DIAGNOSING AND MONITORING OF CRYPTOCOCCAL MENINGITIS IN AIDS PATIENTS DURING TREATMENT BY PCR

ANURAK MUNSRICHOOM

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE (MICROBIOLOGY)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2000

ISBN 974-663-994-3

Thesis

entitled

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Anurak Munsrichoom.

Anurak Munsrichoom

Candidate

Angkana Chaiprasert

Assoc. Prof. Angkana Chaiprasert, Dr.rer.nat.

Major Advisor

Chularut

Assist. Prof. Chularut Prariyachatigul, Ph.D.

Co-advisor,

Lect. Somsit Tunsuphaswadikul, M.D.

Co-advisor,

Prof. Liangchai Limlomwongse, Ph.D.

Dean

Faculty of Graduate Studies

Uraiwan Kositanont

Assoc. Prof. Uraiwan Kositanont, Ph.D.

Chairman

Master of Science Program in Microbiology,

Faculty of Medicine Siriraj Hospital

Thesis

entitled

DIAGNOSING AND MONITORING OF CRYPOCOCCAL MENINGITIS

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was submitted to the Faculty of Graduate Studies, Mahidol University

for the degree of Master of Sciences (Microbiology)

on

May 19, 2000

Anurak Munsrichoom

Anurak Munsrichoom

Candidate

Angkana Chaiprasert

Assoc. Prof. Angkana Chaiprasert, Dr.rer.nat.

Chairman

Chularut

Assist. Prof. Chularut Prariyachatigul, Ph.D.

Menber

Prof. Amorn Leelarasamee,

M.D., M.Med.Sci.

Member

Lt. L.

Prof. Liangchai Limlomwongse, Ph.D.

Dean

Faculty of Graduate Studies

Mahidol University

S. Tunsuphaswadikul

Lect. Somsit Tunsuphaswadikul, M.D.

Member

C. Tuchinda

Prof. Chanika Tuchinda, M.D.

Dean

Faculty of Medicine Siriraj Hospital

Mahidol University

ACKNOWLEDGEMENT

I would like to express my sincere gratitude and appreciation to my advisor, Dr. Angkana Chaiprasert, my co-advisor, Dr. Somsit Tunsuphaswadikul and Dr. Chularut Prariyachatigul for their valuable advice, encouragement, constructive suggestion throughout the studies, laboratory work and preparation of the thesis. I also appreciate to the thesis committee, Dr. Amorn Leelarasami for his kindly advice and valuable comment.

I am also grateful to Jannsen Pharmaceutical Ltd. for providing the financial support on the laboratory supplies for this study and also thank to Miss Siriluk Atamaprasangsa for collecting the specimens, closely follow up the patients in the study and her kindness during the period of study and Miss Nuttaya Suwanapatra for her performing the data statistic analysis. I would like to thank to all graduate students, colleaques and laboratory workers at the Department of Microbiology, Faculty of Medicine, Siriraj Hospital, Mahidol University for their kindness throughout my laboratory work.

Finally, I would like to thank my parents, my family and my good friends for their warm love, supporting, understanding and assist me to reach the final aim.

Anurak Munsrichoom

3836904 SIMI/M : MAJOR: MICROBIOLOGY; M. Sc. (MICROBIOLOGY)
KEY WORDS : CRYPTOCOCCUS NEOFORMANS / PCR / PROPHYLAXIS
ANURAK MUNSRICHOOM: DIAGNOSING AND MONITORING CRYPTOCOCCAL MENINGITIS IN AIDS PATIENTS DURING TREATMENT BY PCR. THESIS ADVISORS: ANGKANA CHAIPRASERT, Dr. rer. nat., CHULURAT PRARIYACHATIGUL, Ph.D., SOMSIT TUNSUPHASWADIKUL, M.D. 126 p ISBN 974-663-994-3.

A PCR system using specific primers CPL1 and CPR4 based on 18S rRNA gene was evaluated for diagnosing cryptococcal meningitis in 160 AIDS patients highly suspected of carrying this disease. When comparing PCR with conventional laboratory methods, it revealed 79.7% (47/59) sensitivity compared to LA with 100% (128/128) sensitivity. Lower sensitivity of PCR was explained by the presence of some *Taq* inhibitors in CSF and/or the inefficient method for lysing yeast cells. *C. neoformans* was isolated from 120 in 128 CSF samples (93.7%) on the first day of patient registration. India ink preparation of pellet from centrifuged CSF showed 96.9% (124/128) positive for encapsulated yeasts. Most of the patients in this study had disseminated cryptococcosis with 80.1% (125/156) positive hemoculture by using automate Bactec 9240 culture system and 8.8% (12/136) positive urine culture.

The patients were divided into two groups and treated with amphotericin B or combination of amphotericin B and itraconazole. The outcome for each patient was determined by both clinical and microbiological examination. In monitoring the patients during treatment and prophylaxis, isolation of the yeast by culture method was the most appropriate criterion for success or failure or relapse. PCR, LA and India ink preparation could not be used solely for this purpose. There were no significant differences in survival rate : 61.6% (37/60) / 68.9% (51/74) ($p = 0.274$), success rate : 26.7% (16/60) / 59.5% (44/74) ($p = 0.268$), mortality rate : 36.7% (22/60) / 31.1% (23/74) ($p = 0.278$) and relapse rate : 16.7% (1/6) / 13.3% (2/15) ($p = 0.435$) in the two groups of these patients. The combination therapy with amphotericin B and itraconazole could be recommended as an alternative regimen for cryptococcal meningitis because it revealed higher success (44/74) than (16/60) in the amphotericin B monotherapy. Fifty-eight patients were given itraconazole in a dose of 400 mg/day as prophylaxis for one year. At the 7th month and 12th month of prophylaxis, there were only 29 patients (50%) came for follow-up. There were 3 relapsed patients (14.3%) during one year. PCR was negative in all specimens obtained from success cases at the end of prophylaxis.

C. neoformans strains in this study were almost sensitive to amphotericin B and to itraconazole. Determination of MIC by E-test revealed the value range from 0.032-0.5 μg/ml for amphotericin B and from 0.003-1.5 μg/ml for itraconazole. The MIC values obtained by E-test were not much different from those obtained by macrotube dilution method from previous studies. The yeast strains isolated from AIDS patients during 1996-1998 revealed predominately A/D serotype (around 99.6%). This figure was the same as the one reported by other investigators both in Thailand and worldwide.

3836904 SIMI/M สาขาวิชา: จุลชีววิทยา; วทม. (จุลชีววิทยา)

อนุรักษ์ มันศรีชุม : การวินิจฉัยและการติดตามการรักษาโรคเยื่อหุ้มสมองอักเสบจากเชื้อคริปโตคอคคัสในผู้ป่วยเอดส์โดยวิธี พีซีอาร์ (DIAGNOSING AND MONITORING CRYPTOCOCCAL MENINGITIS IN AIDS PATIENTS DURING TREATMENT BY PCR) คณะกรรมการควบคุมวิทยานิพนธ์: อังคณา ฉายประเสริฐ, Dr.rer.nat., จุฬารัตน์ ปริยชาติกุล Ph.D., สมสิทธิ์ ตันสุภสวัสดิ์กุล M.D. 126 หน้า. ISBN 974-663-994-3

วิธี PCR โดยใช้ primers ที่จำเพาะ CPL1 และ CPR4 จาก 18S rRNA ยีน ใช้ในการวินิจฉัยโรคเยื่อหุ้มสมองอักเสบจากเชื้อคริปโตคอคคัสในผู้ป่วยเอดส์จำนวน 160 ราย ที่คาดว่าจะเป็นโรคนี้ เมื่อเปรียบเทียบวิธี PCR กับวิธีที่ใช้ในห้องปฏิบัติการโดยทั่วไป พบว่า PCR ให้ผลบวก 79.7% (47/59) ในขณะที่ LA ให้ผลบวก 100%(128/128) PCRให้ความไวต่ำเนื่องจากในน้ำไขสันหลัง มีสารบางตัวยับยั้งการทำงานของเอ็นไซม์ *Taq* หรือขั้นตอนการทำให้เซลล์แตกมีประสิทธิภาพไม่ดีพอ ในวันแรกของการศึกษาสามารถแยกเชื้อคริปโตคอคคัส นีโอฟอร์แมนได้ 120 ตัวอย่างจากน้ำไขสันหลังจำนวน 128 ตัวอย่าง (93.8%) การย้อมตะกอนของน้ำไขสันหลังที่ปั่นแล้วด้วยหมึกอินเดียให้ผลบวก 96.9% (124/128) ผู้ป่วยส่วนใหญ่ในกลุ่มนี้เป็นโรคเยื่อหุ้มสมองอักเสบจากเชื้อคริปโตคอคคัส-ชนิดแพร่กระจายโดยพบเชื้อในเลือด 80.1% (125/156) เมื่อเพาะเชื้อจากเลือดด้วยเครื่องอัตโนมัติ Bactec 9240 และพบเชื้อในปัสสาวะ 8.8% (12/136)

ผู้ป่วยทั้งหมดถูกแบ่งเป็น 2 กลุ่มคือ กลุ่มที่รักษาด้วยยาแอมโฟเทอริซิน บี และกลุ่มที่รักษาด้วยยาแอมโฟเทอริซิน บี ร่วมกับยาไอตร้าโคนาโซล โดยใช้ผลการเพาะเชื้อจากน้ำไขสันหลังเป็นตัวบ่งชี้ว่า ผู้ป่วยรักษาหายหรือรักษาไม่ได้ผล หรือกลับมาเป็นโรคอีกทั้งในระหว่างการรักษาและการติดตามผลการรักษา พบว่าผู้ป่วยทั้ง 2 กลุ่มไม่มีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติทั้งอัตราการรอดชีวิต : 61.6% (37/60) / 68.9% (51/74) (p = 0.274), อัตราการรักษาหาย : 26.7% (16/60) / 59.5% (44/74) (p = 0.268), อัตราการตาย : 36.7% (22/60) / 31.1% (23/74) (p = 0.278) และอัตราการกลับเป็นโรคใหม่ :16.7% (1/6) / 13.3% (2/15) (p = 0.435) การรักษาด้วยยาแอมโฟเทอริซิน บีร่วมกับยาไอตร้าโคนาโซลอาจเป็นอีกทางเลือกหนึ่งในการรักษาผู้ป่วยโรคนี้ เนื่องจากมีจำนวนผู้ป่วยที่รักษาหายสูงกว่าผู้ป่วยที่ได้รับยาแอมโฟเทอริซิน บีอย่างเดียว ผู้ป่วย 58 รายที่รักษาหายจะได้รับยาไอตร้าโคนาโซลขนาด 400 มิลลิกรัมต่อวันติดต่อกันเป็นเวลา 1 ปี พบว่าในเดือนที่ 7 และเดือนที่ 12 ของช่วงนี้มีผู้ป่วยเพียง 29 ราย (50%) ที่มารับการรักษา ในจำนวนนี้มี 3 ราย (14.3%) ที่กลับเป็นโรคอีก ผู้ป่วยทั้งหมดที่รักษาหายเมื่อครบ 1 ปี ให้ผล PCRเป็นลบ

สายพันธุ์คริปโตคอคคัสที่แยกได้จากการศึกษานี้ส่วนใหญ่ไวต่อยาแอมโฟเทอริซินบีและยาไอตร้าโคนาโซล ค่าความเข้มข้นต่ำสุดที่สามารถยับยั้งการเจริญเติบโตของเชื้อได้โดยวิธี E-test พบว่ายา แอมโฟเทอริซิน บี อยู่ในช่วง 0.032-0.5 มิลลิกรัมต่อมิลลิลิตร และช่วง 0.003-1.5 มิลลิกรัมต่อมิลลิลิตร สำหรับยาไอตร้าโคนาโซล ค่าความเข้มข้นต่ำสุดที่ได้โดยวิธีนี้ของยาทั้งสองชนิดไม่ต่างจากค่าที่ได้โดยวิธี macrotube dilution ที่มีผู้ศึกษามาแล้ว และเชื้อที่แยกได้จากผู้ป่วยเอดส์ในช่วงปี 1996-1998 ส่วนใหญ่มีซีโรทัยป์แบบ A/D ซีโรทัยป์ (ประมาณ 99.6%) ซึ่งผลที่ได้เหมือนกับรายงานที่มีผู้ศึกษาไว้ทั้งในประเทศไทยและทั่วโลก

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
bp	base pair
cm	centimeter
CNS	central nervous system
CSF	cerebrospinal fluid
°C	degree Celcius
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetic acid
fg	femtogram
g	gram
h	hour
IgG	immunoglobulin G
kb	kilobase
L	liter
µg	microgram
µm	micrometer
µl	microliter
mM	millimolar
mg	milligram

LIST OF ABBREVIATIONS (Cont.)

ml	milliliter
mm^3	cubic millimeter
min	minute
NYS	New York State Laboratory
PCR	polymerase chain reaction
pg	picogram
pmole	picomole
RAPD	random amplification polymorphic DNA
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
rpm	round per minute
sec	second
TAE	tris-acetate-EDTA
TE	tris-EDTA
UV	ultraviolet
V/V	volume/volume
W/V	weight/volume
YNB	yeast nitrogen base
α	alpha
β	beta
ϕ	phi

CHAPTER I

INTRODUCTION

The incidence of cryptococcosis has increased significantly as the disease, which causes life-threatening infection in approximately 5 to 10% of patients with AIDS (1). In Thailand, cryptococcosis is the third most common opportunistic infection in AIDS patients. There were more than 17,000 cryptococcal infection reported in December 1998 (2) and until September 1999, 21,184 cases of cryptococcosis associated with AIDS were reported (3). It became clear that treatment with amphotericin B (0.3 to 0.7 mg/kg per day) alone or combined with flucytosine (150 mg per day) for 6 to 10 weeks were also recommended for patients with AIDS and cryptococcal infection (4). Approximately 60% of the patients died from this infection and recurrence occurred about 50% of whom who received complete therapy (5).

Cryptococcosis is caused by an encapsulated yeast *Cryptococcus neoformans*. The yeast reproduces by budding and can be classified into four serotypes ; serotype A, B, C and D (6). *C. neoformans* serotype A is the most frequent type that causes disease worldwide (7-11). Laboratory diagnosis of cryptococcosis in AIDS patients relies on isolation of the organism or detection of cryptococcal antigen in a clinical specimen by latex agglutination (LA) test. LA is generally more sensitive and specific than India ink preparation and used to detect antigen at a level of 10 ng ml^{-1} in a few

hours. It has high sensitivity and specificity around 90% (12). Isolation *C. neoformans* by the culture method is the gold standard of laboratory diagnosis with sensitivity range from 80-100% and it is necessary for further antifungal susceptibility testing. Though, it is not too difficult, it consumes more time (about 3-7 days) than microscopic examination and antigen detection.

Varieties of molecular techniques were introduced in the last decade in order to improve the sensitivity, specificity and speed of laboratory detection of *C. neoformans*. One of the most popular method is an *in vitro* DNA amplification or polymerase chain reaction (PCR). In 1996, Prariyachatigul *et al.* (13) have successfully developed PCR method by using primers from 18S rRNA gene sequence for direct detection and identification of *C. neoformans* in CSF samples. The sensitivity and specificity were 100% compared with culture. It is interesting to assess the test again for diagnosing and monitoring cryptococcal meningitis in AIDS patients because of the high sensitivity and specificity of this developed PCR.

It has been reported before AIDS era that patients with cryptococcal meninigitis revealed high mortality rate and relapse rate (14). This situation is worse in the AIDS era because more than 50% of patients died from cryptococcal meningitis in two weeks and about 50% relapse after complete standard regimen (15). Many investigators recommended to improve regimen for treatment of cryptococcosis by using amphotericin B plus triazole drug such as fluconazole or itraconazole (16). In this study outcomes of patients managed with amphotericin B alone and with combination of amphotericin B and itraconazole will be compared.

Prophylaxis for cryptococcosis in these patients is still inconclusive, therefore the value of itraconazole prophylaxis in AIDS patients cured from cryptococcosis will be evaluated. The duration of prophylaxis will be 1 year.

CHAPTER II

OBJECTIVES

The objectives of this study are :

1. To determine the sensitivity of PCR technique using CPL1-CPR4 primers in diagnosis of cryptococcal meningitis compare with the conventional methods.
2. To determine the value of PCR for monitoring AIDS patients during treatment.
3. To compare the outcome of different treatment regimens between the amphotericin B alone and combine amphotericin B with itraconazole by laboratory aspect.
4. To evaluate itraconazole prophylaxis in AIDS patients by laboratory methods.
5. To determine minimal inhibitory concentration of amphotericin B and itraconazole of some *C. neoformans* isolates.

CHAPTER III

LITERATURE REVIEW

I. *Cryptococcus neoformans*

Cryptococcus neoformans was first described as human pathogen by Busse in 1894 (17). It has been named as *Saccharomyces neoformans, Saccharomyces subcutaneous tumefaciens, Cryptococcus hominis, Saccharomyces lithogenes, Torula histolytica* and *Cryptococcus neoformans*. The last name was reported by Lodder and Kreger-Van Rij in 1950 and was at present the accepted name (18).

C. neoformans is a round or oval yeast in the family Cryptococcaceae. A yeast cell is usually 3.5 to 7 μm in diameter and surrounded by a mucopolysaccharide capsule of 1 to 30 μm thickness. It has asexual and sexual reproduction. Asexual reproduction is accomplished by budding of one or two daughter cells connected to the parent cell by a narrow base. Sexual or perfect state of *C. neoformans* can be demonstrated by fungal mating *in vitro* under suitable condition, mycelia (hyphae) and basidiospores on the basidia are produced (19). The perfect state or teleomorph of *C. neoformans* are *Filobasidiella neoformans* (20) and *Filobasidiella basillispora* (21). It can synthesize phenoloxidase that produces melanin-like pigments from phenols, produces urease enzyme that changes urea to ammonia and acquires nitrogen from peptone but can not reduce nitrate. *Cryptococcus* differs from *Candida*

albicans by inability to produce germ tube, chlamydoconidia or hyphae, either *in vitro* or *in vivo*.

I.1 Natural habitat

C. neoformans is a free-living organism that can survive in a variety of environmental niches. The saprophytic nature of *C. neoformans* has been known since 1894 when Sanfelice culture it from peach juice (22). Later, there were reports of isolation of this yeast from milk by Klein in 1901, from soil in 1951 (23) and in pigeon dropping by Emmons in 1955 (24).

In Thailand in 1968, Taylor *et al.*(25) reported the first isolation of *C. neoformans* from natural substrate (avian habitat) such as pigeon dropping in Udonthani, cuckoo dropping in Chanthaburi, pigeon nests and pigeon dropping in Bangkok. Many reports on isolation of *C. neoformans* from birds' manure were later published for examples; pigeon Fancier's House in Dhonburi (26), splotted-neck dove, lebra dove, ring dove, pigeon dropping in Chiang Mai (27) and budgerigar dropping in Bangkok (28).

Survival of *C. neoformans* in avian excreta, soils contaminated by avian excreta and other saprophytic sources can be influenced by several factors, including pH, humidity, temperature, sunlight and biotic factors (29). *C. neoformans* can survive on pigeon excreta for more than 1 year. Humidity can enhance proliferation and survival rate of the fungus in the soil at low temperatures (4 to 26°C) but not at 37°C. Direct sunlight can significantly reduce the survival of cryptococci in the soil and may account for the fact that pigeon excreta from outdoor sites are less contaminated than pigeon

excreta from indoor sites. Pigeon may contribute to the propagation of *C. neoformans* by providing a rich culture medium in the form of pigeon excreta and can disseminate the fungus in the environment by carrying it in their beaks and feet.

C. neoformans strains have been divided into two varieties on the basis of phenotypic, serological, genetic, biochemical and epidemiological criteria. Both varieties are pathogenic for humans. Variety *neoformans* is found worldwide, the type invariably isolated from avian excreta, milk, fruit juice and vegetables (avocado, beet, chayote, stringbean and napal in Mexico) (30), wood and plant debris inside a hallow of *Syzygium jambolona* (31), in living trees *Cassia grandis*, *Ficus microcapa* and *Senna multijaga* (32) and represented the overwhelming majority of isolates recovered from patients with AIDS. Variety *gattii* is found in tropical and subtropical climates i.e, Central Africa, Cambodia, Thailand, Vietnam, Brazil, Australia, Hawaii and Southern California (33). It is associated with the eucalyptus tree (*Eucalyptus camaldulensis* and *Eucalyptus tereticornis*) (34), hallow of *Moquilea tomentosa* in the city of Teresina (35), from bat guano in an attic of an old house in Brazil (31) and is rarely the cause of disseminated infection in patients with AIDS, even in endemic area.

I.2 Capsular polysaccharide

The capsule of *C. neoformans* is composed primarily of polysaccharide, found immediately outside the cell wall and can vary in size, depending on the strain, environment and growth conditions. The main polysaccharide component of the capsule is glucuronoxylomannan (GXM). GXM composes approximately 90% of the capsular polysaccharide (36). Differences in the GXM structure among strains

produce antigenic differences that provide the basis for the classical separation of *C. neoformans* strains into four serotypes known as A, B, C and D. Because some strains display characteristics of both A and D serotypes, AD may constitute a fifth serotype. There is consensus that GXM is composed of a (1→3)-linked linear α-D-mannopyranan (Man*p*) with β-D xylopyranosyl (Xyl *p*), β-D-glucupyranosyl uronic acid (Glc *p*A), and 6-O-acetyl substituents showed in Table I.

Table I Structure of the major capsular polysaccharide of *C. neoformans*

Serotype	Structure	References
A	→3-α-D-Man$_p$(1→3)- -α-D-Man$_p$(1→3)-α-D-Man$_p$(1→ 2 2 2 ↑ ↑ ↑ 1 1 1 β-D-Xyl$_p$ β-D-Xyl$_p$ β-D-Glc$_p$A	37, 38
B	β-D-Xyl$_p$ 1 ↓ 4 →3-α-D-Man$_p$(1→3)- -α-D-Man$_p$(1→3)-α-D-Man$_p$(1→ 2 2 2 ↑ ↑ ↑ 1 1 1 β-D-Xyl$_p$ β-D-Xyl$_p$ β-D-Glc$_p$A	39
C	β-D-Xyl$_p$ β-D-Xyl$_p$ 1 1 ↓ ↓ 4 4 →3-α-D-Man$_p$(1→3)- -α-D-Man$_p$(1→3)-α-D-Man$_p$(1→ 2 2 2 ↑ ↑ ↑ 1 1 1 β-D-Xyl$_p$ β-D-Xyl$_p$ β-D-Glc$_p$A	40, 41
D	→3-α-D-Man$_p$(1→3)- -α-D-Man$_p$(1→3)-α-D-Man$_p$(1→ 2 2 ↑ ↑ 1 1 β-D-Xyl$_p$ β-D-Xyl$_p$	42

I.3 Serotype separation

A simplest medium, creatinine dextrose bromothymol blue agar (CDB) for distinguishing *C. neoformans* var. *neoformans* (A/D serotype) and *C. neoformans* var. *gattii* (B/C serotype) was developed in 1978 by Kwon-Chung (43). One hundred percent (46 isolates tested) of *C. neoformans* var. *gattii* were able to change the color from green to blue in 48 h whereas only 4.4% (4/90) of *C. neoformans* var. *neoformans* could do (44). The biochemical basis of color change described by Polacheck and Kwon-Chung was a pH indicator change from acidity to alkalinity which due to result of degradation of creatinine by creatinine deiminase into methylhydantoin and ammonia. The isolates of var. *neoformans* produced a little amounts of ammonia that can inhibit creatinine deiminase enzyme and had not accumulated ammonia (44).

In 1982 Salkin and Hurd (45) developed a new medium, glycine-cycloheximide phenol red agar (GCP), which composed of 1% glycine, 1.6 µg/ml cycloheximide, 0.6% yeast nitrogen base and phenol red for differentiation *C. neoformans* serotype pairs. On GCP medium var. *gattii* produced a color change from yellow to red in 48 h but not in var. *neoformans*.

Although the glycine-cycloheximide medium was designated to be used with clinical isolates that have identified as *C. neoformans* it has also been investigated with other *Cryptococcus* species. In a study with 177 isolates of *Cryptococcus*;137 isolates of *C. neoformans* (83A, 13B, 16C, 25D), 13 *C. albidus* var. *albidus*, 11 *C. albidus* var. *diffluens*, 10 *C. terrens*, 3 *C. laurentii* and 3 *C. uniguttulatus*, 2 of 3 isolates each

of *C. laurentii* and *C. uniguttulatus* grew and induced a color change within 2 days of inoculation (45).

Fromtling *et al.*(46) tested 90 clinical isolates of *Cryptococcus* in nonendemic areas of serotype B/C on CDB medium. Of the 90 clinical isolates, 12 (13.3%) changed the color of the medium from golden yellow to blue-green within 48 h, indicating rapid assimilation of creatinine which is characteristic of the B/C serotype group. The same 90 isolates were subsequently tests on GCP medium. Only three (3.3%) produced a color change (yellow to red) characteristic of the B/C group. They were confirmed as serotype B by positive agglutination with type specific antisera. The remaining nine isolates were serotype A or D, thus contradicting the results obtained on the CDB medium for this organism. This report indicated that GCP medium had a 100% specificity compared with CDB medium.

In 1982 Kwon-Chung *et al.*(47) developed a modified CDB medium for eliminating the false negative and false positive result of serotyping by using 1% glycine as a carbon and nitrogen source, L-canavanine (30 μg/ml) as arginine-analog, 1 g KH_2PO_4, 1 g of $MgSO_4$ and bromothymol blue as an indicator. By using this medium there was no false negative and false positive in 213 isolates of *C. neoformans* tested for 5 days of incubation, whereas 8 isolates and 9 isolates revealed false negative for B/C serotype in GCP and CDB media respectively.

In 1982, Ikeda *et al.*(48) prepared polyclonal antibody produced by immunization rabbits with whole heat-killed cells and formalinized cells of the yeast for identifying the serotype of clinical isolates by slide agglutination test. The antiserum is cross-

adsorbed with whole yeast cells to produce a polyclonal antiserum specific for a single serotype. At least the eight distinct epitopes are found among all the serotypes. Some epitopes are unique to a single serotype whereas other epitopes are shared by two or more serotypes. The polyclonal antibodies can cross react with *Cryptococcus albidus* and *Candida curvata* corresponded to *C. neoformans* serotype A and *Candida hemicola* demonstrated the same antigenic patterns as serotype D.

In 1987, Eckert & Kozel (49) produced monoclonal antibodies (MAbs) to *Cryptococcus*. One group of BALB/C mice was immunized with serotype D polysaccharide coupled to sheep erythrocytes and the other group immunized with serotype A coupled with sheep erythrocytes. Each group of mice produced antibodies of the IgG1 isotype. These monoclonal antibodies, MAb 439, MAb 1255 and MAb 302 were reactive with polyclonal antigen 1, 2 and 3 which described by Ikeda *et al.* respectively.

Dromer *et al.*(50) presented method for serotyping of *C. neoformans* strains by using only one monoclonal antibody (E1) specific for polysaccharides. The purified monoclonal antibody E1 was conjugated with fluorescein isothiocyanate. For *C. neoformans* var. *neoformans*, a bright, homogeneous staining with several cells aggregates was characteristic of serotype A, whereas only a few serotype D cells were positive. For *C. neoformans* var. *gattii*, a completely negative isolate was serotype C, whereas the population of serotype B included a majority of negative cells but also included positive cells with a speckled pattern. The pattern of fluorescence varied from one serotype to the other when used the same antibody concentration.

Belay *et al.*(51) detected the presence of antigen by using anti-*C. neoformans* monoclonal antibodies in a dot enzyme assay on nitrocellulose membrane strips or absorption of polyclonal rabbit antisera. Four of 40 isolates were untypeable by both factor sera and monoclonal antibodies methods. Sixteen of 40 were serotype A, 4 of 40 serotype A/D, 6/40 serotype D, 6/40 serotype B, 3/40 serotype C and only 1 isolate was serotype B/C. All of the typeable isolates showed the same serotype as described by ATCC.

In 1997 Boekhout & Scorzetti (52) distinguished serotype pairs of C. *neoformans* by using 6 killer yeast strains including ; *C. laurentii* CBS 139, CBS 7235 and CBS 7875 ; *C. podzolicus* 7717 ; *C. humicola* CBS 4218 and *Filobasidium capsuligenum* CBS 4736. Ten different sensitivity patterns occurred among *C. neoformans*, four types were found in var. *neoformans* and six were observed in var. *gattii*. One hundred percent (32 strains) of var. *gattii* were inhibited by killer toxin of *Cryptococcus laurentii* CBS 139 whereas 100% (120 strains) of those var. *neoformans* were not. The killer toxin assay using *C. laurentii* CBS 139 is another rapid method to distinguish the two variety of *C. neoformans* which may be helpful when antisera are not available.

I.4 Virulent factors

In 1977, Kozel (53) demonstrated that cryptococcal polysaccharide adhere to non-encapsulated *C. neoformans* via specific receptor on the cell surface, and inhibit phagocytosis whereas the substance did not adhere to cells of *Candida albicans*, *Candida pseudotropicalis*, *Torulopsis* sp., *Rhodotorula* sp., or *Saccharomyces*

cerevisiae. This result indicated that capsular polysaccharide (glucuronoxylomannan (GXM)) has been identified as specific factor for *C. neoformans* that has antiphagocytic activity.

Fromtling (54) produced 6 nonencapsulated mutants of *C. neoformans* by chemical mutagenesis (N-methyl-N-nitro-N-nitrosogaunidine) of an encapsulated clinical isolates. All of them were avirulent in mice following high dose of intramuscular, intraperitoneal and intravenous inoculations. All animals inoculated by i.m, i.p, and i.v had no symptoms of cryptococcosis within 60 days of infection whereas all of them were killed by parent strain at 12 days after i.v. This evidence showed that the capsular polysaccharide is one of the virulent factors of *C. neoformans*.

Kwon-Chung *et al.* (55) showed that all mice which received $5x10^5$ or $5x10^6$ cells of melanin-lacking and temperature sensitive *C. neoformans* strains (mel^-Tem^-) remained healthy beyond 60 days whereas all received wide type cells were 100% killed within 30 days. From this result it was concluded that phenoloxidase and the ability to grow at 37°C are the virulent factors of *C. neoformans* which correlated with the data from Polacheck *et al.* (56). Since the growth at 37°C , ability to form melanin pigment and production of capsular polysaccharide are unique to *C. neoformans*, these three features may play an important role in its pathogenicity.

II Cryptococcosis

Cryptococcosis is caused by *C. neoformans*, an opportunistic fungal pathogen, that conferred infection by inhalation or ingestion. Infection in humans or animals may be localized or disseminated. It is the most common life-threatening fungal infection in patients who are immunocompromised especially in patients with AIDS otherwise infection may occur in healthy individuals. Although cryptococcosis commonly occurs by inhalation of dust, debris, avian dropping that contaminated with viable yeast cells, it can in addition occur by ingestion of this organism. Later evidence is demonstrated by Takos *et al.* (57), who studied cryptococcosis in marmosets. The monkeys ingested virulent *Cryptococcus* contaminated in food and developed lesions only in gastrointestinal tract such as stomach, small and large intestines and caecum. The manifestation of cryptococcosis in brains and lymph nodes in the monkey occurred after feeding large dose of *Cryptococcus* contaminated food.

Irokanulo and Akueshi (58) found that cryptococcosis mice showed necrotic foot-pad, inflamed spleen and granulomatous of lungs in some of the infected mice when inoculated 10^7 organisms per 0.2 ml into the left hind foot-pad of the mice and resulting in more invasive in *C. neoformans* var. *neoformans* than var. *gattii* but not significant.

Cryptococcosis in wild and domestic mammals, with sporadic case in cats, dogs, goats, horses and sheep has been reported (59). Outbreaks in bovine and caprine livestocks has also been identified (60). Information regarding the variety of

C. neoformans that causes infections in animals is scarce (61). In Europe, isolation of *C. neoformans* var. *gattii* is exceptional until 1990 (62).

There were five outbreaks of severe pulmonary disease in goats which had suffered from epizootic from 1990 to 1994 from different area in Spain. Three from five of outbreaks, symptoms were associated with cachexia as the most common clinical manifestation, involvement of central nervous system, subacute or chronic respiratory symptoms, some cases had systemic disease. *C. neoformans* var. *gattii* could be isolated from 13 autochthonous, and all isolates were serotype B. They were isolated from lung (10 samples), liver (1 sample) and brain (2 samples). This report was the first identification of autochthonous *Cryptococcus neoformans* var. *gattii* from goats with predominantly severe pulmonary disease (63).

II.1 Clinical manifestations

The most common form of cryptococcosis in patients with AIDS and non AIDS is meningitis. In patients with AIDS, headache and fever are the most common symptoms that occur about 60 to 100% of patients (64). Others clinical manifestations are confusion, seizure but is often atypical, presumably due to the minimal inflammatory response to the infection, some patients have headache even in the absence of other neurological sings or symptoms. CT scans could reveal cerebral atrophy or hydrocephalus or normal. Nausea or vomiting have been reported in about 40%. Clinical symptoms are usually present for 2 to 4 weeks before diagnosis, although they may be present symptom for up to 4 months (5).

Table II. Incidence of symptoms signs, and laboratory values in patients with cryptococcal meningitis (5).

Characteristic	Incidence (%)
Headache	67-100
Fever	62-95
Meningeal signs	25-30
Seizures	4-9
Focal neurologic abnormality	6-17
Positive serum cryptococcal antigen	94-100
Positive CSF cryptococcal antigen	91-100
Positive CSF India ink	64-88
Positive CSF culture	95-100
Abnormal CSF glucose	17-64
Abnormal CSF protein	35-69
Abnormal CSF leukocyte count	13-35

Cryptococcosis in patients with non AIDS is predominately caused by *C. neoformans* var. *neoformans* serotype A in The United States, Canada, Brazil, Argentina, Belgium, Holland, United Kingdom, New Zealand, Japan and Thailand (33). Isolates of serotype D were frequently found in Italy, France, and Germany whereas var. *gattii* serotype B was found in Southern California (33).

The first reported of isolation of *C. neoformans* var. *gattii* from AIDS patient was described in Zairean patient (65). The second strain was found in Canadian patient (66) and the third was isolated from patient in the USA (67). The first Brazilian case was reported in 1990 (68). The fifth case occurred in Australia (69). The sixth and the seventh cases were reported in Rwanda (70) and the eighth case was in a Zambian patient (71). The first Mexican (72) and Indian (73) cases were reported in 1997.

Among opportunistic infections complications of AIDS patients the most second common is cryptococcosis. In New York city all 24 clinical isolates of *C. neoformans* from patients with AIDS were serotype AD (7). This finding is similar to the situation found since 1984 with patients without AIDS, in which 75% of cryptococcal isolates are serotype A (74).

In Los Angeles where the incidence of var. *gattii* was as high as 40% (33) but all of the isolates from patients with AIDS were *C. neoformans* var. *neoformans* (8). Swinne (9) found that various isolates recently obtained from patients with AIDS in Zaire were of *C. neoformans* serotype A. This data was the same as in Southern California the endemic area of var. *gattii* but most etiologic agent from AIDS were var. *neoformans* (8).

In Thailand, Imwidthaya *et al.*(10) reported that all 26 clinical and environmental isolates in Bangkok were serotype AD. In 1996, Sethakorn found that 98.1% (104/106) of *C. neoformans* were serotype A by using both LA with monoclonal antibody and PCR fingerprinting method, therefore, *C. neoformans* var. *neoformans* serotype A was the most common serotype found in AIDS patients in Thailand and worldwide (11).

II.2 Disease form

The most common cryptococcosis occurred by inhalation of air or debris that contaminated with viable yeast cells and entry in pulmonary alveoli of the lung. If patients have adequate host defense, they can eliminate the organisms and have no disease but if host defense is defect, they will produce lesion in the lung that may be

asymptom or localized lesion in the lung, some organisms disseminated via blood circulation to the brain. Although experimental disease has been produced by feeding large amounts of yeast cells to animals (57), it is doubtful that human infection via the alimentary tract occur with any frequency. Organism enters the route of skin and nasopharyngeal mucosa is possible but also rarely occurred. The clinical types of cryptococcal disease include pulmonary, central nervous system, cutaneous and mucocutaneous, and disseminated cryptococcosis (18).

II.2.1 Pulmonary cryptococcosis

Pneumonia is the most common extraneural presentations that found 4 to 10% of patients with cryptococcosis without meningitis. Primary infection in the lung usually has no diagnostic symptoms and most cases are probably asymptomatic. Frequently present symptoms included cough, low-grade fever, seldomly occur symptoms are night sweats, pleuritic pain, malaise and weight loss. Scanty mucoid sputum is produced and rarely there may be hemoptysis (75). Lesion in pulmonary cryptococcosis rarely produced cavitation, fibrosis, or calcification, many lesions heal without forming granulomas (cryptococcomas) and leave no residual history of infection. In rare cases, the disease was fulminant. There were pulmonary consolidation, pronounced fever, and present with acute respiratory distress syndrome (ARDS) (76).

The pathogenesis of *C. neoformans* pulmonary infection in the rat was studied after intratracheal inoculation. *C. neoformans* could penetrate the lung parenchyma shortly after infection. Immunocompetent rat controled pulmonary cryptococcosis

efficiency, with minimal extrapulmonary dissemination and low levels of serum GXM and macrophage activation is likely to play a crucial role in limiting *C. neoformans* infection in the rat lung (77).

Culture of sputum and lung biopsy specimens are frequently negative in patients with pulmonary cryptococcosis. The presence of the yeasts in sputum in the absence of radiographic abnormalities is mostly due to colonization.

II.2.2 Central nervous system cryptococcosis

The most common site of infection with *C. neoformans* in both AIDS and non-AIDS patients is the central nervous system (CNS). There are meningitis, meningoencephalitis and expanding cryptococcoma. The first is the most common (18).

Examination of cerebrospinal fluid (CSF) in AIDS patients with cryptococcal meningitis usually reveals a large organisms burden but often a minimal inflammatory response. Ten to 35% of patients, a CSF leukocyte count is higher than 20 cells/mm^3, 35 to 70% have an increased CSF protein and 17 to 64% have a low glucose level (78)

C. neoformans usually infected both the brain and the meninges diffusely that cause meningitis and encephalitis. The reason for the predilection of *Cryptococcus* in the central nervous system is probably due to that central nervous system has less cellular hosts defense, it has nutritional factors for yeast cell growth such as asparagine and creatinine and the absence of inhibitory factor to eliminate the yeast cells.

Most patients with cryptococcal meningitis have only symptoms of headache (87%) or mental change for long periods before any manifestations occur. They included fever (60%) (79), stiffness, nausea, vomiting, confuse and usually had cerebral edema. About 10% produced additional lesion in another area but lesion in the lung was absent. Approximately 10% of patients were asymptomatic (80). In late stage of disease, the yeasts are spread hematogenously. Patients with cryptococcemia usually have concomitant CNS involvement but in one study as many as 20% of patients with cryptococcemia had no evidence of meningitis. Cryptococcosis may involve other extraneural site including the skin, kidney, spleen, pancreas, adrenals, ovaries, lymph node, skeletal muscle, liver and gastrointestinal tract (81).

II.2.3 Cutaneous and mucocutaneous cryptococcosis

Cutaneous and mucocuaneous cryptococcosis in human is usually a manifestation of disseminated disease and occur about 10% to 15% of cases. The lesions of the skin produced as papules, acneform pustules or abscesses that ulcerate with time. There are two types, primary and secondary cutaneous cryptococcosis. The primary cryptococcosis are more superficial and ulcerative than deep and necrotic that resolve spontaneously. The secondary cutaneous cryptococcosis occur in a patients with underlying disease and is a common manifestation of wider dissemination (18).

II.2.4 Other forms of cryptococcosis

Osseous cryptococcosis presents about 5% to 10% of reported cases that less than in blastomycosis, coccidioidomycosis and actinomycosis. Differential diagnosis of

osseous cryptococcosis must included other diseases of the bone. Swelling and pain may be present.

The eye infection is resulting in the formation of multiple lesions in the uveal tract, retina and vitreous (cryptococcal chorioretinitis) (18).

Life-threatening myocarditis due to fungal infection has been rarely reported in AIDS patients, fungi may be the etiologic agents of severe cardiac dysfunction. Clinical manifestations represented in one AIDS patient were pulmonary edema, fever, cough and diarrhoea. At necropsy *C. neoformans* was found in the lungs, liver, spleen and pancreas (82).

In Thailand 1960, Satitnimankarn *et al.* (83) reported the first case of cryptococcal meningitis at Siriraj Hospital. The patient was a 47 year old female. Clinical manifestations included localized amyloidosis, fever, headache, vomiting, weakness and concious. The last week of admission, patient had a severe headache, high grade fever, stiff-necked, vomiting and semiconcious 2 days before dead. From autopsy cryptococci were found in intracellular and extracellular in alveoli, adrenal glands, renal tubules, subarachnoid in mononuclear or histiocytes or giant cells and stalk of pituitary gland.

In 1982 Leelarasamee *et al.* (84) reported 19 cryptococcosis patients (10 males, 9 females) at Siriraj Hospital from 1974 to 1982. Seven patients had underlying diseases such as SLE, lymphoma, acute myeloblastic anemia. Eleven patients were cured but 3 had recurrent infection.

In 1990, the first case of cryptococcal meningitis in AIDS patient at Siriraj Hospital was found. In 1992, 30 isolates of *C. neoformans* were collected from clinical specimens obtained from 26 male and 4 female patients at Siriraj Hospital. Twenty-seven patients (90%) were AIDS, 1 patient was diagnosed as SLE and the other two were suffered from acute lymphoblastic anemia. There were 16 patients died and 14 improved. In 1993, 57 cryptococcosis patients were reported from Siriraj Hospital and 49 of them were associated with AIDS (85). Until December 1998 there were more than 17,000 cases of cryptococcosis infection in Thailand (2), and lastly in September 1999, there were 21,184 cases of cryptococcosis with AIDS reported by MOPH (3).

III Treatment outcome and recurrent

Cryptococcal meningitis was a uniformly fatal disease before the introduction of amphotericin B (86). Amphotericin B is a polyene antibiotic which mediates antifungal effects by binding to cell membrane sterols, damaging the cell membrane and resulting in leakage of essential metabolites (87). Although cures are obtained in 53% to 58% of patients with amphotericin B therapy (0.3-0.7 mg per kilogram of body weight per day), it requires prolonged treatment for 10 weeks and toxicity is substantial (88). In addition, many patients develop fever, chill, rigor, nausea and vomiting which each dose of amphotericin B (18). Treatment results did not correlate with sex or total dose of amphotericin B. Initial CSF finding (frequency of positive India ink preparation and the mean opening pressure, protein and glucose level, and leukocyte counts) in patients who failed after completion of amphotericin B treatment have equal value to cure patients whereas there were several striking differences in death patients before or

during amphotericin B treatment compared with cure patients. Compared with those who were cured, patients who died received little or no therapy (less than 4 weeks) were more likely to have a positive India ink preparation, high lumbar opening pressure, lower CSF glucose levels and low CSF leukocyte counts (89).

Flucytosine can also cure this infection, but acquired drug resistance and a low proportion of cure rate make this drug no use as a single therapeutic agent (90). Flucytosine combine with amphotericin B was more effective than amphotericin B alone. The ability to manage a favorable therapeutic results in only six weeks is important, in view of the costs of hospitalization whereas amphotericin B alone result in 10 weeks of treatment.

Among non AIDS patients with cryptococcal meningitis, the administration of amphotericin B combined with flucytosine over a period for six weeks led to success rate of 75% to 85% (18), whereas AIDS patients, whose success rate with 40% to 50% and drug intolerance is common, especially when combine with flucytosine. Treatment with amphotericin B alone or combined with flucytosine, although prevent death and serious neurological impairment in some patients, is associated with significant morbidity, particularly renal and hematological injury (14, 18).

New therapeutic approaches for the treatment of *C. neoformans* infection are urgently needed. Potential strategies to improve therapy include combination of antifungal drugs and/or use of immunomodulators such as anticryptococcal antibodies to enhance the ability of the host to clear the infection (91).

Fluconazole is a triazole which inhibits membrane sterol synthesis. Since azole block the synthesis of ergosterol, the target of amphotericin B, there has been concerned about using these two types of antifungal drugs together (11). Fluconazole offers many advantages in the treatment of cryptococcal disease. It is well absorbed even in AIDS patients, is tolerable at high dose (400mg per day at the first day and 200 mg per day in thereafter) has a long haft life (30 h) and has good CSF penetration up to 80% of serum concentration (92).

Response rate for both fluconazole and amphotericin B were similar and disappointingly low in the study by Saag *et al.* (88), possibly because low dose of both drugs were used. The median time to negative CSF culture was 62 days for fluconazole and 42 days for amphotericin B compare with 21 days in patients that reported by Haubrich *et al.* (92). The mean serum level with 800 mg per day was 45 μg/ml compared with 18.9 μg/ml was obtained with fluconazole at 400 mg per day. The observed CSF levels achieved with 800 mg doses approximately the MICs for the patients isolates. Thus, the higher levels of fluconazole obtained with the 800 mg dose may have accounted for the faster CSF sterilization. Adverse reactions of fluconazole are elevated levels of hepatic enzymes, agranulocytosis, seizure, minor nausea, a rise in liver function test result in serum glutamic pyruvic transaminase and serum glutamic oxaloacetic transaminase. Fluconazole was tolerated much better than amphotericin B, 73% of fluconazole recipients reported no adverse effects, as compare with 36% of amphotericin B. In addition, severe toxicity requiring discontinuation of the drug was more frequent in the amphotericin B group, treatment failure due to drug toxicity

occurred in 8% of amphotericin B recipients as compare with 2% of fluconazole recipients (16).

Itraconazole is an oral triazole antifungal drug. It is poorly soluble in water and the resulting erratic absorption makes it an unreliable agent in acutely ill patients. A few studied suggested that no dose adjustment was necessary in patients with liver impairment or renal insufficiency (93). Van der Horst and Saag (94) studied the treatment of 408 cryptococcal meningitis in AIDS patients with itraconazole. They found that in step two of treatment at 10 weeks, 72% (109/151) of the patients receiving fluconazole had negative CSF culture whereas only 60% (93/155) of patients receiving itraconazole showed negative CSF culture. With regard to clinical efficiency 68% of fluconazole and 70% of itraconazole recipients had complete resolution of symptoms. During prophylaxis treatment, there were 2 deaths among the 151 fluconazole recipients and 5 among the 155 itraconazole recipients. There were no significant differences of outcome between using these two drugs, itraconazole may be a suitable alternative for patients who cannot take fluconazole.

During the last decade, research has led to the development of the lipid formulation of amphotericin B safer alternative to the standard deoxycholate preparation. The various products are amphotericin B lipid complex or Abelect® (95), amphotericin B colloidal dispersion or Amphocil® / Ampholec® (96) and liposomal amphotericin B or AmBisome® (97) have been used in many rather small studies during the last 5 years.

Recurrent

Leelarasamee *et al.* (84) found that recurrence of treatment may be because of inadequate level of antifungal drug in serum and CSF and/or the patients were complicated with other diseases or both antifungal drugs (amphoteicin B and flucytosine) can eliminate *Cryptococcus* but cannot decrease recurrent of disease.

Relapse of cryptococcal meningitis in AIDS patients is reported in approximately 50% of those who received complete therapy (98). Reasons for the high rate of relapse may include an inadequate host response to assist therapeutic (14). The patients who had persistent urinary cryptococcosis were clinically indistinguishable from those who had not but the former group of patients have relapse of systemic disease. So the urinary tract may be the focus from which dissemination occurs in recurrent cryptococcal disease. The active urinary cryptococcal infection in patients results in continuation of antifungal therapy beyond that planned for primary treatment. This strategy may have reduced the rate of central nervous system relapse among patient with urinary cryptococcosis. The optimal clinical management of patients with persistent urinary cryptococcosis was unclear and based on limited experience i.e, prolong weekly treatment of intravenous amphotericin B or oral fluconazole treatment (99).

Recurrent cryptococcal meningitis results from persistence of the initial infection and relapse *C. neoformans* (12).

The initial and relapse *C. neoformans* isolates are susceptible to amphotericin B and fluconazole *in vitro* and *in vivo* strongly suggests that recurrence are not due to

drug resistance but recurrence of cryptococcal meningitis may reflect either poor compliance with the suppression drug regimen or further deterioration of the immune system. Patients who died early apparently had rapidly progressive disease (89).

Five patients who had AIDS, their outcome were equally poor; only one was free of relapse in one year after the discontinuation of therapy. Because of the high relapse rate of cryptococcosis in AIDS and the high mortality associated with relapse, most investigator recommended maintaining anticryptococcal treatment with weekly amphotericin B (14).

Diamond and Bennett found that patients who had a lymphoreticular cancer or who were taking corticosteroids were found to be not a higher risk of dying of active disease during treatment (89). Equally persistent to the management of AIDS patients with meningitis, fluconazole therapy also successfully prevented relapse for an average of 25 weeks in 13 of 14 patients (relapse rate = 7.1% (1/14)). AIDS patients with cryptococcal infection not only require a long course of amphotericin B treatment for control of their initial infection, but also require long-term suppressive therapy to prevent clinical relapse (14).

IV. Laboratory diagnosis

The conventional methods for detection and isolation *C. neoformans* such as microscopic examination by India ink preparation for detection of encapsulated yeasts, polysaccharide antigen detection by latex agglutination and cultivation at 37°C are the gold standard laboratory diagnosis of cryptococcosis.

IV.1 India ink preparation

The India ink preparation was prepared by mixing a drop of cerebrospinal fluid (CSF) or CSF sediment or other specimens with a drop of India ink. Encapsulated yeast cell was searched with medium and high power objective lens. Be careful not to confuse with lymphocytes, tissue cells, fat droplets, aggregated particles of India ink or even contaminant cryptococcal yeast in India ink. The last event must be avoided by always viewing the aged India ink for the absence of encapsulated yeast cells before using it. Confirming the presence of typical small single or double budding should be observed by Gram stain. This test has been found to be positive in 30% to 50% of patients with non-AIDS cryptococcal meningitis and positive in more than 80% of patients with AIDS and cryptococcal meningitis (100). The sensitivity of an India ink preparation generally allows detection between 10^3 and 10^4 CFU of yeasts per ml or greater concentration. It is likely that the heavily positive results of India ink preparation in many AIDS patients with cryptococcal meningitis represent concentration between 10^5 and 10^7 CFU/ml in CSF (101).

IV.2 Serological examination

The only useful serological examination for diagnosing cryptococcosis at any sites is the serum cryptococcal antigen test that can be performed in a few hours and it is positive in more than 90% of patients with meningitis and most infected patients without meningitis (12). The agglutination of latex particles coated with antibody raised against the cryptococcal capsule occur when antigen is presents in the samples. False positive tests are unusual unless the patients produces rheumatoid factor that also

agglutinated the antibody-coated latex. Although false positive tests are rare, *C. neoformans* is usually easy to grow and the diagnosis of cryptococcal infection should always be confirmed by culture.

Tests for detection cryptococcal antibody are not useful in the diagnosis of cryptococcal infection because some people have antibody and most patients with cryptococcal infection do not. During the course of successful treatment, the titer of cryptococcal polysaccharide should fall but this may not be accompanied by the development of cryptococcal antibody (102).

Cryptococcal skin test preparation are useful for epidemiological surveys but are of no value for diagosis.

IV.3 Culture and identification

Culture is the gold standard for detection of *C. neoformans*. Respiratory secretions, CSF, urine, blood, and any tissue obtained by biopsy should be examined microscopically and cultured at least on Sabouraud dextrose agar. It is helpful, especially if the specimen is contaminated, to cultivate a portion of the specimen on media that contain antibacterial antibiotic such as chloramphenical, but cryptococci are sensitive to cycloheximide (actidione) and media containing this antibiotic should be avoided. As many slants as possible should be inoculated and incubated at room temperature or 25°C to 30°C. Some tubes may also be placed at 37°C for guide differentiation from saprophytic cryptococci. Growth often appears within 48 to 72 h but may be delayed for seven days or more if the inoculum is small. For this reason,

cultures should be kept for six weeks before being discarded as negative and tube media should be used to avoid drying.

When colonies appear, they are usually convex, mucoid, cream-colored to light tan or very rarely a light pink. Individual cells are more round than oval.

In vitro cultural identification of *C. neoformans* can be definitely established by mouse pathogenicity. If mice are inoculated intracerebrally, intravenously or intraperitoneally with 10^5 to 10^7 cells of *C. neoformans*, a rapid progressively fatal infection follows within one to three weeks. One to two mice sacrificed at weekly intervals up to one month and the brain culture and microscopic examination for the presence of round encapsulated yeast should be performed (58).

The culture should be identified by biochemical tests to confirm species of *C. neoformans* by using DOPA and urea media. *C. neoformans* is the only species in the genus *Cryptococcus* that can produce 3,4-dihydroxyphenylalanine phenoloxidase.. This enzyme will react with L-β-3,4-dihydroxyphenylalanine. In the presence of ferric ion it was converted to O-diphenol and was later oxidized to melanin. *C. neoformans* has enzyme urease that can change urea to ammonia which affects the pH of media by changing from acid to base and result in changing color media from light orange to margenta red. This test can separate *C. neoformans* from *Candida albicans* (103).

In a 12 months study of 19,457 blood culture conducted by Hopfer *et al.* (104) radiometric measurement was superior for early (12-24 h) detection than subculture and Gram's stain of broth medium. Because 73% of all blood cultures positive for

yeast were first detected radiometrically, as compared with 22% first detected by subculture and 5% by Gram's stain.

Prevost & Bannister (105), in their study of 668 biphasic fungal blood cultures and 38,324 Bactec blood cultures, found that radiometric system revealed a shorter detection time (2.4 VS 8.3 days. Reimer *et al.* (106) confirmed the rapid detection time of fungemia for the BACTEC 6B broth with supplemented peptone broth. The Bactec ® 460 radiometer failed to detect *Cryptococcus neoformans* in eight aerobic BACTEC 6B culture bottles inoculated with the patient's blood. The diagnosis of cryptococcemia was established by terminal (seven-day) subculturing of 6B broth to chocolate agar, which was positive for all eight radiometer negative blood culture bottles (107).

IV.4 Molecular technique

Because of the increasing cryptococcosis due to raising prevalence of AIDS infection, many investigators recommended the improve method for rapid detection and identification of *C. neoformans*. In recent year the molecular technique could play an important role for rapid diagnosis of diseases, early detection of relapse cases and typing etiologic agents both isolated from patients and from environments. All these technique were intensively studied with cryptococcosis and *C. neoformans*.

IV.4.1 Detection and identification

4.1.1 DNA probe

Middle-repetitive DNA sequence from *C. neoformans* that are species and variety specific could be used as hybridization probes to distinguish *C. neoformans* from other yeastlike fungi such as *Candida albicans* which are often abundant in the respiratory

secretions of patients with AIDS. Polacheck *et al.* (108) developed DNA probes for diagnosis and epidemiological study of cryptococcosis in AIDS patients by isolation of middle-repetitive DNA sequences from *C. neoformans* that are species and variety specific. These probes were used for assessing strain relatedness among cryptococcal isolates from patients with and without AIDS from Zaire and the United States. Five distinct hybridization patterns were observed for the 60 isolates examined, regardless of the restriction enzymes (*Eco*RI and *Hind* III) used for digestion. The most common pattern among the isolates from the patients with AIDS who were from United States was the same as patients with AIDS who were from Zaire.

The species-specific probe showed a high specificity, gave signals only with *C. neoformans*, and did not cross hybridize with DNA from a number of yeastlike fungi. The variety-specific probe demonstrated that there was no hybridization with isolates of *C. neoformans* var. *gattii*, while a typical hybridization pattern was obtained only with isolates of *C. neoformans* var. *neoformans*.

Accuprobes are commercial DNA probes for identifying many important pathogenic microorganisms recovered from clinical specimens including *C. neoformans*. It is used to identify culture on cultivation medium. Gen-Probe's chemiluminescence-labeled DNA probes are complementary to rRNA sequences. Labeled DNA-RNA hybrids are differentiated from nonhybridized probe. After specific hybridization occurred in liquid phase according to manufacturer's instructions, the fluorescence was measured in a luminometer. A positive result is based on a manufacturer's cutoff value of greater than or equal to 1,500 PAL light units (PLU). The results were obtained with the species-specific probe for *C. neoformans*. All 42

isolates of *C. neoformans* react with the probe whereas RNA from the other 56 yeast isolates did not hybridize with the probe. Positive and negative controls, as recommended by the manufacturer, include *H. capsulatum* var. *capsulatum* ATCC 38904 and *C. neoformans* ATCC 32045, as well as *Blastomyces dermatitidis* ATCC 60916 and *Candida albicans* ATCC 18804 respectively. The sensitivity and specificity of this probes were 100% (109).

A major advantage in using Accuprobe is the rapidity. The test is technically easy to perform, a small inoculum size is required. Positive and negative results are easily differentiated and short time period needed to perform the test (approximately 2 h) but required the expensive instrument and a probe shelf-life of 6 months, which may be insufficient for small laboratories that received few specimens (109).

4.1.2 Polymerase chain reaction (PCR)

The use of PCR seems particularly suited for detection specific DNA or RNA segments from pathogens that either grow very slowly, are difficult to culture or have never been cultured *in vitro*. Many pathogenic fungi including *C. neoformans* have been investigated using PCR technique.

Genes coding for ribosomal RNA are attractive targets for PCR based detection schemes as (i) sensitivity is likely to be higher than in schemes designed to detect single copy gene since rDNA genes are present in multiple copies in each organism and (ii) due to slow divergence of small subunit rRNA sequence over evolutionary time, regions of sequence conservation across kingdom have been identified, thereby enabling amplification of fungal specific DNA.

Unique primers were developed to amplify segments of the genes coding for rRNA of *C. neoformans*. These primers were designed on the basis comparative analysis the sequenes of the 5.8S and internal transcribed spacer (ITS) regions of *Filobasidiella neoformans*. The ITS1 and ITS4 (produce of approximately 600 to 650 bp) were designed for universal fungal primers and the three primers (CN primers) were designed for *C. neoformans* specific primers. Various combination of these primers were tested in PCR for their ability to amplify DNA from 37 strains of *C. neoformans* and 31 other isolates representing 18 species of yeasts. It was found that primers CN4 and CN5 amplified DNA (product 136 bp long) from both varieties of *C. neoformans*, other primers CN5-CN6 (product is 116 bp long), CN4-ITS1 and CN6-ITS1 amplified DNA only from *C. neoformans* and from *Filobasidiella depauperata*. The sensitivity of primer combination was 100% but it was found that higher-molecular-weight bands were generated with DNA from unrelated yeasts. These specific oligonucleotides may be used as primers in PCR to identify *C. neoformans* (110).

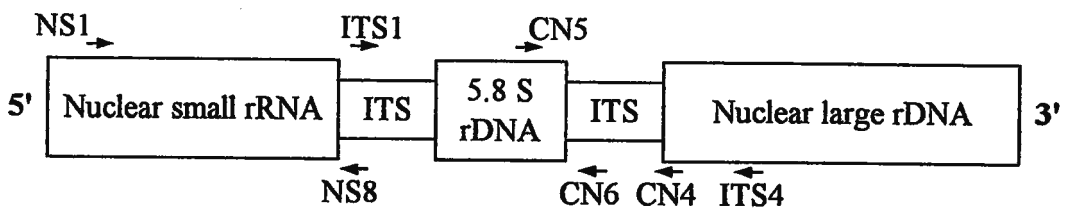

FIG 1. The approximate locations (arrows) of the CN primers, as well as useful ITS and NS primers, are indicated on the diagram of the rDNA genes. CN4 and CN6 are 3' primers; CN5 is a 5' primer. Arrowheads approximate the 3'end of each primer 16).

Maiwald *et al.* (111) designed primer from the gene coding for the small ribosomal subunit 18S rRNA that contains universally conserved as well as highly variable

sequences and allowed the discrimination of individual fungal species to developed PCR method for rapid presumptive differentiation of a panel of 12 clinically yeasts. By using six restriction enzymes *Alu*I, *Ban*I, *BbS*I, *Dra*II, *Eco*1471 and *Nhe*I, characteristic-PCR-restriction enzyme patterns were obtained for *Candida albicans*, *Candida tropicalis*, *Candida krusei*, *Candida kefur*, *Candida lusitaniae*, *Candida guilliermondii*, *Candida glabrata* and *Saccharomyces cerevisiae*, as well as the pairs *Candida parapsilosis* / *Candida viswanathii* and *Trichosporon beigelii* / *Cryptococcus neoformans*. The study on restriction analysis revealed that *Alu*I was predicted to cut only DNA of *C. neoformans* at position 942 and thus differentiated it from all other yeast species but this restriction enzyme is unable to cut the DNA fragment from *C. neoformans* serotype D. Possible explanation for the failure of *Alu*I to cut the fragment of serotype D include (i) an error in the publish sequence of the 18S-rRNA gene of *C. neoformans* at position 944 or (ii) a different 18S-rRNA gene sequence of the *Cryptococcus* strain that was used.

In 1996, Prariyachatigul *et al.* (13) assessed a PCR technique for the detection and identification of *C. neoformans* targets at 18S rRNA gene. The primers CPL1 and CPR4 were used for amplification DNA from *C. neoformans* strains and CSF specimens. It was found that the primers CPL1 and CPR4 revealed specific 343 bp amplified product from *C. neoformans* DNA and no amplified DNA products from other microorganisms or humans except *Trichosporon* and *Klebsiella pneumoniae* that gave product of 343 bp and 490 bp respectively. These PCR products from *Trichosporon* and *Klebsiella pneumoniae* could not hybridize with INSR4 the specific *C. neoformans* probe. The lowest amounts DNA of *C. neoformans* that could give

positive result for PCR were 1 pg and at least 100 fg after Southern blot hybridization with INSR4 specific probe. The sensitivity of PCR for detection of *C. neoformans* in simulated CSF was 5 cells/ml. Assessment of the sensitivity and specificity of PCR in direct detection of *C. neoformans* from CSF of 27 patients were both found to be 100% compared with the culture method. The sensitivity of PCR was only 88.9% and 84.2% compared with LA test and India ink preparation respectively. The one sample with clinical diagnosis of HIV positive and tuberculous meningitis were negative for all tests. So the CPL1 and CPR4 primers were useful in the diagnosis of cryptococcal meningitis in AIDS patients by direct detection of *C. neoformans* DNA by PCR and revealed no cross amplification with HIV DNA.

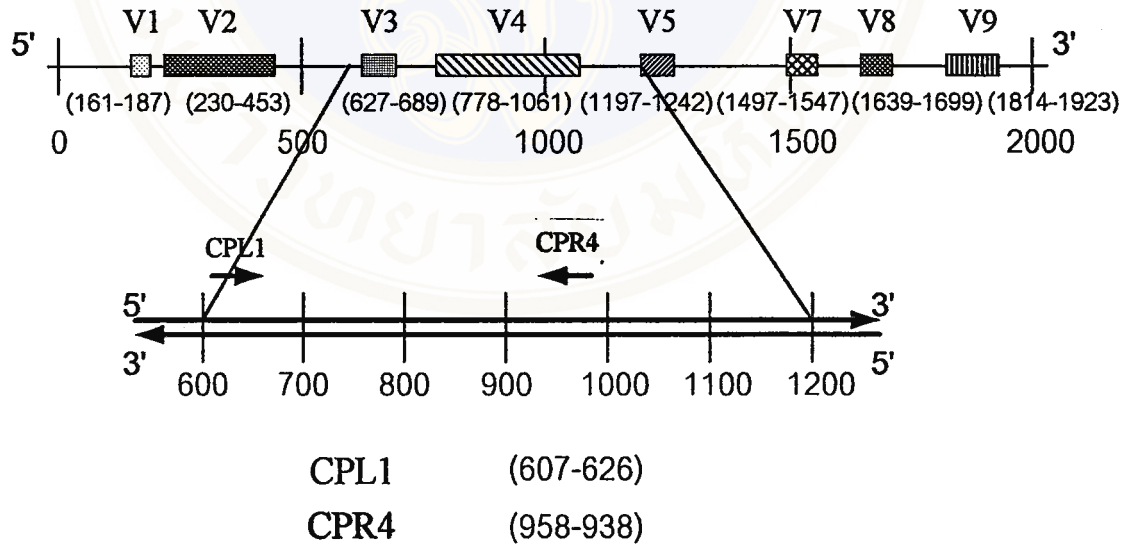

Figure 2 The location of primers sequences.

IV.4.2 Molecular typing

More thorough study of the epidemiology of cryptococcosis is needed to define risk factors and to recommend appropriate prevention strategies. One obstacle to such epidemiologic studies has been the lack of sensitive and reliable method to differentiate strains of *C. neoformans*. Serologic reactivity of the capsular polysaccharide and biochemical differences have historically been used for separating cryptococcal strains into two varieties. However the usefulness of capsule serology, has a limited value as a typing method. There were only 5 serotypes determined by this technique. In recent year, there were many studies performed in order to improve this limitation. One way among various technique was a using of molecular procedure in studying epidemiology of *C. neoformans*.

There are at least 6 different published methods for molecular typing of *C. neoformans*. They are as the following ; allele locus sequencing, multilocus enzyme electrophoresis (MEE), random amplify polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) and karyotyping.

4.2.1 Allele locus sequencing

Despite the medical important of *C.neoformans*, relatively little is known about the genetic variation of clinical isolates. Casadevall *et al.* (112) studied the OMPPase gene locus of 13 *C. neoformans* var. *neoformans* strains, including 10 from clinical isolates, by using RFLP and nucleotide sequence analysis. They found that the 779 bp *URA* 5 gene is coding for OMPPase that has a single copy, highly polymorphic and at least 6 alleles were identified. The nucleotide sequence of some alleles differed by up

at the third codon positions and were silent. The low frequency of nucleotide replacement relative to silent substitutions implied that there was strong selection against amino acid changes in OMPPase. The allelic variation suggested that there was extensive genomic diversity among *C. neoformans* clinical isolates from one geographic area. The veriants alleles of OMPPase were potentially useful marker in the study of the epidemiology, pathogenesis of *C. neoformans* strains and investigation whether patients were infected with more than one strain. It could be used to determine whether clinical relapses were a result of relapse of infection with the original strain or a new infection, and to investigate the relationship between environmental and clinical isolates.

4.2.2 Multilocus enzyme electrophoresis (MEE)

MEE has been employed in epidemiology studies of many bacteria and fungi (113). In previous study of cryptococci, variation among strains was seen in the electrophoresis mobilities of four enzymes. One study evaluated the usefulness of MEE as a tool for subtyping of *C. neoformans*. The cytoplasm of *C. neoformans* contains many enzymes such as ; alcohol dehydrogenase and glucose-6-phosphate dehydrogenase. Electrophoretic mobility was determined by comparison with the mobility of a cryptococcal standard electrophoresed on the same gel. Each unique combination of electrophoretic variants was designated as an electrophoretic type (ET). The degree of relatedness between two strains was estimated by the relatedness index (RI). The lower RI, the more closely related were the two strains.

Brandt *et al.* (113) studied 34 cryptococcal clinical isolates included all five serotypes and different geographic origins. The cytoplasmic extracts of *C. neoformans* were characterized on starch gel electrophoresis and strained for 10 enzyme activities. They found that the two cryptococcal varieties could be readily distinguished from each other (RI of 0.94). Serotype A and D isolates were separated at an RI of 0.58 by 4 enzymes and serotype A strains could be further divided into four enzyme types (RI of 0.52). MEE identified the acapsular mutant cap 67-2 as a serotype D strain. This strain was derived from a serotype D parent and cannot be serotyped by antisera and monoclonal antibody. All 4 isolates of serotype AD could be distinguished from those of either serotype A or D. All isolates were divided into 5 groups, differing in mobility of glutamate dehydrogenase. These isolates were tightly clustered, however displaying relatedness at an RI of 0.2. Serotype B and C isolates demonstrated greater diversity in ET patterns, no two among 8 of 9 *C. neoformans* var. *gattii* isolates tested displayed the same profile. Heterogeneity among these isolates was demonstrated for all enzymes except glumamate dehydrogenase.

4.2.3 Random amplify polymorphic DNA

RAPD was first described by William *et al.* (114) in 1990 by using arbitrary primer (AP)-PCR method in the study of bacteria and fungi. RAPD dose not require exhaustive characterization of the sequence of genomic DNA or the performance of various isotope technique.

Yamamoto (115) used RAPD analysis to study the epidemilogy of 8 environmental (all of them were serotype A) and 21 (20 serotype A and 1 serotype B)

clinical isolates of *C. neoformans* in the southern Japanese prefecture of Nagasaki. Using 3 primers revealed 6 patterns among 21 clinical isolates and 3 patterns among 8 environmental isolates. Two environmental isolates from two locations strongly associated with two isolates of patients by demonstration of identical RAPD patterns. Their results suggested that clinical and environmental isolates belong to the same pool of *C. neoformans* and that these isolates have certain geographic locations, although the number of isolated strains was limited.

Brandt *et al.* (116) compared MEE and RAPD for molecular subtyping of *C. neoformans*. RAPD improved the discriminatory power of MEE for isolates within var. *neoformans* whereas less sensitive than MEE for isolates of *C. neoformans* var. *gattii.*

Chen *et al.* (117) analyzed RAPD by using 3 primers combinations of 20-22 oligonucleotides (CN1/MYC1, MYC1/5SOR,and SOR/CN1) and 2 primers combinations of 12 oligonucleotides (FPK1-01/FPK1-05 and FPK1-05/FPK1-07) to discriminate 30 cryptococci from AIDS patients and 30 cryptococci from non AIDS patients. DNA preparation made from 2 separate cultures of each isolate were run in parallel in RAPD analysis. RAPD can discriminate effectively between strains of *C. neoformans* var. *neoformans* (41 isolates were serotyped; 38 of serotype A, 1 was serotype D, 1 was serotype AD and 1 was untypeable) by one primer pairs but to discriminate yeasts strains from HIV patients and non-HIV patients need more than one primer pairs.

4.2.4 Restriction fragment length polymorphism (RFLP)

RFLP analysis was obtained by hybridization segments of the chromosome with a radio or non-radio labeled repetitive element present in multiple copies in the genome.

Currie *et al.* (118) examined 8 environmental isolates and 17 clinical isolates in New York City by using RFLP analysis. The genomic DNA was digested with *Hae*II, *Hind* III or *Sst*I and hybridized with two DNA probes; *URA* 5 gene or the CNRE-1 labeled with [α-^{32}P] dCTP. They found that *Sst*I-digested DNA hybridized with CNRE-1 probe revealed 16 RFLP patterns more than *Hind*III digested DNA with *URA* 5 probe (revealed 5 patterns), whereas *Hae*II digested DNA hybridized with *URA* 5 probe revealed 4 patterns. RFLP for all isolates showed 16 different patterns and *C. neoformans* var. *neoformans* from 8 environmental sites revealed 6 patterns.

The CNRE-1 probe was significantly more discriminatory in typing *C. neoformans* var. *neoformans* isolates than the *URA* 5 probe (CNRE-1 revealed 6 patterns whereas the latter revealed 3 patterns). Two strains were found at environmental sites and were isolated from two patients as well. Strains shared by patients and environmental samples were separated by approximately 6 km in the study area when the patients address was used as a potential marker for geographic location of exposure.

Haynes (119) studied *C. neoformans* strains from 5 separate AIDS patients in London isolated in a single episode by using DNA digested with restriction enzyme *Eco*RI hybridized with 5 [γ-^{32}P] dATP labeled oligonucleotide probes. Unique type was observed in each patient. A total of 8 different unique types were found in these

patients. Two of isolate pairs from single episode within 1 day were genotypically indistinguishable. The other three pairs of isolates were all distinguishable. One of these isolate pairs was obtained from a single episode of cryptococcosis while the other two obtain from recurrent infections.

4.2.5 karyotyping

Karyotyping method was base on a feature of chromosome size variability of pathogenic organisms. This technique of typing may helpful in some pathogenic yeasts, such as *Candida* spp. which contained approximately 7 to 8 chromosomes (120) but may be less useful for other pathogenic fungi such as *Coccidioides immitis*, which contains only three large chromosomes (121). This is the limited of karyotyping method.

In studies performed with environmental isolates from geographically separated areas, there were marked similarities among isolates in the size and number of their chromosomes (122). However, clinical isolates of *C. neoformans* var. *gattii* did show significant differences between strains but var. *neoformans* did not. If this finding was also true for var. *neoformans*, the variety causing most clinical disease, then karyotyping may not be particularly useful for distinguish different strains for epidemiological purposes.

Also, regarding chromosome stability, in one strain of *C. neoformans* var. *neoformans*, it has been shown that selection for fluoro-orotic acid resistance produced karyotype instability. Perfect *et al.* (123) studied karyotyping by using minichromosome which produced by Varma and Kwon-Chung (124). From 40 clinical

isolates (from CSF and blood of HIV and non-HIV infected patients) and 6 environmental isolates, DNAs were extracted and the DNA bands were separated in the gel by pulsed-field electrophoresis. There was no conserved patterns associated with body site of infection, geographical location of the isolate, or HIV status. Karyotypes of individual isolates remained stable during *in vitro* passage and *in vivo* infection. Karyotype was used to excluded the possibility of nosocomial spread of *C. neoformans* in one clinical situation and supported relapse in two other cases. There were three patient isolates which appeared to possess a conserved patterns and two patient isolates which had similar karyotypes. Each of them appeared to have different amplified DNA patterns by polymerase chain reaction fingerprinting using the core sequence of the wild-type M13 phage.

Although karyotyping was useful for studying epidemiology of *C. neoformans*, there were many factors affected difference in karyotypes such as the length of the electrophoretic run and altering voltage of the contour-clamped homogeneous electric field. Karyotyping of each species should be performed only with fixed and standardized protocol.

CHAPTER IV

MATERIALS AND METHODS

MATERIALS

1. Microorganisms

1.1 Reference strains of *C. neoformans*

Species	Strains	Source
C. neoformans	NYS 152, serotype A	Dr. Salkin
C. neoformans	NYS 155, serotype B	Dr. Salkin
C. neoformans	NYS 151c, serotype C	Dr. Salkin
C. neoformans	NYS 151d, serotype D	Dr. Salkin

These strains were obtained from Dr. Ira F Salkin, Albany New York, USA and were kindly provided by Associate Prof. Dr. Pannakorn Imwidthya.

1.2 *C. neoformans* MCC E- CAT and the DNA was obtained from Division of Mycology and Mycobacteriology, Department of Microbiology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Thailand.

2. DNA

Hae III ϕX 174 RF DNA, New England BioLabs, USA

3. Enzyme

Taq DNA polymerase, Pharmacia, Sweden.

4. Latex Agglutination Kit (LA-Kit, Center for Immunodiagnostic Production, Ramathibodi Hospital, Mahidol University, Thailand and Meridian diagnostics, Cincinnati, Ohio,USA).

5. Primers

5.1 The primer CPL1 5′ GGAGGTAGTGACAATAAAT 3′ nucleotide (459-478 from 18S rRNA gene).

5.2 The primer CPR4 5′ TGCTAATGTATTCGGGCGATT 3′ nucleotide (801-781 from 18S rRNA gene).

These primers were synthesized by Bio-Service Unit, Mahidol University.

6. Chemicals

Chemicals	Molecular weight	Source
Acetic acid, glacial (CH_3COOH)	60.05	E.Merck
Agarose, Nusieve®	-	FMC
Bromophenol blue		

2-Deoxynucleotide-5'-triphosphate, dNTP	-	Pharmacia
Ethanol, absolute (C_2H_5OH)	46.07	E. Merck
Ethidium bromide ($C_{21}H_2ON_3Br$)	394.31	Sigma
Ethylenediaminetetraacetic acid disodium salt, dihydrate, EDTA ($C_{10}H_{14}N_2O_8.2H_2O$)	372.24	E. Merck
Gelatin	-	Difco
Glycerol ($C_3H_5(OH)_3$)	92.10	Fluka
Hydrochloric acid (HCl)	36.46	BDH
Magnesium chloride ($MgCl_2$. $6H_2O$)	203.31	E. Merck
Mineral oil, Light white oil, M 5904	-	Sigma
Phenol red	-	M&B
Potassium chloride (KCl)	74.56	Mallinckrodt
Potassium dihydrogen phosphate (KH_2PO_4)	136.09	E. Merck
Sodium acetate (CH_3COONa)	136.08	E. Merck
Sodium hydroxide (NaOH)	40.00	J.T.Baker
Tris(hydroxymethyl)-aminomethane ($C_4H_{11}NO_3$)	121.14	E. Merck
Tritron X- 100	-	Fluka

7. Instruments

7.1 Analytical balance, Precisa 80A, Switzerland

7.2 Autoclave, Huxley Speedy HL-300, Taiwan

7.3 Automatic pipette, Gilson, France

7.4 Biological safety cabinet class II, Gelman BH2000 series, Gelman Science, Australia

7.5 Freezer -70°C, Forma Scientific, USA

7.6 Horizontal gel electrophoresis, Mupid II, Japan

7.7 Light microscopy, PM-7 Olympus, Japan

7.8 pH meter, ORION 520A, Orion Research Inc., USA

7.9 Pipet-aid®, Drummond Scientific Co., USA

7.10 Polaroid camera, Polaroid MP4^{+}, USA

7.11 Refrigerated centrifuge, Hermle ZK 380, Germany

7.12 Shaking waterbath, Lauda MT/2, Germany

7.13 Stir plate, thermolyne nuova II, USA

7.14 Thermal cycler 480, Perkin- Elmer Cetus, USA

7.15 Top- bench microcentrifuge, Fotodyne, USA

7.16 UV-Transilluminator Spectroline® TC-321A, USA

7.17 Vacuum pump, Oil Rotary vacuum pump, Japan

7.18 Colorimeter, USA

8 Miscellaneous

8.1 Bubble plastic rack, Scienceware, USA

8.2 Centrifuge tube 5 ml, Sarstedt, Germany

8.3 Centrifuge tube 15 ml, Sarstedt, Germany

8.4 Centrifuge tube 50 ml, Sarstedt, Germany

8.5 Filtered tip, Elkay, USA

8.6 Glass beads 425-600 μm in diameter, Sigma, USA

8.7 Microcentrifuge tube 0.6 ml, Robbin, USA

8.8 Microcentrifuge tube 1.5 ml, USA/ Scientific plastic®, USA

8.9 Pipette tips, USA/ Scientific plastic®, USA

8.10 Polaroid film, Polaroid 667, UK

8.11 Timer, SEIKO MT 603F, Japan

8.12 E-Test strip for Amphotericin B, Itraconazole, AB Biodisk, Sweden

9. Media

9.1 Brain Heart Infusion Medium

BHI agar base (Difco)	37	g
Agar granulated (BBL)	20	g
Distilled water	1,000	ml

Sterilized by autoclave 121°C 15 min.

9.2 Sabouraud Dextrose Agar

Dextrose (Difco)	20	g
Agar (BBL)	20	g
Peptone (Difco)	10	g
Distilled Water	1,000	ml

Sterilized by autoclave 121°C 15 min.

9.3 Glutamine-Glycine-Asparagine DOPA Medium

Solution A

KH_2PO_4	4	g
$MgSO_4.7H_2O$	2.5	g
Thiamine hydrochloride (Fluka)	10	mg
Biotin (Fluka)	20	µg
3,4 Dihydroxyphenylamine (Sigma)	200	mg
Dextrose (Difco)	5	g
Glutamine (Fluka)	1	g
Glycine (E. Merck)	1	g
Asparagine (Fluka)	1	g
Chloramphenicol (M&H)	50	mg
Distilled water	500	ml

The pH was adjusted to 5.5 with 1 M KH_2PO_4, Sterilized by membrane filtration.

Solution B

Agar (BBL)	20	g
Distilled water	500	ml

Sterilized by autoclave 121°C 15 min., allow to cool at 56°C mix with solution A.

9.4 Glycine-cycloheximide-phenol red medium

Solution A

Glycine (E. Merck)	1 %	
Cycloheximide (Fluka)	1.6	µg/ml
YNB (Difco)	0.6 %	

The pH was adjusted to 5.6, sterilized by membrane filtration.

Solution B

Agar (BBL)	2 %	
0.5% solution of phenol red	30	ml/L

The solution was sterilized by autoclave 121°C 15 min., allow to cool at 55-56°C, mix with solution A.

9.5 Urease Medium

Solution A

Urease agar base (BBL)	29	g
Distilled water	100	ml

Sterilized by membrane filtration,

Solution B

Agar granulated (BBL)	15	g
Distilled water	900	ml

Sterilized by autoclave 121°C 15 min, allow to cool at 55-56°C, mix with solution A.

9.6 Bactec/Mycosis-IC/F (Becton Dickinson)

Processed Water	40 ml
Brain Heart Infusion Broth	1.0%W/V
Soybean-Casein Digest Broth	0.5%W/V
Yeast Extract	0.035%W/V
Sucrose	0.6%W/V
Dextrose	0.1%W/V
M-Inositol	0.05%W/V
Ferric Ammonium Citrate	0.0001%W/V
Sodium Polyanetholesulfonate	0.05%W/V
Saponin	0.24%W/V
Chloramphenicol	0.0037%W/V
Tobramycin	0.001%W/V
Antifoaming Agent	0.01%W/V

10. Reagents

10.1 Reagents for DNA extraction by modified physical rupture method

- 10x TE buffer 100 mM Tris-base
  10 mM EDTA
  Adjusted pH to 8.0 with 1N HCl
- TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0)
- 3M CH_3COONa
- Absolute ethanol

- 70% ethanol

10.2 Reagents for polymerase chain reaction (final concentration)

- 1x reaction buffer : 10mM Tris-HCl pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 0.001% (W/V) gelatin
- 200 μM dNTP
- 25 pmole primers

10.3 Reagents for agarose gel electrophoresis

- Agarose gel (4% Nusieve agarose in 1x TAE)
- 1x TAE buffer (0.04 M Tris-acetate, 1 mM EDTA)
- 4x gel loading buffer (50% glycerol, 0.05% bromophenol blue)
- 1 μg/ml ethidium bromide solution

11. Clinical specimens

Total numbers of 861 CSFs, 206 bloods and 157 urines from 160 patients suspected of having cryptococcal meningitis were collected from April 1996 to June 1998 by Dr. Somsit Tunsuphaswasdikul at Bamrasnaradura Hospital and sent to Medical Mycology Laboratory, Siriraj Hospital, Mahidol University for performing laboratory diagnosis.

Three to five milliliter of each CSF sample were collected and added into sterile 5 ml centrifuge tube then sent on the same day or on the next day by keeping the tube at room temperature to be processed in Medical Mycology Laboratory.

Two hundred and six peripheral blood samples were collected and separated into three parts. Five milliliter of them were put in sterile tube containing 0.5 M EDTA (100 µl) for DNA extraction. Two to three milliliter were added into a sterile centrifuge tube for detection of antigen by latex agglutination (LA) test and three to eight milliliter of them were added into Bactec/Mycosis-IC/F bottle. EDTA and clotted blood were kept at 4°C, Bactec/Mycosis-IC/F bottle was kept at room temperature before incubation in the Bactec 9240 automated hemoculture system.

A total of one hundred and fifty-seven urine samples were collected in amount about forty to forty-five milliliter in a sterile centrifuge tube and then kept the tube at 4°C until isolation of *C. neoformans* by culture.

CSF was collected at weekly intervals until two successively continous no growth of *C. neoformans* during treatment. Thereafter CSFs were collected at the 1st, 7th, and 12th month or at anytime if the patients had a headache because of the high pressure of CSF during prophylaxis treatment. Culture from blood and urine would be performed on the first day of treatment and in the 1st, 7th and 12th month in prophylaxis period.

Methods

Cultivation of *C. neoformans* from CSF

One milliliter of CSF at the 1st week to the 3rd week was pipetted in 1.5 ml microcentrifuge tube and spinned at 12,000 rpm 15 min, in case of treated patients,

three milliliters of CSF were used for isolation of *C. neoformans*. Supernatant was collected to a new sterile 1.5 ml microcentrifuge tube. Cell pellet was mixed gently with the rest of supernatant. Twenty microliter each of suspension were dropped on BHI agar plate for pentaduplicate. The plate was incubated at 37°C and examined for appearance of yeast colony everyday until 1 month.

Cultivation of *C. neoformans* from blood samples

Three to eight milliliter of peripheral blood were inoculated into Bactec/Mycosis IC-F bottle and incubated in Bactec 9240 automatic hemoculture detection system (Becton Dickinson, USA). The result was read automatically every 10 minute by detection of increasing amount of CO_2 in the medium by CO_2-sensor deposite at the bottom of the bottle. After positive growth detection by chemiluminescence, each bottle should be processed further by conventional method. The culture broth was smeared on slide and stained for microscopy. When budding yeast cells were presented, 100 µl of the broth were inoculated on BHI agar and incubated at 37°C for identification.

Cultivation of *C. neoformans* from urine samples

Forty to forty five milliliter of urine were centrifuged at 3,500 rpm (rotor 1617 of Hettich Universal 16) for 15 min. The supernatant was discarded and the cells pellet was resuspended with 3 ml TE buffer and mixed by vortexing. One hundred microliter of cell suspension were cultured on BHI agar and incubated at 37°C. The

plate was examined daily until one month. The remaining cell suspension was pipetted in 1.5 ml microcentrifuge tubes and stored at -70°C.

India ink preparation

One drop of cell suspension from centrifuged CSF was mixed with one drop of India ink on glass slide. The slide was examined for encapsulated budding yeast under light microscope with 600x magnification.

Latex agglutination (LA) test for detection of cryptococcal antigen

One hundred microliter of supernatant fraction of CSF were pipetted in two wells of black slide (supplied with commercial LA kit). Glycine buffer diluent was added into the wells. Normal globulin reagent was added to the first well and anticryptococcal globulin reagent was added into the other. Solutions in each well were mixed with applicator sticks. The slide was rotated by hand or rotator for 5 min. The results of agglutination or nonagglutination were evaluated with naked eyes compared with negative and positive controls in each test.

Identification of *C. neoformans*

All yeast isolates were identified as *C. neoformans* by positive capsule in India ink preparation, positive phenoloxidase and urease on glutamine-glycine-asparagine DOPA medium and urease medium within 5 days at room temperature.

Serogrouping of *C. neoformans.*

All isolates were serotyped for either AD or BC serogroup by using the glycine-cycloheximide medium with a dye indicator, phenol red (GCP). A loopful of 48-72h yeast growth from Sabouraud dextrose chloramphenicol medium (SDA) slant of each isolates was streaked on a slant of GCP agar and incubated at room temperature for 5-7 days. Cultures were daily examined for positive growth and color change of the medium. Reference strains of *C. neoformans* serotype A, B, C and D (Salkin *et al.*) were employed for control.

DNA preparation from clinical specimens

CSF

One milliliter of CSF was pipetted into 1.5 ml microcentrifuge tube and centrifuged at 12,000 rpm 15 min, 4°C to collect the cells. Five hundred and fifty microliter of supernatant were discarded and fifty microliter of 10x TE were added in the cells suspension, mixed well. The suspension was transferred into a new 1.5 ml microcentrifuge tube with 0.5 ml sterile siliconized glass beads. The tube was vortexed in order to break yeast cells for 1 min and alternately placed on ice for 1 min. This step was repeated 5 times. The tube was boiled in boiling water on hot plate for 5 min and then centrifuged at 12,000 rpm for 1 min. Supernatant was transferred to a new 1.5 ml microcentrifuge tube. Ten microliter of 3 M sodium acetate were added in 300 µl suspension. Twice volume of absolute alcohol were added and then mixed thoroughly by inverting the tube. It was kept at -20°C for at least 30 min. The tube

was centrifuged at 12,000 rpm 15 min, 4°C. Pour off the supernate carefully in order to undislodge the DNA pellet. The DNA pellet was washed with 500 µl 70% alcohol. The tube was vortexed and centrifuged at 12,000 rpm 5 min, 4°C. The supernatant was discarded and the DNA pellet was dried at 56°C. DNA was resuspended in 10-20 µl TE buffer and stored at -20°C until used.

Blood

Five to eight milliliter of EDTA blood was lysed with twice volume of lysis buffer and centrifuged at 3,500 rpm 15 min (rotor 1628 Hettich Universal 16) then discarded the supernatant. Repeat this step until there was no sign of hemolysis. The cells pellet was resuspended with 2 ml of TE buffer and then transferred into two tubes of 1.5 ml microcentrifuge tube for DNA extraction as mentioned above.

DNA amplification by polymerase chain reaction (PCR)

Each reaction mixture contained 50 mM KCl, 10 mM Tris HCl (pH 8.4), 2.5 mM $MgCl_2$, 0.001% gelatin, 25 pmole of each primers, 200 µM of each deoxynucleotide triphosphate, 1 unit of *Taq* polymerase (Pharmacia, Sweden) and deionized distilled water. Forty five microliter of reaction mixture were aliquoted into 0.6 ml microcentrifuge tube and overlaid with 50 µl of mineral oil to prevent water evaporation and stored at -20°C. Each PCR-run consisted of reagent control (reaction mixture without DNA), positive control (1 pg, 100 fg purified DNA of *C.neoformans*) and tests (5 µl of DNA solution extracted from CSFs). PCR was performed in a DNA thermal cycler (Perkin-Elmer Cetus 480, USA.) by denaturing at

94°C for 5 min 1 cycle at first and followed by 50 cycles of denaturing at 94°C 45 sec, annealing at 62°C 45 sec and extension at 72°C 45 sec.

Agarose gel electrophoresis

The amplified product was detected by running 5 µl PCR reaction mixture with 3 µl 4x gel loading buffer, electrophoresed in 4% nusieve agarose gel that (left to be harden before used at least 30 min) in 1x TAE buffer at 100 volts for 30 min in Mupid II electrophoretic apparatus. The gel was stained with 0.5 µg/ml ethidium bromide, and destaining with distilled water, visualized on a UV transilluminator and photographed by a Polaroid camera. *Hae* III-digested ϕX 174 DNA was used for standard DNA marker to estimate the molecular size of DNA fragments.

Antifungal susceptibility testing of *C. neoformans*

Suspend well isolated 48-72 hour colonies of *C. neoformans* from Sabouraud dextrose agar in sterilized distilled water to achieve 1 McFarland turbidity. Dip a sterile swab (not too tightly spun) into the inoculum suspension and press out excess fluid. Swab carefully in three directions to obtain even growth on the entire agar surface (dry wet plate before inoculation). Allow the moisture to be absorbed for at least 15 minutes. Apply the E test strips to the agar surface with an applicator or sterile forceps (ensure that inoculated agar surfaces are completely dry before applying the strips). Use a template to position 2 strips on a 90 mm plate. Once applied, do not move the strips. Incubated at 35°C in an incubator until growth is clearly seen after 48 to 72 hour. Using *Candida parapsilosis* ATCC 22092 as a reference strain.

CHAPTER V

RESULTS

A. Laboratory investigations

The clinical samples from patients in this study were obtained from Bamrasnaradura Hospital. They were HIV positive patients with clinically suspected of having cryptococcal meningitis. The patients have never been treated with antifungal or antituberculous drugs. The CSF, blood and urine were collected from the patients. Three to five milliliter of CSF were collected in 5 ml sterile centrifuge tube. Two to three milliliter of peripheral blood were collected in 12 ml sterile centrifuge tube for latex agglutination, 5 milliliter were collected in EDTA blood tube for DNA isolation and 3-8 ml were used for hemoculture with Bactec 9240 automate system. Forty to fifty milliliter of urine were collected for culture. All of specimens were sent to Division of Mycology and Mycobacteriology, Department of Microbiology, Faculty of Medicine, Siriraj Hospital, Mahidol University for laboratory diagnosis, which included culture, India ink preparation, latex agglutination and PCR.

India ink preparation was prepared from one portion of the centrifuged CSF by mixing it with India ink and investigated under a light microscope. Single or groups of encapsulated yeast cells with or without budding could be seen microscopically.

Latex agglutination test prepared from inactivated CSF reacted with anticryptococcal antibody coated-latex particles. The agglutination was interpreted

positive and no agglutination was negative for latex agglutination test. The degree of agglutination was defined in 1+ to 4+. At the first day or first week of treatment, almost all of the CSF revealed degree of agglutination in 3+ to 4+.

The sediment of CSF and urine were cultured on BHI medium and incubated at 37°C. The colonies appeared white and mucoid within 48-72 h of the enrolled day or within 14 days of treatment. The color of colony was white at first and turn to pale yellow when incubated for a long time at 37°C or at room temperature. The colonies of *C. neoformans* from culture on BHI medium, that appeared within 2-3 days were purified by subculture on SAB with chloramphenicol medium and were identified by testing its ability to produced phenoloxidase and urease on glutamine-glycine-asparagine DOPA medium and urea medium respectively. The colony of *C. neoformans* was brown or black on DOPA medium and the yeast could assimilate urea and change the color of medium from o-rose to pink or margenta red.

PCR using specific primers of *C. neoformans*, CPL1 and CPR4 primers was able to amplify 18S rRNA gene sequence and revealed a specific 343 bp product from the isolated DNA of CSF and blood. Negative PCR was reported when there was no band of specific amplified product from the electrophoresed gel.

I. Treatment period

From April 1996 through June 1998 a total of 845 CSF, 206 Blood and 145 urine samples from 160 patients were collected and investigated.

On the first enrolled day (Do) of studied, the patients were screened first for cryptococcal infection by positive for India ink from CSF samples and followed by CSF culture, hemoculture and urine culture. In addition other diagnostic tests such as LA, PCR from CSF and clotted blood samples were performed.

When compared among different laboratory methods for diagnosing cryptococcosis on the first enrolled day, India ink preparation revealed positive in 96.9% (124/128), LA was the most sensitive and revealed 100% (128/128) positive result and culture revealed positive in 93.7% (120/128). PCR could detect DNA of *C. neoformans* from 79.7% (47/59) of CSF samples (see Table 3). There were 79.3% (46/58) of culture positive revealed positive in PCR and 44 of 47 (93.6%) CSF samples had positive results for PCR, culture and India ink. There was one CSF sample which PCR positive but CSF culture and India ink negative and 12 of 58 patients showed positive in culture and India ink but PCR were negative in Table 4.

On the first day, there were 128 CSF, 156 blood and 136 urine samples (128 of 160 patients were collected CSF samples to performed laboratory investigation but 32 patients were not) were tested. Five samples from CSF culture were contaminated with bacterial organism after incubated at 37°C. Table 5 showed the success rate of isolation of *C. neoformans* from different clinical samples. CSF revealed positive in 93.7% (120/128), hemoculture was positive in 80.1% (125/156) and urine revealed positive in 8.8% (12/136). There were 6 of 123 patients (4.9%) revealed positive cultures from CSF, blood and urine. Another 6 patients showed positive culture of *C. neoformans* from only CSF and urine. Three of five patients whose CSF cultures

were contaminated with bacteria revealed positive hemoculture whereas the others 2 (patients) were not. In 2 of 3 patients whose CSF showed no growth, *C. neoformans* was isolated from blood samples. In 117 of 120 positive CSF culture had confluent growth of *C. neoformans*. The others, CSF of one patient had 2 colonies of *C. neoformans* and he revealed two successively negative culture after the 2nd week of treatment, the other patient was excluded because of pulmonary TB, his CSF grew 4 colonies of *C. neoformans*, and the last one, isolation of yeast showed 6 colonies, culture turn negative at the 6th week of treatment.

Table 3 Comparison among different laboratory methods for diagnosing cryptococcal meningitis on the first enrolled day of patients.

Laboratory methods	No. of CSF Positive/ No tested	Percentage of positive
India ink preparation	124/128	96.9
Latex agglutination	128/128	100
Cultivation	120/128*	93.7
Polymerase chain reaction (PCR)	47/59	79.7

* ; heavy bacterial contaminations in 5 samples (3 samples revealed no growth)

Table 4 Results of culture and India ink preparation of CSF samples that were performed PCR on the first enrolled day of patients.

No. of patients	laboratory investigations		
	Culture	PCR	India ink
44	+	+	+
2	+	+	-
12	+	-	+
1	-	+	-
59	58	47	56

Table 5 Results of isolation of *C. neoformans* from CSF, blood and urine samples on the first enrolled day by cultivation method.

Specimens	Amount of positive culture /Total amount tested	% positive
CSF	120/128*	93.7%
Blood	125/156	80.1%
Urine	12/136	8.8%

* ; heavy bacterial contaminations in 5 samples (3 samples revealed no growth)

During the first enrolled day to the 1st week, 26 patients were excluded from the studied (13 from each group of patients). Table 6 showed the excluding factors of the patients from the studied. The exclusion criteria involved high liver enzyme, refused treatment, high serum creatinine, transferring to other hospitals, CSF revealed negative culture at the first enrolled day and previous experience of antifungal drugs therapy. The remainder 134 patients, 60 patients in amphotericin B group (0.7-1 mg/kg/day) and 74 patients in amphotericin B combined with itraconazole (400 mg/day) group, were enrolled at the first week.

At the first week, there were 134 enrolled patients and 113/134 patients were followed up and investigated. Only CSF samples from all of them were collected for culture and India ink preparation. Culture revealed positive in 84.1% (95/113) and 59.3% (32/54) positive for PCR. There were 96.3% (52 of 54) of patients who performed for PCR revealed positive in culture. Thirty of 54 patient's CSF showed positive result in all three methods (culture, PCR and India ink). Thirty one CSF revealed positive in culture and PCR. There were 21 CSFs revealed positive result in culture and India ink preparation but PCR were negative. The last patient showed positive result in PCR and India ink preparation but negative in culture. At this week, there were 12 patients died in the treated group with amphotericin B alone and 14 patients died in combine amphotericin B with itraconazole group. One hundred and eight patients (48 patients in amphotericin B group and 60 patients in amphotericin B with itraconazole group) were followed up after the first week.

The second and consecutive weeks of treatment, the number of CSF positive cultures were continuously decreased. During this period, 26 patients did not respond

to antifungal treatment. In this group, some patients still revealed a large number of *C. neoformans*, some patients converted to negative but the next week converted to positive again which a few growth of *C. neoformans*. There were 20 patients died (11 in amphotericin B and 9 in amphotericin B with itraconazole group), 25 patients revealed failure of treatment (19 from amphotericin B), and in 6 patients the treatment was stopped (3 of each group).

There were 16 cured patients in amphotericin B group and 44 in amphotericin B with itraconazole group as shown in table 8. The patients who successively revealed two consecutive weeks of negative culture from CSF were recognized as cured and recruited in prophylaxis period. Almost of them had received amphotericin B for an accumulation dose of at least 1 g. They were only four patients showed conversion of culture at the first, second, third and forth week of treatment and obtained amphotericin B less than 1 g. They were also checked for negative culture from blood and urine samples before entering the prophylaxis period.

Table 6 Number of dropping out and exclusion criteria in each group of patients.

Exclusion criteria	Am B	Am B+Itra
Lab fault	-	1
High serum creatinine	-	1
Refused Tx	6	4
High liver enzyme	1	5
Pulmonary TB	1	1
Transferred	3	1
Unconcious	1	-
Receive antifungal drug before	1	-
Total	13	13

Am B = Amphotericin B, Itra = Itraconazole, Tx = treatment

Table 7 Results of laboratory methods for diagnosing cryptococcal meningitis on the first week of treatment patients.

No. of patients	Laboratory investigations of CSF		
	Culture	PCR	India ink
30	+	+	+
1	+	+	-
21	+	-	+
1	-	-	-
1	-	+	+
54	52	32	52

Culture : + = positive *C. neoformans*, Culture : - = No growth or negative *C. neoformans*
PCR : + = revealed a specific 343 bp amplified product of 18S rRNA gene sequence,
PCR : - = no band of specific amplified product of 18S rRNA gene sequence
India ink : + = encapsulated yeast cells were seen India ink : - = encapsulated yeast cell was not seen

II. Prophylaxis period

Sixty patients who were mentioned above were enrolled in prophylaxis period. The prophylaxis period should be one year, all patients were treated with itraconazole (400 mg/day). CSF's would be examined at the 1st month, 7th month and 12th month or when patients have had symptoms of headache. In the success treatment to prophylaxis period, there were 2 patients died. One patient who was successively treated with amphotericin B plus itraconazole at the 8th week died from pulmonary TB and the other one in amphotericin B group died with unknown cause. Fifty-eight patients were enrolled. Only 29 of 58 patients came for followed-up (total 48 CSF and 11 blood samples). There were 3 relapse.

In the 1st month, 9 of 58 patients were investigated and 1 of them (11.1%) converted to positive with numerous growth of *C. neoformans* but hemoculture was not performed. In this case the volume of CSF was inadequate for PCR. The other 8 patients (89.9%) CSF culture were still negative, CSFs from 4 patients had enough volume for PCR and all of them revealed negative result.

During the 1st month to the 7th month, 6 patients came for followed up, only one of them used to be investigated at the 1st month and revealed negative culture. Other 5 (83.3%) patients were newly followed up, only one patient had recurrent with numerous growth of *C. neoformans* both from CSF and hemoculture, in addition only this patient had enough CSF volume for PCR and its revealed positive too. India ink was negative in 1 of 5 culture negative patients.

At 7th month, 21 patients were examined. Eight of them used to be investigated (6 of 8 patients used to be followed up at the 1st month). Only one of 21 patients had recurrent and revealed numerous growth of *C. neoformans* from CSF and hemoculture. Five of 21 CSF samples could be performed PCR and all of them result in negative.

During the 7th month to 12th month, 5 patients were investigated, all of CSF obtained were culture negative and only one was performed for PCR which was negative too. Only one of 5 patients positive for India ink and this case was 1 of 2 patients who did not come for follow up at the previous month.

At the 12th month, 8 patients were investigated. All of them used to be followed up and revealed negative both by CSF culture and India ink but PCR couldn't performed because of inadequate amount of CSF.

B. Treatment and outcome

One hundred and thirty four patients were separated into two groups by antifungal drug treatment. The first group comprised of 60 patients were treated with amphotericin B (0.7-1 mg/kg/day) alone and the second group consisted of 74 patients were treated with amphotericin B combined with itraconazole (400 mg/day). These two groups were comparable in the age, body weight, hemoglobin and hematocrit value, sodium potassium level and $CD4^+$ count. Table 9 showed the survival rate of patients during treatment period, 37 of 60 patients (61.6%) were alive and 23 patients died in the group treated with an amphotericin B alone and in the amphotericin B combined with itraconazole group, 51 of 74 patients (68.9%) were alive. Table 8 showed the status of patients in two groups. In amphotericin B group, 22 patients died, 13 patients were excluded, 19 patients with failure treatment (1 case failure of treatment and died with TB), 3 patients stop treatment and 16 of 60 (26.7%) succeed. In the 2nd group, there were 21 patients died, 13 were excluded, 6 with treatment failure, stop treatment in 3 patients and 44 of which 74 patients (59.5%) succeeded (2 cases success of treatment and died with pulmonary TB). Differences of survival rate and success rate in two groups were not significance (P value = 0.274 in survival and P value = 0.268 in success rate). The mortality rate was 36.7% (22/60) and 31.1% (23/74) in the 1st and the 2nd group respectively and this result showed non significant

difference (P value = 0 .278). In the prophylaxis period there were 3 recurrent patients, 2 of them were treated with combination drugs one recurrent in the 2nd and the other recurrent at the 7th month. The 3rd patient who was recurrent at the 1st month was treated with amphotericin B alone.

Table 8 Status of patients in each group during treatment period.

Status of patient	No. of patient	
	Am B alone	Am B+ Itra
dead	22	21
excluded	13	13
failure of treatment	19[a]	6
stop treatment	3	3
success	16	44[b]
Total	73	87

a ; 1 case failure of treatment but dead by TB
b ; 2 cases success of treatment but died
Am B = Amphotericin B, Itra = Itraconazole

Table 9 Result of treatment and outcome in two groups of patients.

Treatment and outcome	No. of patient		P value
	Am B alone	Am B+ Itra	
survival	37/60 (61.6%)	51/74 (68.9%)	0.274
successful	16/60 (26.7%)	44/74 (59.5%)	0.268
mortality	22/60 (36.7%)	23/74 (31.1%)	0.278
recurrent	1/6(16.7%)	2/15 (13.3%)	0.435

Am B ; Amphotericin B, Itra ; Itraconazole

Table 10 Amount of positive *C. neoformans* from CSF culture, India ink preparation, latex agglutination and PCR method during treatment and prophylaxis periods.

Treatment period	Time	CSF Culture		India ink preparation		latex agglutination		PCR	
		amount	%	amount	%	amount	%	amount	%
	Do	120/128	93.7	124/128	96.9	128/128	100	47/59	79.7
	Wk1	95/113	84.1	103/113	91.1	17/17	100	32/54	59.3
	Wk2	70/108	64.8	93/108	86.1	12/12	100	20/54	37.0
	Wk3	56/98	57.1	88/98	89.8	11/11	100	23/53	43.4
	Wk4	38/87	43.7	78/87	89.7	8/8	100	12/46	21.4
	Wk5	31/75	41.3	67/75	89.3	1/1	100	8/39	20.5
	Wk6	28/58	48.3	52/58	89.7	3/3	100	12/35	34.3
	Wk7	21/42	50	37/42	88.1	1/1	100	6/21	28.6
	Wk8	18/29	62.1	28/29	96.5	1/1	100	7/17	41.2
	Wk9	13/22	59.1	19/22	86.4	-		6/9	66.7
	Wk10	10/13	76.9	10/13	76.9	-		1/8	12.5
	Wk11	3/6	50	5/6	83.3	-		0/2	0
	Wk12	1/4	25	1/4	25			0/3	0
Prophylaxis period		No of pos.	No. of Pt					PCR pos	PCR neg
	Mo. 1	2	7	5/9	55.6			0	4
	Mo. 2	1	3	4/4	100			-	-
	Mo. 3	0	2	2/2	100			-	-
	Mo. 4	0	3	3/3	100			-	-
	Mo. 5	0	2	2/2	100			-	-
	Mo. 6	0	1	1/1	100			-	-
	Mo. 7	1	21	12/22	54.5	1:128 (2 pt) 1:256 (1 pt) 1:512 (1 pt)		0	5
	Mo. 8	0	1	0/1	0	1:128 (1 pt)		0	1
	Mo. 9	0	1	0/1	0			-	-
	Mo. 10	-	-	-	-			-	-
	Mo. 11	0	1	0/1	0			-	-
	Mo. 12	0	8	2/8	25			-	-

Table 11 Result of dead patients at interval week of treatment in two group of patients.

Week	No. of patients				Accumulation Percentage of Dead
	Amphotericin B		Amphotericin B + Itraconazole		
	Dead	Alive	Dead	Alive	
0	0	60	0	74	0.0
1	12	48	14	60	60.9
2	3	45	2	58	67.4
3	3	42	1	57	76.1
4	1	41	3	54	84.8
5	2	39	1	53	91.3
6	0	39	0	53	91.3
7	1	38	0	53	93.5
8	0	38	1	52	95.6
9	0	38	1	51	97.8
10	1	37	0	51	100.0
11	0	37	0	51	100.0
Total	23		23		100

Table 12 Results of laboratory investigations of succeed patients on the first enrolled day, at the week of negative culture of treatment and at the 7th month during prophylaxis period.

Patients/ laboratory method		Period of laboratory investigations		
		First enrolled day	Treatment	prophylaxis
SIMI-CN 27	Culture	num[a]	- (W3)	- (M7)
	LA titer	>1024	>1024	512
	India ink	-	0-1[b]	5-10[b]
	PCR	+	-	-
SIMI-CN 84	Culture	num	- (W5)	- (M7)
	LA titer	>1024	512	128
	India ink	5-10[b]	1-3[b]	-
	PCR	+	-	-
SIMI-CN 90	Culture	num	- (W3)	- (M7)
	LA titer	>1024	-	128
	India ink	1-3[b]	0.1[b]	0-1[b]
	PCR	+	+	-
SIMI-CN 108	Culture	30	- (W6)	- (M8)
	LA titer	>1024	1024	128
	India ink	3-5[b]	0-1 (W3)[b]	0-1[b]
	PCR	+	-	-
SIMI-CN 122	Culture	num	- (W6)	- (M7)
	LA titer	>1024	512	256
	India ink	5-10[b]	0-1[b]	0-1[b]
	PCR	-	-	-

a = numerous , b = cells / high power field, W = week, M = month

Table 13 The result of $CD4^+$ cell and drug dosage in over all patients between the group treated with amphotericin B and the group treated with combination drugs : amphotericin B with itraconazole

Over all patients	N	Mean	SD	2-tail Sig
CD4				
A	45	21.18	20.52	p=0.396
A+I	57	27.74	53.25	
Dose Ampho				
A	60	1.178	0.754	p=0.620
A+I	74	1.115	0.704	

CD4 unit = cells/ml A unit = g
A = Amphotericin B, I = Itraconazole

Table 14 The result of $CD4^+$ cell and drug dosage in dead patients between the group treated with amphotericin B and the group treated with combination drugs : amphotericin B with itraconazole.

Dead	N	Mean	SD	2-tail Sig
CD4				
A	7	12.86	4.88	p=0.506
A+I	6	10	8.96	
Dose Ampho				
A	22	0.414	0.416	p=0.364
A+I	21	0.306	0.36	

CD4 unit = cells/ml A unit = g
A = Amphotericin B, I = Itraconazole

Table 15 The result of $CD4^+$ cell and drug dosage in failure treated patients between the group treated with amphotericin B and the group treated with combination drugs : amphotericin B with itraconazole.

Failure	N	Mean	SD	2-tail Sig
CD4				
A	19	21.74	18.457	p=0.557
A+I	6	16.67	17.512	
Dose Ampho				
A	19	2.036	0.198	p=0.60
A+I	6	2.244	0.205	

CD4 unit = cells/ml A unit = g
A = Amphotericin B, I = Itraconazole

Table 16 The result of $CD4^+$ cell and drug dosage in successfully treated patients between the group treated with amphotericin B and the group treated with combination drugs : amphotericin B with itraconazole.

Success	N	Mean	SD	2-tail Sig
CD4				
A	16	25.625	26.825	p=0.582
A+I	44	31.841	59.73	
Dose Ampho				
A	16	1.317	0.261	p=0.395
A+I	44	1.394	0.404	

CD4 unit = cells/ml A unit = g
A = Amphotericin B, I = Itraconazole

C. Pair-serotyping of *C. neoformans*

In this study the glycine-cycloheximide phenol red medium (GCP) was used for paired-serotyping of *C. neoformans*. After the isolated yeasts from clinical samples were identified as *C. neoformans*, they were serotyped by incubation on GCP medium for 5- 7 days at room temperature. The positive result showed a change in color of GCP medium from yellow to pink or red which conferred by *C. neoformans* serotype BC. Negative result revealed no color change on GCP medium which was characteristic of *C. neoformans* serotype AD. Table 17 showed the results of pair-srotype of 514 *C. neoformans* isolates from 158 patients. There were 512 isolates (410 isolates from CSF, 99 isolates from blood and 3 isolates from urine) from 157 patients revealed no change in color of medium. They were AD serotype . There were only 2 isolates (one from CSF and the other from urine) from one patient could change the color of medium from yellow to pink or red, and were classified in serotype BC. All isolates of *C. neoformans* from the same patient at the first and consecutive weeks or months were the same serotype.

Table 17 Pair-serotyping of 514 *C. neoformans* strains isolated from 158 patients on GCP medium.

Specimens	Serotype AD	Serotype BC	Total isolates
CSF	410	1	411
Blood	99	0	99
Urine	3	1	4
Total	512	2	514
Percentage	99.6%	0.4%	100%

D. *In vitro* susceptibility test by E test method

Eighty-nine strains of *C. neoformans* isolated from the patients in this study were subcultured from stock's stored at -70°C for performing antifungal drugs susceptibility test to determine minimum inhibitory concentration (MIC) by E test method. The tested drugs were amphotericin B and itraconazole. *Candida parapsilosis* ATCC 22092 was used as tested reference strain. Sixty-five strains were obtained from successfully treated group of patients (33 cases), 9 strains from patients who died, the other 9 strains came from patients who were non-response to treatment even the accumulative doses of amphotericin B were more than 2 gm and the last six strains were isolated from relapse cases. There were 2 strains from one dead patient and one non-response patient revealed no growth on subculture, so eighty-seven strains were investigated for MIC. The MIC value of all strains from cure patients were range from 0.012-0.50 μg/ml and 0.032-1.5 μg/ml for amphotericin B and itraconazole respectively. The MIC range for amphotericin B was 0.032-0.25 μg/ml and for itraconazole was 0.064-1.5 μg/ml in *C. neoformans* strains obtained from patients who died. In cases of strains from patients who were non-response to treatment (even the accumulative doses of amphotericin B were more than 2 gm) were 0.032-0.38 μg/ml and itraconazole was 0.003-0.50 μg/ml. The last six strains in relapse cases, the MIC value were 0.012-0.25 μg/ml and 0.004-0.50 μg/ml for itraconazole as shown in Table 18.

Table 18 The MIC of amphotericin B and itraconazole antifungal drugs in each group of patients.

Group of patients	Antifungal drugs				Number of strains
	Amphotericin B unit		Itraconazole unit		
	MIC range	$\bar{X} \pm SD$	MIC range	$\bar{X} \pm SD$	
• Successfully treated group	0.012-0.5	0.177 ± 0.117	0.032-1.5	0.435 ± 0.323	65
• Death from cryptococcal meningitis	0.032-0.25	0.141± 0.081	0.064-1.5	0.649 ± 0.476	8
• Received amphotericin B more than 2 gm	0.032-0.38	0.146 ± 0.128	0.003-0.5	0.133 ± 0.167	8
• Relapsed cases	0.012-0.25	0.180 ± 0.087	0.004-0.5	0.358 ± 0.386	6
Reference strain: *Candida parapsilosis* ATCC 22092	0.125		0.016		1

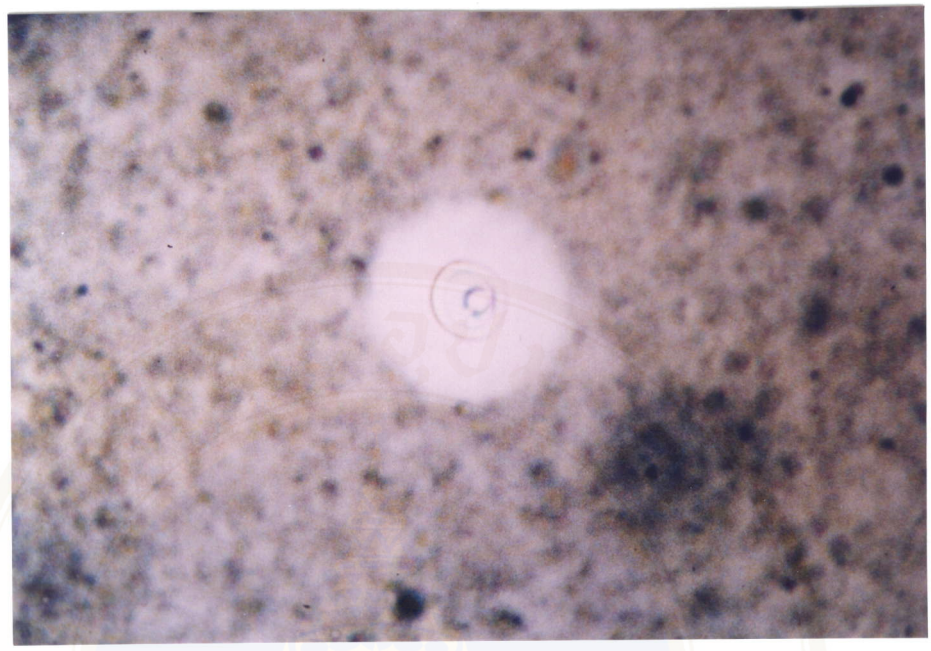

Figure 3 : Encapsulated yeast cell of *C. neoformans* from CSF sediment seen by India ink preparation under a light microscope (x 600 magnification).

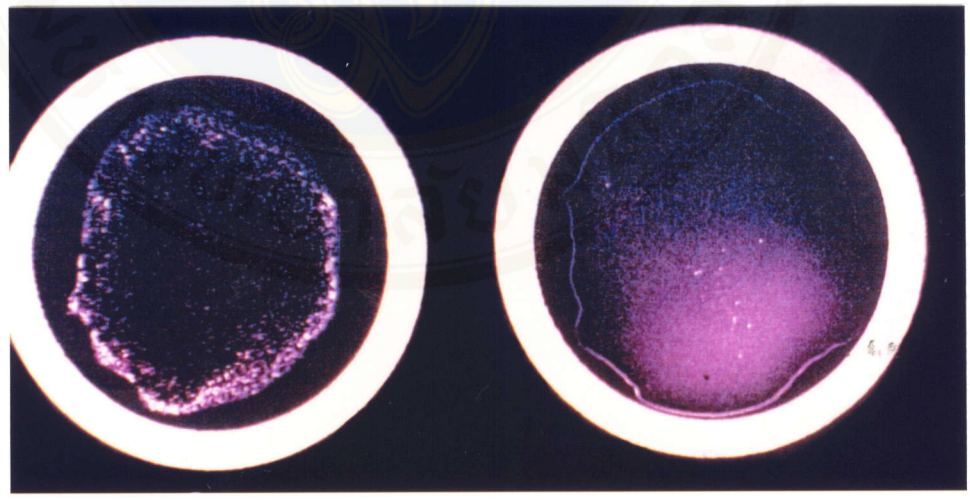

Figure 4 : Latex agglutination test for detection of cryptococcal antigen. (left) agglutination show positive result , (right) no agglutination show negative result.

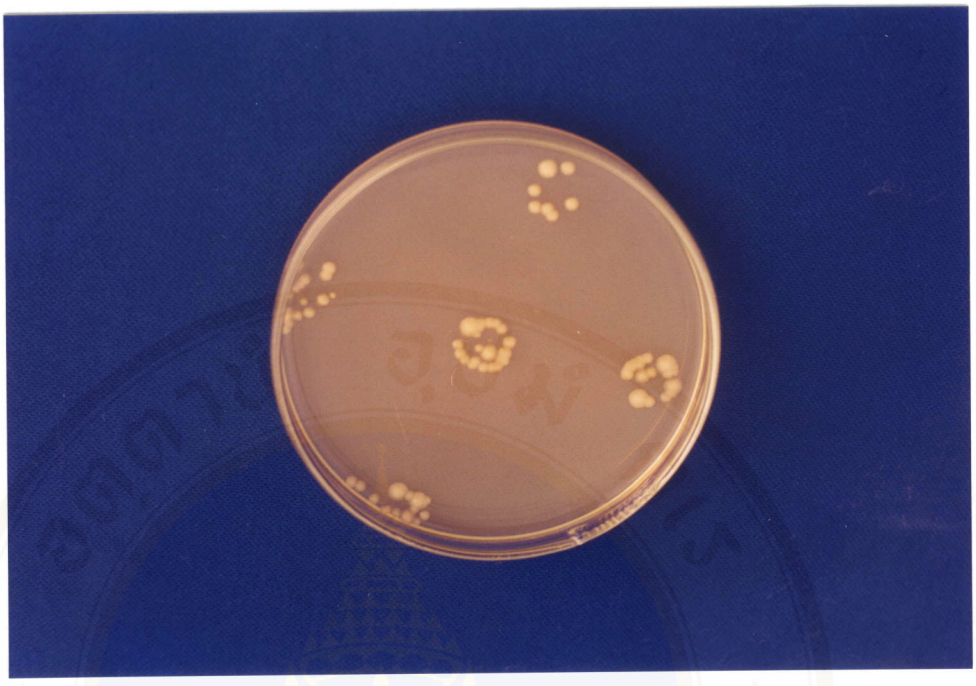

Figure 5 : The colonies of *C. neoformans* from CSF sample on BHI plate incubation at 37°C, after 3 days. The cultivation of *C. neoformans* on BHI medium in pentaduplicate fashion done with some modification from the method that described by Supanwong and Pichai.

Figure 6 : The result of urease production; (left) the colorless media show negative result, (right) the pink or red media show positive result.

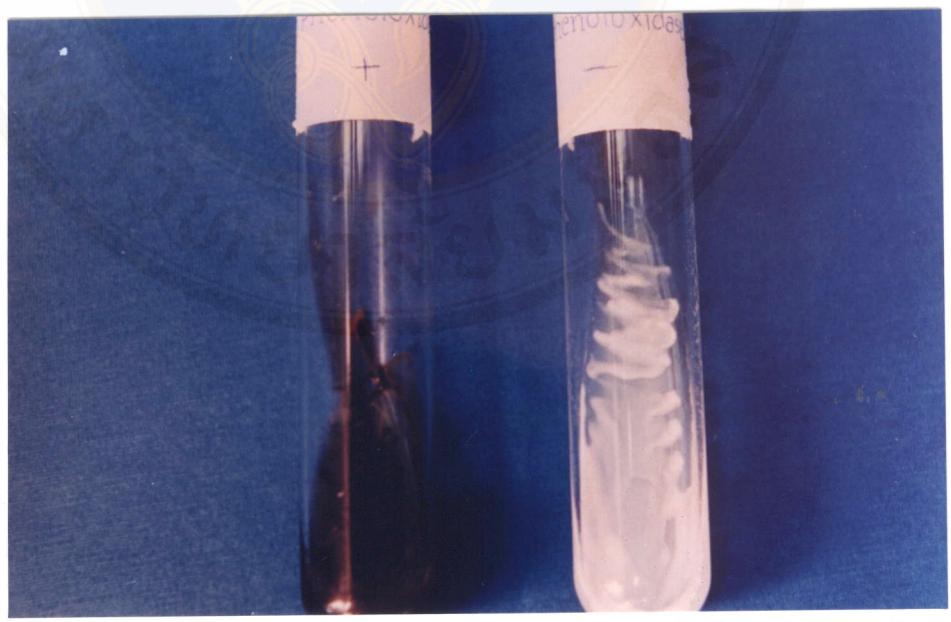

Figure 7 : The result of phenoloxidase test; (left) the black colonies of *C. neoformans* after 5 days on DOPA medium, (right) the white colonies of *Candida albicans.*

Figure 8. Agarose gel electrophoresis of PCR product of18S rRNA gene sequence of *C. neoformans* obtained from CSF of AIDS patients during treatment period.

Lane 1 : ∅X DNA-*Hae* III digested DNA marker

Lane 2 : Strain SIMI-CN 66 Do

Lane 3 : Strain SIMI-CN 84W1

Lane 4 : Strain SIMI-CN 88W3

Lane 5 : Strain SIMI-CN 83W1

Lane 6 : Strain SIMI-CN 141W4

Lane 7 : Strain SIMI-CN 141W5

Lane 8 : Strain SIMI-CN 141W6

Lane 9 : Strain SIMI-CN 141W8

Lane 10 : Strain SIMI-CN 142W4

Lane 11 : Strain SIMI-CN 143W4

Lane 12 : Strain SIMI-CN 143W5

Lane 13 : Strain SIMI-CN 156W5

Lane 14 : Strain SIMI-CN 157W5

Lane 15 : DDW control (blank control)

Lane 16 :*C. neoformans* DNA 1 ng (positive control)

Lane 17 : *C. neoformans* DNA 1 ng (positive control)

CHAPTER VI

DISCUSSION

Laboratory investigation

Comparison among different laboratory methods for diagnosis of cryptococcal meningitis, serological tests (LA) for the diagnosis of cryptococcosis was one of the true success stories for rapid and accurate diagnosis of this fungal infection. The latex agglutination test is now approximately 95% sensitive and specific for identification of invasive cryptococcosis (125). Most latex agglutination test kits can detect at least 10 ng of polysaccharide per ml of body fluid. The popularity of the diagnostic test for cryptococcal polysacccharide antigen is readily apparent when it is noted that there are at least 5 commercial latex agglutination kits for cryptococcal polysaccharides. There are four kits : Crypto-LA, CALAS, Myco-Immune and IMMY in the USA. And a kit made by Sanofi Diagnostic Pasteur in Europe. In this study, CALAS was used for detection of cryptococcal antigen in the CSFs and revealed the sensitivity of 100% (128/128 samples) for laboratory method in diagnosing cryptococcal meningitis. There was no false negative found which might be due to thick capsulated strains of all isolated *C. neoformans*. The high sensitivity could be derived solely from good quality of the LA kit.

The India ink examination is another useful and rapid diagnostic test for cryptococcal meningitis. This simple test has been found to be positive in 30 to 50% of

patients with non-AIDS cryptococcal meningitis and positive in more than 80% of patients with AIDS and cryptococcal meningitis (100). The sensitivity of an India ink preparation generally allows detection between 10^3 and 10^4 CFU of yeasts per ml or greater concentration. Before the AIDS epidemic the quantity of yeast in CSF during meningitis ranged from 10^3 to 10^7 CFU/ml (101). It is likely that the heavily positive results of India ink preparation in many AIDS patients with cryptococcal meningitis represent concentration between 10^5 and 10^7 CFU/ml in CSF. Furthermore, the sensitivity of the examination may ever be improved by centrifuging the CSF specimen (500 rpm for 10 min) and using the pellet for staining.

In this investigation, direct examination by India ink preparation showed the second most sensitive of 96.9% in diagnosing cryptococcal meningitis. This figure was higher than reported in the past which should be according to the centrifugation of sample before taken the pellet for staining and experience of the observer. The specificity of the examination occasionally can be reduced by false positive associated with leukocytes, myelin globules, fat droplets and tissue cells. Although not as sensitive or specific as the serological tests for cryptococcal meningitis, India ink preparation is a rapid test that can often deliver an immediately diagnosis within minutes of the lumbar puncture and give the clinician an appreciation for the burden of yeasts. A heavily positive examination may identify a patient with a higher risk for sudden increase in intracranial pressure during early administration of antifungal drugs. Therefore, India ink preparation should be performed as a screening test on all initial CSF specimens from patients highly suspected of having cryptococcal meningitis.

Cultivation method is the gold standard method for detection and identification *C. neoformans.* Usually it should be as sensitive as LA for the diagnosis of cryptococcal meningitis. The sensitivity was lower than LA and India ink preparation because of some contamination. Cultivation technique is a reliable method for detection *C. neoformans* and for monitoring the results of treatment. The cultivation of *C. neoformans* from CSF, blood and urine on BHI medium in pentaduplicate fashion were done with some modification from the method that described by Supanwong and Pichai (126). The number of colonies of *C. neoformans* were nearly equal in each drop on the same plate. This method was easy to do and accurate. It should be used with caution with samples from nonsterile sites because bacteria would grow over the yeast. For avoidance of bacterial contamination some antibacterial antibiotics should be added into the medium and daily observed the plate and subcultured on a new plate in a streak fashion. *C. neoformans* was isolated from CSF in 93.7% (120/128 samples), from blood in 80.1% (125/156 samples) and from urine only in 8.8% (12/136 samples). On the first admission day (on the first enrolled day), the number of colonies of *C. neoformans* were numerous in almost all of the patients (117 patients) except 3 patients there were a few yeast colonies.

There were some differences in culture results at the beginning of the study between two laboratories (Siriraj Hospital and NIH) which were due to the volume of CSF used for cultivation. The large amount of CSF revealed more positive culture and was recommended by Richardson and Warnock (127) that at least 3 ml should be obtained for isolation of fungal pathogens. The most appropriated procedure, all CSF samples should be centrifuged, the supernatant can be used for serological tests and

the sediment can be employed for culture and India ink preparation. At the first enrolled day of this study 0.5 ml of CSF was used for culture, at the 1st week to the 3rd week 1 ml was used and the later weeks or months at least 3 ml of CSF were used for culture in order to obtain more reliable culture result.

Three of 120 patients' CSF revealed a few colonies of *C. neoformans* on the first enrolled day and showed two successively negative culture after the 2nd week of treatment. The other patient was excluded because of pulmonary TB, his CSF grew 4 colonies of *C. neoformans* and the last one, isolation of yeast showed 6 colonies, culture turned negative at the 6th week of treatment, all of them survived. Whereas 31 of 134 (23.1%) patients who revealed numerous growth of *C. neoformans* died within two weeks.

Cryptococcemia has become a common occurrence with disseminated cryptococcosis in AIDS patients or others immunocompromised host such as SLE and malignant lymphoma. In several series of disseminated cases in AIDS, the rate of positive blood culture range from 35-68% of cases. They used a radiometric BACTEC system(107), a lysis-centrifugation methods (128) and BACTEC NR660 (129). In this study, the BACTEC 9240, (a non radiometric system) was employed for blood culture. Positive rate of *C. neoformans* from hemoculture was as high as 80.1% which had never been reported before. The continuous agitation of BACTEC 9240 blood culture bottles for the full incubation time significantly improve the detection of CO_2 and recovery of *C. neoformans* from blood cultures and also could be a reflection of high numbers of yeasts in the blood of these studied patients. Hemoculture may be reliable

used for diagnosis of *C. neoformans* when lumbar puncture could not be performed in disseminated cryptococcosis. In this study the volume of urine of 45 ml were centrifuged before culture but revealed positive only in 8.8%. The positive rate in urine culture was low. It might reflect the absence of *C. neoformans* in urine in these patients.

The PCR method revealed positive in 79.7%(47/59) in this study whereas Prariyachatigul *et al.* showed sensitivity of PCR equal to 100% compared with CSF culture. The sensitivity of PCR in this study was lower than conventional methods. This might be due to incomplete lysis of the yeast cell in the step of cell breaking and resulted in small amount of released DNA from the yeast cells. There were 46 of 58 patients whose CSF culture positive revealed positive in PCR and 44/56 whose India ink preparation positive showed positive in PCR. For the culture negative CSFs which showed PCR positive, there might be three possible explanations. Firstly, amphotericin B or itraconazole might partially damage cryptococci in CSF and make them unable to grow on BHI medium although they were still alive. Secondly, the yeast cells in the CSF were dead but their cells and DNA were not degraded and finally it might be false positive result. So these would produce negative results in culture but positive results in the PCR. The culture positive but PCR negative should explain by having inhibitor in the CSF or blood which inhibit the reaction of PCR. One simple way for avoiding false negative result was to dilute the extracted DNA 10-20 fold before performing PCR (130).

In the last few years, there have been a series of studies that used rapid PCR based identification schemes to identify yeast strains (16, 131). At present PCR was

adapted directly to clinical specimens. The strategies generally utilized specific primers from the gene encoding the 18S rRNA. With the used of specific PCR, it could rapidly distinguish *C. neoformans* from other yeasts in a mix sample or in tissue.

PCR was a rapid method. The assay need only 4 hour counting from receiving CSF until detection of the specific DNA band in agarose gel. This conferred a rapid laboratory method for diagnosis of cryptococcal meningitis. in this study, PCR revealed disappointed lower sensitivity in this study for diagnosing cryptococcosis than LA, India ink and culture. Its value in monitoring the patients during treatment and prophylaxis period was also remained to be proved because of too small number of samples studied at this moment. An application of PCR could be used to confirm or identify *C. neoformans*.

In prophylaxis period, all of 5 CSF samples from 5 patients showed low cryptococcal antigen titer. All of them showed cryptococcal antigen of 1024 at the first enrolled day. The cryptococcal antigen tests were still positive through the end of prophylaxis period with only 2 to 4 folds decreasing in titer eventhough CSF culture showed negative results over 12 months. The diagnosis of cryptococcal meningitis in AIDS patients could not base solely on LA test but need the information of previous treatment and also titer of antigen.

Treatment and outcome

In cryptococcal meningitis, " cure or success" means the eradication of the organism and elimination of symptoms. In both non-AIDS and AIDS patients, the two

most important factors that suggest relapse and require reinstitution of treatment are a positive culture and development of new or persistent neurological symptoms (132). The initial treatment regimens for cryptococcal meningitis in non-AIDS patients have ranged from 4 to 10 weeks with relapse rates can approach 15 to 20% with these regimens (19). In AIDS patients, the length of therapy and follow up is indefinite. Relapse rates of 50 to 60% were reported with a corresponding reduction in life expectancy (12).

The success rate of treatment in this study was 26.7% in amphotericin B group and 59.5% in amphotericin B plus itraconazole group. These were a little bit lower than average range of 55 to 75% success rate (132). The poor immune status of hosts ($CD4^+$ less than 100 cells/ml) might be the factors for higher dead rate and/or failure rate in these patients. Another reason should be due to a heavy load of the yeasts at the initial treatment and unability for the drug to sterilize the etiologic agent. In combined group, the success rate was higher, itraconazole might have role in elimination the yeast cells as well. There was no significant difference between the two groups in over all mortality rate (P= 0.278) amphotericin B and combined group (36.7% and 31.1%). Comparison in dead rate, on the first week the mortality rate was highest in both groups (52.2% for amphotericin B and 60.9% for combined group). The mortality rate during the first two weeks of therapy was higher in combined group (69.6%) and 65.2% in amphotericin B group, this result was the same as reported by Saag. Saag *et al.*(88) noted that in 194 patients, 131 received fluconazole and 63 received amphotericin B (mean daily dose 0.4 mg per kilogram of body weight in patients with successful treatment and 0.5 mg per kilogram in patients with treatment

failure; P= 0.34). Treatment was successful in 25 of the 63 amphotericin B recipients (40%) and in 44 of the 131 fluconazole recipients (34%) (P=0.40). There was no significant difference between the groups in over all mortality due to cryptococosis (amphotericin B and fluconazole, 9 of 63 (14%) and 24 of 131 (18%); (P=0.48); however, mortality during the first two weeks of therapy was higher in the fluconazole group (15% and 8% ; P= 0.25). The median length of time to the first negative CSF culture was 42 days in the amphotericin B group and 64 days in the fluconazole group (P= 0.25). Clark *et al.*(64) reported that among cryptococcal meningitis with AIDS, in whom success rates with administration of amphotericin B (0.3 to 0.5 mg per kilogram of body weight per day) with or without flucytosine are only 40% to 50% and intolerance is common, especially when flucytosine is used. By contrast, Larsen *et al.*(99) reported success in all 6 patients treated with higher doses of amphotericin B (0.7 mg per kilogram per day for the first two weeks of therapy) plus flucytosine. Although, the success rate and mortality rate in both groups of patients in this study were not significantly different, the more success and less failure patients in combined group might be able to support a combination drugs regimen for a treatment of cryptococcosis in AIDS patients.

The recurrence rate of amphotericin B was 16.7% (1/6) and 13.3% (2/15) in combined group (6 patients in amphotericin B and 15 patients in combined group were followed up at the 7th month of prophylaxis period). In non-AIDS patients relapse rates can still approach 15 to 20%. Prolonged consolidation treatment with triazole for 3 to 6 months and possibly up to a year should be considered and should be closely followed for 1 year. In AIDS patients, oral fluconazole therapy at a dosage

of 200 mg/day for 1 year could reduce relapse rate to less than 5% (133). Although the concept of chronic indefinite suppressive therapy for cryptococcosis in AIDS has been established by the medical community, it has not actually been proved to be effective for longer than 1 year after treatment. Primary isolates of *C. neoformans* for all patients should ideally be stored for at least 1 year after diagnosis. Although relapse isolates might remain susceptible *in vitro* to the treatment agents, drug-resistant isolates were increasing. It was important to compare the MIC of the relapse isolate to the original isolate in all cases of relapses (102).

Pair-serotyping of *C. neoformans*

The serotype of *C. neoformans* was determined by streaking the isolate on glycine-cycloheximide phenol red agar (GCP medium). This medium distinguishes *C. neoformans* var. *neoformans* from *C. neofornans* var. *gattii* but cannot distinguish serotype A from D or B from C (45). Clancy *et al.*(67) reported that cryptococcosis in AIDS patients in Southern California was always caused by *C. neoformans* var. *neoformans.* This result was the same as reported by Shimizu *et al.*(8) *C. neoformans* var. *neoformans* predominated in AIDS patients in Los Angeles which is an endemic area of *C. neoformans* var. *gattii.*

In 1989, there was a report of serotype pair from clinical and environmental isolates of *C. neoformans* in Bangkok, Thailand by Imwidthaya *et al* (10).. They found that all 13 strains from cryptococcosis patients and 13 strains from feces of pet birds cultured on glycine-cycloheximide medium were A/D serotype.

C. neoformans strains in this study were belong to A/D serotype 99.6% (512/514) and only 0.4% (2/514) were B/C serotype. This result correlated with the typing of *C. neoformans* isolates from patients in Thailand using slide agglutination with Crypto Check (Iatron, Inc. Tokyo) was published in 1996 by Sukroongreung *et al.* (134). One hundred and sixty nine clinical isolates obtained between January 1993-March 1995 (AIDS era) were tested. Serotype A was outstandingly predominant 93% (157/169) (134) and the same as reported in 1997 by Sethakorn (11). They found that 98.2% (104/106) of *C. neoformans* isolates were serotype A and only 0.9% (1/106) were belong to serotype B and 0.9% (1/106) were serotype D both by reacting with specific monoclonal antibodies and by using PCR fingerprinting method. Therefore, *C. neoformans* var. *neoformans* serotype A was the most common serotype found in AIDS patients in Thailand and worldwide.

***In vitro* susceptibility test by the E-test method**

An E-test is a rapid method for determination the MIC of organism. In this study the MIC of almost strains of success patients falled in the susceptible range to amphotericin B and itraconazole. There were 3 strains (from 3 patients) resisted to amphotericin B (MIC = 0.5 μg/ml) whereas all were susceptible to itraconazole. All of these three patients were cured by amphotericin B and itraconazole. They also revealed no relapse until 7th month of itraconazole prophylaxis. In dead patients there was only one *C. neoformans* isolate obtained among 8 patients was resist to itraconazole (MIC = 1.5 μg/ml). This patient was dead at the third week when the accumulate dose of amphotericin B was 0.71 g. The death should due to the severity

of the disease and the low host immune response that couldn't eliminate the organism. The MIC of all strains of failure patients and relapse patients showed susceptible to both antifungal drugs. Therefore, the cause of failure in these patients could not be explained by resistant *C. neoformans*. Sagg *et al.* (88) reported that the patients who have disseminated cryptococcosis, the progressive of disease was more rapid than those who were complicated with only cryptococcal miningitis. The patients in this study were good examples because 80.1% revealed positive hemoculture and more than 60% of death occurred in 2 weeks after administration of therapy.

In the susceptibility study, the MIC range of amphotericin B and itraconazole were 0.012-0.5 μg/ml and 0.003-1.5 μg/ml respectively. In 1996, Sethakorn (11) reported the MIC range of amphotericin B were 0.5-1 μg/ml and itraconazole were 0.25-0.5 μg/ml by macrodilution method. The MIC value of *C. neoformans* for amphotericin B and itraconazole antifungal drugs by macrodilution method and E-test method were not significantly different. Because of laborious technique and the lack of purified drugs, macrodilution method was not routinely undertaken in general mycological laboratory. The E-test might be an alternative method for determination of MIC mycological laboratory. However, the high cost of the test and the lack of clinical correlation with the obtained MIC would not make it to become a routinely standard test at present.

CHAPTER VII

CONCLUSION

1. Although PCR is a rapid method which the result can be obtained within 4 hours, it is not the best method for laboratory diagnosis of cryptococcal meningitis in AIDS patients. It is more expensive than India ink preparation, LA and culture and reveals less sensitivity (only 79.7% whereas LA gives 100% sensitivity). India ink preparation of pellet from centrifuged CSF should be recommened as the most rapid and economic test for diagnosis of cryptococcal meningitis. Its value should be used as an alternative rapid method for followed up during treatment and during prophylaxis period.

2. Isolation of *C. neoformans* from blood of AIDS patients showed positive result in 80.1% and about 8.8% from urine samples. This indicated that disseminated cryptococcosis in AIDS patients was common and hemoculture should be an alternative procedure where CSF culture could not be performed. High percentage of positive hemoculture in this study can be due to the correct clinical diagnosis and the use of automate BACTEC 9240 culture system.

3. The treatment regimens between monotherapy with amphotericin B and combined drugs of amphotericin B and itraconazole revealed no significant differences in survival rate, success rate, mortality rate and relapse rate. The group of patients received a combination treatment showed higher percentage

of success around 59.5% (44/74) than 26.7% (16/60) in the monotherapy group. Amphotericin B combined with itraconazole might be recommened as an alternative treatment regimen for cryptococcal meningitis

4. Itraconazole prophylaxis in a dose of 400 mg/day could protect the AIDS patients from relapse cryptococcosis around 50% (relapse rate was about 14.3% of the followed up patients compare to approximately 50% in the nonprophylactic group). Recent guideline recommends a life-long prophylaxis with triazole compound for these patients. In the prophylaxis period, isolation of *C. neoformans* from CSF or blood should be done when it was possible to confirm for the recurrent disease. PCR and LA could be use only as supplementary tests and should be interpreted with other informations especially the history of disease and medical treatment.

5. *C. neoformans* strains isolated from AIDS patients during 1996-1998 revealed predominately A/D serotype (around 99.6%) which was the same as reported by other investigators both in Thailand and worldwide.

6. *C. neoformans* strains in this study were almost sensitive to amphotericin B (MIC value < 0.5 μg/ml) and to itraconazole (MIC value < 1.0 μg/ml) The usage of these antifungal drugs for treatment of cryptococcal meningitis should have some clinical value here. Other regimens should be tried in order to increase the success rate and reduce the mortality rate in patients with cryptococcal meningitis.

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APPENDIX

Table 1 : Information of all patients recruited in the study.

No	Lab no.	Regimen	Total dose of Am (g)	First CSF cul Neg at wk	pt. status	dead at wk	serotype
1	SIMI-CN 2	A+I	0.05		dead	W1	
2	SIMI-CN 3	A	0.65		stop		AD
3	SIMI-CN 4	A	2.2		failure Tx		AD
4	SIMI-CN 5	A+I	1.025	W2	success Tx		AD
5	SIMI-CN 6	A+I	0.05		dead		AD
6	SIMI-CN 7	A+I	2.11		failure Tx		AD
7	SIMI-CN 8	A+I	1.3		excluded		AD
8	SIMI-CN 9	A	1.54	W2	success Tx		AD
9	SIMI-CN 10	A	0.06		dead	W1	AD
10	SIMI-CN 11	A+I	0.24		stop		AD
11	SIMI-CN 12	A+I	0.05		excluded		AD
12	SIMI-CN 13	A+I	0.05		dead	W1	AD
13	SIMI-CN 14	A+I	2.485		failure Tx		AD
14	SIMI-CN 15	A	1.92		failure Tx		AD
15	SIMI-CN 16	A	1.15	W2	success Tx		AD
16	SIMI-CN 17	A+I	1.35	W4	success Tx		BC
17	SIMI-CN 18	A+I	2.325	W8	success Tx		AD
18	SIMI-CN 19	A+I	0.4		stop		AD
19	SIMI-CN 20	A	0.52		dead	W2	AD
20	SIMI-CN 21	A+I	1.125	W3	success Tx		AD
21	SIMI-CN 22	A+I	0.2		dead	W1	AD

Table 1 (cont.)

No	Lab no.	Regimen	Total dose of Am (g)	First CSF cul Neg at wk	pt. status	dead at wk	Serotype
22	SIMI-CN 23	A+I	2.15		failure Tx		AD
23	SIMI-CN 24	A	0.12		dead	W1	AD
24	SIMI-CN 25	A+I	1.05		dead	W1	AD
25	SIMI-CN 26	A+I	0.64		excluded		AD
26	SIMI-CN 27	A+I	1.24	W1	success Tx		AD
27	SIMI-CN 28	A	0.76		excluded		AD
28	SIMI-CN 29	A	2		success Tx		AD
29	SIMI-CN 30	A+I	0.64		stop		AD
30	SIMI-CN 31	A+I	1.15	W3	success Tx		AD
31	SIMI-CN 32	A+I	1.2		dead	W4	AD
32	SIMI-CN 33	A	0.12		dead	W1	AD
33	SIMI-CN 34	A+I	1	W1	success Tx		AD
34	SIMI-CN 35	A+I	0.17		dead	W1	AD
35	SIMI-CN 36	A	0.76		dead	W3	AD
36	SIMI-CN 37	A	1.42		dead	W5	AD
37	SIMI-CN 38	A+I	0		excluded		AD
38	SIMI-CN 39	A+I	1.6	W2	success Tx		AD
39	SIMI-CN 40	A+I	1.76	W4	success Tx		AD
40	SIMI-CN 41	A	0.55		stop		AD
41	SIMI-CN 42	A	1.225	W2	success Tx		AD
42	SIMI-CN 43	A+I	1.52	W3	success Tx		AD
43	SIMI-CN 44	A+I	0.98		dead	W4	AD
44	SIMI-CN 45	A	2.42		failure Tx		AD
45	SIMI-CN 46	A+I	2.5		failure Tx		AD
46	SIMI-CN 47	A+I	1.71	W5	success Tx		AD
47	SIMI-CN 48	A	0.04		dead	W1	AD
48	SIMI-CN 49	A	1.35	W5	success Tx		AD

Table 1 (cont.)

No	Lab no.	Regimen	Total dose of Am (g)	First CSF cul Neg at wk	pt. status	dead at wk	Serotype
49	SIMI-CN50	A+I	1.49	W3	success Tx		AD
50	SIMI-CN 51	A+I	0.1		dead		AD
51	SIMI-CN 52	A+I	2	W5	success Tx		AD
52	SIMI-CN 53	A	2.55		failure Tx		AD
53	SIMI-CN 54	A+I	1.96	W6	success Tx		AD
54	SIMI-CN 55	A+I	2.08	W6	success Tx		AD
55	SIMI-CN 56	A	2.1		failure Tx		AD
56	SIMI-CN 57	A+I	2.22		failure Tx		AD
57	SIMI-CN 58	A+I	0.05		dead		AD
58	SIMI-CN 59	A+I	0.12		dead		AD
59	SIMI-CN 60	A+I	0.04		excluded		AD
60	SIMI-CN 61	A	0.71		dead		AD
61	SIMI-CN 62	A	1.32	W1	success Tx		AD
62	SIMI-CN 63	A	1.83		failure Tx		AD
63	SIMI-CN 64	A+I	0.87	W5	success Tx		AD
64	SIMI-CN 65	A+I	0.825		dead		AD
65	SIMI-CN 66	A+I	1.64	W8	success Tx		AD
66	SIMI-CN 67	A	2.02		failure Tx		AD
67	SIMI-CN 68	A	0.24		dead		AD
68	SIMI-CN 69	A+I	1.23	W3	success Tx		AD
69	SIMI-CN 70	A+I	0.08		excluded		AD
70	SIMI-CN 71	A+I	2		failure Tx		AD
71	SIMI-CN 72	A+I	1.5	W7	success Tx		AD
72	SIMI-CN 73	A	0.04		dead		AD
73	SIMI-CN 74	A+I	0.16		dead		AD
74	SIMI-CN 75	A+I	0.96		excluded		AD
75	SIMI-CN 76	A	0.04		excluded		AD

Table 1 (cont.)

No	Lab no.	Regimen	Total dose of Am (g)	First CSF cul Neg at wk	pt. status	dead at wk	Serotype
76	SIMI-CN 77	A+I	0.03		excluded		AD
77	SIMI-CN 78	A+I	0.28		dead	W1	AD
78	SIMI-CN 79	A	1.18	W5	success Tx		AD
79	SIMI-CN 80	A	0.76		excluded		AD
80	SIMI-CN 81	A+I	1.95	W3	success Tx		AD
81	SIMI-CN 82	A+I	0.54		dead	W3	AD
82	SIMI-CN 83	A	0.68		dead	W3	AD
83	SIMI-CN 84	A+I	1.02	W4	success Tx		AD
84	SIMI-CN 85	A+I	1.14	W3	success Tx		AD
85	SIMI-CN 86	A+I	0.32		dead	W2	AD
86	SIMI-CN 87	A+I	0.04		excluded		AD
87	SIMI-CN 88	A	2		failure Tx		AD
88	SIMI-CN 89	A	2.025		failure Tx		AD
89	SIMI-CN 90	A+I	1.5	W4	success Tx		AD
90	SIMI-CN 91	A	0.3		excluded		AD
91	SIMI-CN 92	A	1.2		excluded		AD
92	SIMI-CN 93	A+I	0.21		excluded		AD
93	SIMI-CN 94	A	2		failure Tx		AD
94	SIMI-CN 95	A+I	1.2	W5	success Tx		AD
95	SIMI-CN 96	A	0.08		excluded		AD
96	SIMI-CN 97	A+I	0.67		excluded		AD
97	SIMI-CN 98	A	1.14	W4	success Tx		AD
98	SIMI-CN 99	A+I	1.72	W4	success Tx		AD
99	SIMI-CN 100	A	0.64		excluded		AD
100	SIMI-CN 101	A+I	1.36	W6	success Tx		AD
101	SIMI-CN 102	A+I	1.21	W3	success Tx		AD
102	SIMI-CN 103	A+I	0.98	W5	success Tx		AD

Table 1 (cont.)

No	Lab no.	Regimen	Total dose of Am (g)	First CSF cul Neg at wk	pt. status	dead at wk	Serotype
103	SIMI-CN 104	A+I	0.03		dead	W1	AD
104	SIMI-CN 105	A	2.05		failure Tx		AD
105	SIMI-CN 106	A	1.29	W4	success Tx		AD
106	SIMI-CN 107	A	2.025		failure Tx		AD
107	SIMI-CN 108	A+I	0.93	W3	success Tx		AD
108	SIMI-CN 109	A	1.44	W4	success Tx		AD
109	SIMI-CN 110	A	2.07		failure Tx		AD
110	SIMI-CN 111	A+I	0.84	W4	success Tx		AD
111	SIMI-CN 112	A+I	2.14	W7	success Tx		AD
112	SIMI-CN 113	A	0.64		dead	W4	AD
113	SIMI-CN 114	A+I	1.135	W7	success Tx		AD
114	SIMI-CN 115	A+I	0.33		dead	W2	AD
115	SIMI-CN 116	A	0.28		dead	W1	AD
116	SIMI-CN 117	A+I	0		dead	W1	AD
117	SIMI-CN 118	A	0.2		dead	W1	AD
118	SIMI-CN 119	A+I	1.145		failure Tx		AD
119	SIMI-CN 120	A	0.32		dead	W2	AD
120	SIMI-CN 121	A+I	0.8		excluded		AD
121	SIMI-CN 122	A	1.37	W5	success Tx		AD
122	SIMI-CN 123	A	1.17		failure Tx		AD
123	SIMI-CN 124	A	0.2		excluded		AD
124	SIMI-CN 125	A	2.08		failure Tx		AD
125	SIMI-CN 126	A+I	0.03		excluded		AD
126	SIMI-CN 127	A	0.08		excluded		AD
127	SIMI-CN 128	A	1.17	W3	success Tx		AD
128	SIMI-CN 129	A+I	2.04	W5	success Tx		AD
129	SIMI-CN 130	A+I	0.87		dead	W5	AD

Table 1 (cont.)

No	Lab no.	Regimen	Total dose of Am (g)	First CSF cul Neg at wk	pt. status	dead at wk	Serotype
130	SIMI-CN 131	A	0.15		dead	W1	AD
131	SIMI-CN132	A	0.125		dead	W1	AD
132	SIMI-CN 133	A	1.75		failure Tx		AD
133	SIMI-CN 134	A	0.9		dead	W5	AD
134	SIMI-CN 135	A+I	1.83	W6	success Tx		AD
135	SIMI-CN 136	A+I	0.04		dead	W1	AD
136	SIMI-CN 137	A	0.03		excluded		AD
137	SIMI-CN 138	A	2.01		failure Tx	W10	AD
138	SIMI-CN 139	A	1.36		dead	W7	AD
139	SIMI-CN 140	A	0.03		excluded		AD
140	SIMI-CN 141	A	1.99		failure Tx		AD
141	SIMI-CN 142	A+I	1.2	W2	success Tx		AD
142	SIMI-CN 143	A	1.94		failure Tx		AD
143	SIMI-CN 144	A+I	1.01	W3	success Tx		AD
144	SIMI-CN 145	A	1.2	W2	success Tx		AD
145	SIMI-CN 146	A	0.69		excluded		AD
146	SIMI-CN 147	A+I	1.07	W4	success Tx		AD
147	SIMI-CN 148	A	0.6		stop		AD
148	SIMI-CN 149	A	0.03		dead	W1	AD
149	SIMI-CN 150	A	0.03		excluded		AD
150	SIMI-CN 151	A+I	1.56	W3	success Tx		AD
151	SIMI-CN 152	A	0.77	W2	success Tx		AD
152	SIMI-CN 153	A+I	0.56	W1	success Tx		AD
153	SIMI-CN 154	A	0		dead	W1	AD
154	SIMI-CN 155	A+I	1.46	W4	success Tx		AD
155	SIMI-CN 156	A+I	1.17	W4	success Tx		AD
156	SIMI-CN 157	A+I	1.51	W4	success Tx		AD
157	SIMI-CN 158	A	1.57	W5	success Tx		AD
158	SIMI-CN 159	A	1.36	W4	success Tx		AD
159	SIMI-CN 160	A+I	1.08	W3	success Tx		AD
160	SIMI-CN 161	A	0.4		dead	W2	AD

A = Amphotericin B, I = Itraconazole

Table 2 : Information of all patients included in prophylaxis period.

No.	Lab No.	Month of followed of Laboratory investigation									Drug
		M1	M2	M4	M6	M7	M8	M9	M11	M12	
1	SIMI-CN 5					NG, {-}, (0-1)				NG (-)	A+I
2	SIMI-CN 9					NG, (0-1)					A
3	SIMI-CN 16					NG, (-)		NG, (-)			A
4	SIMI-CN 17				NG (0-1)						A+I
5	SIMI-CN 18	NG (1-2)			NG (1-2)	NG, (0-1)					A+I
6	SIMI-CN 21			NG (0-1)		NG, {-}					A+I
7	SIMI-CN 27					NG, {-}, (5-10)			NG (-)	NG (-)	A+I
8	SIMI-CN 31					NG, (30-50)					A+I
9	SIMI-CN 42					NG, (-)					A
10	SIMI-CN 50	NG (0-1)				NG, (-)					A+I
11	SIMI-CN 52		NG, (1-2)								A+I
12	SIMI-CN 55	NG (0-2)				NG, {-}, (-)					A+I
13	SIMI-CN 62						NG, (-)				A
14	SIMI-CN 66		num, {+}, (30-50)								A+I
15	SIMI-CN 72					NG, (0-1)				NG (-)	A+I

Table 2 (Cont.)

No.	Lab No.	Month of followed of Laboratory investigation									Drug
		M1	M2	M4	m6	M7	M8	M9	M11	M12	
16	SIMI-CN 79	NG (-)				NG (-)					A
17	SIMI-CN 84					NG, {-}, (-)					A+I
18	SIMI-CN 85	NG {-} (5-10)									A+I
19	SIMI-CN 90					NG, {-} (0-1)				NG, (-)	A+I
20	SIMI-CN 99					NG, (-)				NG, (-)	A+I
21	SIMI-CN 101					NG, (-)					A+I
22	SIMI-CN 102	NG, (2-4)				NG, (0-1)					A+I
23	SIMI-CN 103					num, H+					A+I
24	SIMI-CN 106					NG, (-)	NG, (-)				A
25	SIMI-CN 108						NG, {-}, (-)				A+I
26	SIMI-CN 109	num (0-1)									A
27	SIMI-CN 112		NG (0-1)				NG, (0-1)				A+I
28	SIMI-CN 122					NG. {-} (0-1)					A
29	SIMI-CN 151	NG (1-2)					NG, (-)				A+I

num = numerouse, NG = No growth for CSF culture, ( ) = a moint of encapsulated yeast cells per high power field under light microscopic by India ink preparation, { } = the result of specific band 343 bp by PCR, h = hemoculture, + = positive, - = negative, A = Amphotericin B, I = Itraconazole

Table 3 : The *in vitro* MIC value by E test method of the isolation of *C. neoformans*

No.	Lab No.	MIC of Amphotericin B		MIC of Itraconazole	
		48 hour	72 hour	48 hour	72 hour
SUCCESS PATIENTS					
1	SIMI-CN 5 Do	0.047	0.047	0.125	0.125
2	SIMI-CN 9 Do	0.19	0.19	1.0	1.0
3	SIMI-CN 16 Do	0.25	0.25	1.0	1.0
4	SIMI-CN 16 W1	0.064	0.064	0.25	0.25
5	SIMI-CN 17 Do	0.064	0.0.64	0.75	0.75
6	SIMI-CN 17 W1	0.19	0.19	0.25	0.25
7	SIMI-CN 17 W2	0.25	0.25	0.50	0.50
8	SIMI-CN 18 Do	0.008	0.008	0.094	0.094
9	SIMI-CN 18 W1	0.032	0.032	0.047	0.047
10	SIMI-CN 18 W2	0.064	0.064	0.125	0.125
11	SIMI-CN 18 W3 (1)	0.19	0.19	0.50	0.50
12	SIMI-CN 18 W3 (2)	0.094	0.094	1.0	1.0
13	SIMI-CN 21 Do	0.094	0.094	1.0	1.0
14	SIMI-CN 21 W1	0.15	0.15	0.50	0.50
15	SIMI-CN 27 D0	0.094	0.094	0.094	0.094
16	SIMI-CN 29 Do	0.25	0.19	0.25	0.25
17	SIMI-CN 29 W1	0.094	0.094	0.25	0.25
18	SIMI-CN 29 W2	0.25	0.25	0.50	0.50
19	SIMI-CN 29 W3	0.125	0.125	0.25	0.25
20	SIMI-CN 29 W4	0.19	0.125	0.125	0.125
21	SIMI-CN 29 W5	0.125	0.125	0.25	0.25
22	SIMI-CN 29 W6	0.094	0.094	0.19	0.19
23	SIMI-CN 31 Do	0.064	0.064	1.0	1.0
24	SIMI-CN 31 W1	0.25	0.25	0.50	0.50
25	SIMI-CN 31 W2	0.094	0.094	0.38	0.38

Table 3 (Cont.)

No.	Lab No.	MIC of Amphotericin B		MIC of Itraconazole	
		48 hour	72 hour	48 hour	72 hour
26	SIMI-CN 40 W1	0.19	0.19	0.50	0.50
27	SIMI-CN 47 Do	0.19	0.19	0.38	0.38
28	SIMI-CN 47 W1	0.125	0.125	0.38	0.38
29	SIMI-CN 47 W2	0.50	0.50	0.50	0.50
30	SIMI-CN 47 W3	0.19	0.19	0.50	0.50
31	SIMI-CN 47 W7	0.19	0.19	1.0	1.0
32	SIMI-CN 49 Do	0.19	0.19	1.0	1.0
33	SIMI-CN 50 W2	0.50	0.50	0.75	0.75
34	SIMI-CN 54 Do	0.19	0.19	0.10	0.10
35	SIMI-CN 54 W1	0.19	0.19	0.19	0.19
36	SIMI-CN 54 W3	0.125	0.125	0.50	0.50
37	SIMI-CN 54 W5	0.125	0.125	0.125	0.125
38	SIMI-CN 55 W1	0.19	0.19	0.132	0.032
39	SIMI-CN 55 W2	0.50	0.50	0.75	0.75
40	SIMI-CN 55W3	0.25	0.25	0.75	0.75
41	SIMI-CN 55 W4	0.25	0.25	0.10	0.10
42	SIMI-CN 64 W1	0.25	0.25	0.25	0.25
43	SIMI-CN 64 W3	0.19	0.19	0.125	0.125
44	SIMI-CN 69 Do	0.064	0.064	0.50	0.50
45	SIMI-CN 95 W2	0.125	0.125	0.38	0.38
46	SIMI-CN 99 W2	0.19	0.19	1.0	1.0
47	SIMI-CN 99 W3	0.125	0.125	0.38	0.38
48	SIMI-CN 101 W1	0.094	0.094	0.064	0.064
49	SIMI-CN 101 W2	0.064	0.064	0.50	0.50
50	SIMI-CN 108 W1	0.125	0.125	0.75	0.75
51	SIMI-CN 111 Do	0.19	0.19	0.75	0.75

Table 3 (Cont.)

No.	Lab No.	MIC of Amphotericin B		MIC of Itraconazole	
		48 hour	72 hour	48 hour	72 hour
52	SIMI-CN 111 W1	0.38	0.38	0.125	0.125
53	SIMI-CN 111 W2	0.50	0.50	0.125	0.125
54	SIMI-CN 114 W4	0.125	0.125	0.006	0.006
55	SIMI-CN 122 W1	0.25	0.25	1.0	1.0
56	SIMI-CN 122 W5	0.125	0.125	0.19	0.19
57	SIMI-CN 129 W1	0.064	0.064	1.0	1.0
58	SIMI-CN 138 W1	0.38	0.38	0.19	0.19
59	SIMI-CN 142 Do	0.023	0.023	0.75	0.75
60	SIMI-CN 144 Do	0.19	0.19	0.75	0.75
61	SIMI-CN 147 W1	0.094	0.094	0.25	0.25
62	SIMI-CN 150 Do	0.032	0.032	0.008	0.008
63	SIMI-CN 150 W1	0.064	0.064	1.0	1.0
64	SIMI-CN 151 Do	0.19	0.19	0.38	0.38
65	SIMI-CN 152 Do	0.19	0.19	0.38	0.38
DEAD PATIENTS					
66	SIMI-CN 32 W2	NG	NG	NG	NG
67	SIMI-CN 33 Do	0.125	0.125	0.38	0.38
68	SIMI-CN 44 W2	0.125	0.125	0.50	0.50
69	SIMI-CN 48 Do	0.25	0.25	1.0	1.0
70	SIMI-CN 61 Do	0.25	0.25	1.5	1.5
71	SIMI-CN 61 W1	0.064	0.064	1.0	1.0
72	SIMI-CN 65 Do	0.19	0.19	0.50	0.50
73	SIMI-CN 68 Do	0.032	0.032	0.064	0.064
74	SIMI-CN 113 W3	0.094	0.094	0.25	0.25

Table 3 (Cont.)

No.	Lab No.	MIC of Amphotericin B		MIC of Itraconazole	
		48 hour	72 hour	48 hour	72 hour
FAILURE PATIENTS					
75	SIMI-CN 94 W1	0.25	0.25	0.19	0.19
76	SIMI-CN 105 W9	a few amount of colonies of *C. neoformans*			
77	SIMI-CN 110 W7	0.064	0.064	0.023	0.023
78	SIMI-CN 123 W6	0.032	0.032	0.016	0.016
79	SIM -CN 133 W3	0.032	0.032	0.003	0.003
80	SIMI-CN 133 W5	0.032	0.032	0.023	0.023
81	SIMI-CN 138 W1	0.38	0.38	0.19	0.19
82	SIMI-CN 143 W1	0.19	0.19	0.125	0.125
83	SIMI-CN 143 W4	0.19	0.19	0.50	0.50
RELAPSE PATIENTS					
84	SIMI-CN 66 W1	0.25	0.25	0.50	0.50
85	SIMI-CN 66 W2	0.19	0.19	0.032	0.032
86	SIMI-CN 66 W4	0.012	0.012	0.032	0.032
87	SIMI-CN 66 W5	0.25	0.25	0.50	0.50
88	SIMI-CN 103 W1	0.19	0.19	1.0	1.0
89	SIMI-CN 103 W2	0.19	0.19	0.004	0.004
90	Control ATCC 22019	0.125	0.125	0.016	0.38

Commercial latex agglutination kits for cryptococcal polysaccharide are :

1. Crypto-LA : International Biological Laboratories, Cranbury, N.J., USA.
2. CALAS : Meridian Diagnostics, Cincinnati, Ohio, USA.
3. Myco-Immune : American MicroScan, Rahway, N.J., USA.
4. IMMY : Immuno-mycologies, Norman, Okla, USA.
5. A kit is made by Sanofi Diagnostics Pasteur, Marnes la Cogvetti, France.

BIOGRAPHY

NAME	Mrs. Anurak Munsrichoom
DATE OF BIRTH	23 Aug 1965
PLACE OF BIRTH	Nakornrachasima, Thailand
INSTITUTIONS ATTENDED	Khonkaen University, 1984-1988 B. Sc. (Medical Technology) Mahidol University, (1995-2000) M. Sc. (Microbiology)
OFFICE & POSITION	Microbiology Laboratory, Department of Pathology, Queen Sirikit National Institute of Child Health, Bangkok, Thailand. Position : Medical Technologist.