford) and were adjusted with spectrophotometer in saline suspension to match transmittance at 530 nm. Then, suspensions were transferred 20 µl of the inoculum into 11 ml of YeastOne® inoculum broth (1.5-8x10^3 CFU/ml). The antifungal drugs were used such as The Sensititre® YeastOne™ Test Panel (Trek Diagnostic Ltd., West Sussex, England). The wells were reconstituted by the addition of 100 µl of inoculum suspension. A check of colony count should be done by removing 10 µl from the positive control well and plating onto SDA and cover all wells with the adhesive seal. After incubation at 35 °C for 72 h, MICs were determined by observe the lowest antifungal concentration preventing to develop of red colour (first blue well) and quality control using *Candida* spp.

Results

All of 70 clinical isolates were positive for *C. neoformans* by Nigrosin staining, microscopic characteristic and urease production. The cryptococcal cells were generally appearing as round shape cells, surrounded by a large polysaccharide capsule. After testing by the urease enzyme of *C. neoformans* isolates showed that all *C. neoformans* create enzyme urease and hydrolyses urea to ammonia, which leads to higher pH and color change indicator (Phenol red), from yellow to pink.

Among the 70 *C. neoformans* clinical isolates from both Hospitals, 41 (58.57%) clinical isolated from Khon Kaen hospital were derived into male 27 (38.57%) and female 14 (20%) and 29 (41.43%) clinical isolated from Srinagarind hospital divided into male 18 (25.71%) and 11 (15.71%) from female as shown in table1.

To examine the molecular typing by using PCR-RFLP, all the isolates were amplified with primer pair *URA5-SJ01*. A fragment of 750 bp was showed in figure 1. The specific 750 bp was digested with the restriction enzyme *Sau96I* and *Hhal* in double digestive condition. From 70 *C. neoformans* clinical isolates, 69 (98.57%) isolates were presented molecular type VNI and 1 isolate was presented molecular type VGII (1.43%) show in figure 2.

Table 1 Epidemiological characteristic and molecular group of patients with *C. neoformans*

Molecular type	Sex	HIV status	Hospital	Age (range)	Biology sample					Total (n)
					Cerebrospinal fluids (CSF)	Blood	Pus	Bone marrow	Tracheal suction	
VNI	Male	positive	Khon Kaen	0-15	-	-	-	-	-	-
				16-30	6	1	-	1	-	8
				31-45	6	3	-	-	-	9
				46-60	-	-	-	-	-	-
				>60	-	-	-	-	-	-
			Srinagarind	0-15	-	-	-	-	-	-
				16-30	1	1	-	-	-	2
				31-45	-	3	-	-	-	3
				46-60	1	-	-	-	-	1
				>60	-	-	-	-	-	-
	Male	Negative	Khon Kaen	0-15	-	1	-	-	-	1
				16-30	-	-	-	-	-	-
				31-45	3	3	-	-	-	6
				46-60	1	1	-	-	-	2
				>60	-	-	-	-	-	-
			Srinagarind	0-15	-	-	-	-	-	-
				16-30	1	2	-	-	-	3
				31-45	1	3	-	-	-	4
				46-60	3	2	-	-	-	5
				>60	-	-	-	-	-	-
	Female	Positive	Khon Kaen	0-15	-	-	-	-	-	-
				16-30	2	1	-	-	-	3
				31-45	1	-	-	-	-	1
				46-60	1	-	-	-	-	1
				>60	1	-	-	-	-	1
			Srinagarind	0-15	-	-	-	-	-	-
				16-30	-	-	-	-	-	-
				31-45	-	-	-	-	-	-
				46-60	-	-	-	-	-	-
				>60	-	-	-	-	-	-
		Negative	Khon Kaen	0-15	-	-	-	-	-	-
				16-30	1	1	-	-	-	2
				31-45	1	1	-	-	-	2
				46-60	-	1	-	-	-	1
				>60	-	3	-	-	-	3
			Srinagarind	0-15	-	-	-	-	-	-
				16-30	1	-	-	-	-	1
				31-45	1	1	1	-	-	3
				46-60	1	3	-	-	-	4
				>60	-	1	1	-	1	3
VGII	Male	Negative	Khon Kaen	31-45	1	-	-	-	-	1
Total (%)					34 (48.57)	32(45.71)	2(2.86)	1(1.43)	1(1.43)	70(100)

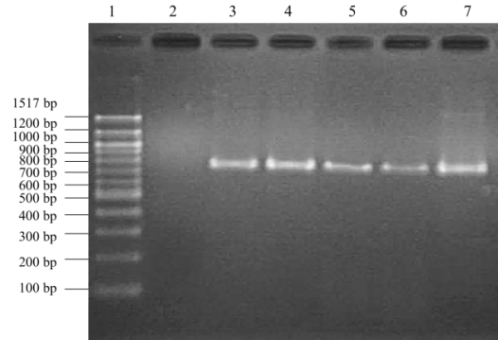

Figure 1 show the amplification products of *URA5* gene, *lane* 1 is the molecular marker (DNA ladder 100 bp), *lane* 2 is the negative control (DDW), *lane* 3 is the positive control (References strain WM 148) and *lans* 4, 5, 6 , 7 are clinical isolates of *C. neoformans*.

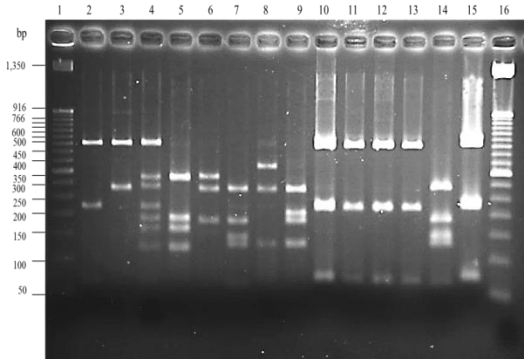

Figure 2 The PCR- RFLP profile of the *URA5* gene from *Cryptococcus* spp. Obtained double digested with *Sau*96I and *Hha*I. *Lanes* 1 and 16 are the molecular marker (DNA ladder 50 bp), *lanes* 2, 3, 4, 5 are genotype VNI, VNII, VNIII, VNIV, respectively. *Lanes* 6, 7, 8, 9 are the genotype VGI, VGII, VGIII and VGIV and *lanes* 10, 11, 12, 13, 14, 15 are *C. neoformans* clinical isolates from patients.

The *in vitro* susceptibility profile of *C. neoformans* 36 isolates to six antifungal agent drugs, MIC range, MIC_{50}, MIC_{90} and MIC geometric mean (µg/ml) were showed in table 2. We found that the susceptible to fluconazole, voriconazole, and posaconazole were 100% inhibition by concentration of 8 µg/ml, 0.12 µg/ml and 0.25 µg/ml, respectively and the results of itraconazole and 5- flucytosine were presented 97.22 (35/36) and 94.44% (34/36) respectively. In addition, there are twenty-nine from thirty-six isolates were susceptible to amphotericin B but only 7 resistant isolates.

Discussion and conclusion

The molecular epidemiology has been studied in worldwide. The main etiology agents cryptococcosis are two species *C. neoformans* and *C. gatti* that present numerous differences geographical distribution, epidemiology and molecular character (Kaocharoen et al., 2013). Genotyping of pathogen species complex have been recognized VNI, VNII, VNB, VNIII and VNIV among *C. neoformans* isolates and VGI, VGII, VGIII and VGIV among *C. gattii* isolates from previous studies (Bertout et al., 2013; Ferreira-Paim et al., 2011). At that time, the molecular types VNI and VGII were founded the most common genotype worldwide. In this study, molecular type VNI (98.57%) being the most common molecular type and the rare molecular type VGII (1.43%) which only one case was founded. Previously study described *C. gattii* isolates belong to the VGII genotype (Trilles et al., 2008). This genotype was reported as the cryptococcosis outbreak that occurred on Vancouver Island (BC, Canada)

Table 2 MIC value, geometric mean and susceptibility profile of *C. neoformans* isolates *N* =36

Antifungal agent	MIC range (µg/ml)	MIC_{50} (µg/ml)	MIC_{90} (µg/ml)	MIC geometric mean (µg/ml)	Susceptibility profile % (isolates)			
					S	S-DD	I	R
Amphotericin B	0.5-1	0.5	1	0.583	80.56 (29/36)	0	0	19.44 (7/36)
Fluconazole	2-8	4	8	4.16	100 (36/36)	0	0	0
Itraconazole	0.03-0.25	0.12	0.25	0.084	97.22 (35/36)	2.78 (1/36)	0	0
Voriconazole	0.015-0.12	0.12	0.12	0.049	100 (36/36)	0	0	0
5-Flucytosine	1-8	4	8	2.774	94.44 (34/36)	0	5.56 (2/36)	0
Posaconazole	0.03-0.25	0.12	0.25	0.090	100 (36/36)	0	0	0

MIC, Minimum Inhibitory Concentration MIC_{50}, MIC at which 50% of the isolates are inhibited MIC_{90}, MIC at which 90% of the isolates are inhibited *S*, susceptible *S-DD*, susceptible dose-dependent *I*, intermediate *R*, resistant

(Byrnes et al., 2009). In Brazil reported of this VGII genotype, it's potential to cause severe disease in immunocompetent host (Matos et al., 2012). The determination of susceptible profile revealed *C. neoformans* isolates to antifungal drugs, studies conducted in several part of the world have shown low MIC_{50} and MIC_{90} values for fluconazole against *C. neoformans* VNI (Tay et al., 2006). In this study no evidence of resistant to fluconazole (100% susceptible at MIC_{90} 8 µg/ml), posaconazole, voriconazole (100% susceptible at MIC_{90} 0.25, 0.12 µg/ml) but itraconazole (97.22% susceptible at MIC_{90} 0.25 µg/ml, 2.78% susceptible, 5-flucytosine (94.44% susceptible at MIC_{90} 8 µg/ml). The majority of studies addressing *in vitro* susceptibility testing for amphotericin B against *C. neoformans* and *C. gattii* had showed that most of these isolates susceptible at MIC values $\leq$ 1 mg/L. Lozano-Chui and coworker (1998) reported MIC values of 3-4 mg/L, which were associated with treatment failure.

Identification of the species by PCR-RFLP and antifungal susceptible analysis are important to determining the prevalent of *C. neoformans* including therapy in patients. Moreover, we have knowledge the importance of performing molecular typing to give finding of difference in the *in vitro* susceptibility profile in Khon Kaen, Thailand.

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