

**EVALUATION OF
CRYPTOCOCCUS NEOFORMANS ANTIBODY
IN CRYPTOCOCCOSIS BY IMMUNOFLUORESCENCE**



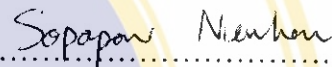
**A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE
(CLINICAL PATHOLOGY)
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Thesis
entitled
**EVALUATION OF
CRYPTOCOCCUS NEOFORMANS ANTIBODY
IN CRYPTOCOCCOSIS BY IMMUNOFLUORESCENCE**

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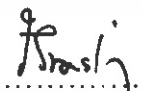
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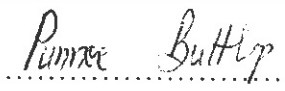
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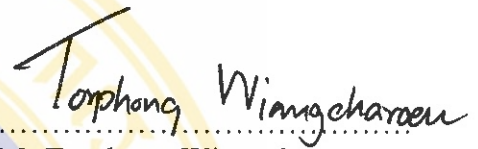
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**EVALUATION OF
CRYPTOCOCCUS NEOFORMANS ANTIBODY
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was submitted to the Faculty of Graduate Studies, Mahidol University
For the degree of Master of Science (Clinical Pathology)

on
20 March 2007



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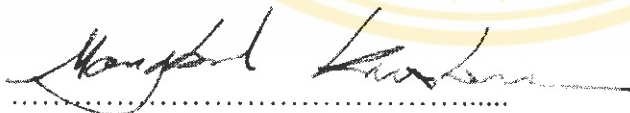
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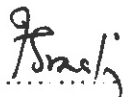
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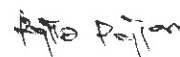
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Torphong Wiangcharoen

EVALUATION OF *CRYPTOCOCCUS NEOFORMANS* ANTIBODY IN CRYPTOCOCCOSIS BY IMMUNOFLUORESCENCE**TORPHONG WIANGCHAROEN 4536252 RACP/M****M.Sc. (CLINICAL PATHOLOGY)****THESIS ADVISORS : SOPAPORN NIEMHOM, Ph.D., MONGKOL KUNAKORN, M.D., KUMTHORN MALATHUM, M.D.****ABSTRACT**

Cryptococcosis is a common opportunistic infection in patients with late-stage HIV infection and occurs worldwide without any defined endemic areas. The clinical presentations of cryptococcosis vary depending on the host and site(s) of infection. The most commonly involved organs are lungs and the central nervous system. The infection is commonly initiated by inhalation of *Cryptococcus neoformans* into the lung, causing pulmonary cryptococcosis. Discrimination of pulmonary cryptococcosis by clinical manifestations may be difficult from other types of pneumonitis. Laboratory diagnosis specific to *Cryptococcus neoformans* in combination with clinical symptoms is important for the early diagnosis and treatment of pulmonary cryptococcosis before dissemination of the disease which could develop to a more severe type of cryptococcosis. In cases of pulmonary infection, *Cryptococcus neoformans* is localized in the lung and could induce the host to produce a specific antibody which can be detected in the serum. In this study, indirect immunofluorescence for *Cryptococcus neoformans* antibody detection was developed and evaluated in patients with pulmonary cryptococcosis as well as in those with CNS cryptococcosis and extrapulmonary-nonmeningeal cryptococcosis. In 8 patients with pulmonary cryptococcosis, the titers of *Cryptococcus neoformans* specific antibody ranged from 1:40 to more than 1:1280 in the patient's sera. *Cryptococcus neoformans* antibody was not found in 10 patients with CNS cryptococcosis but found in 2 of the total of 8 cases with extrapulmonary-nonmeningeal cryptococcosis with the titer of 1:40 and 1:80. In 50 sera obtained from donated blood from healthy subjects, *Cryptococcus neoformans* antibody was found in 5 sera with the titer range from 1:80 to 1:320. The 5 sera that were positive for *Cryptococcus neoformans* antibody also gave positive results by tube agglutination assay with the titer of 1:2 and 1:4. The antibody by IF was not detected in the patients with diseases other than cryptococcosis. The sensitive and specific IF could be suitable for use in laboratory diagnosis, the test is valuable particularly in the purpose of early diagnosis of primary pulmonary cryptococcosis before the disease is disseminated to the central nervous system.

KEY WORDS : *Cryptococcus neoformans*/INDIRECT IMMUNOFLUORESCENCE/CRYPTOCOCCOSIS/*C. neoformans* ANTIBODY

การประเมินระดับของแอนติบอดีต่อเชื้อราคริปโตคอกคัสนีโอฟอร์แมนส์ในผู้ป่วยโรค
คริปโตคอกโคซิสโดยวิธีอิมมูโนฟลูออเรสเซนซ์ (EVALUATION OF
CRYPTOCOCCUS NEOFORMANS ANTIBODY IN
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บทคัดย่อ

โรคติดเชื้อราคริปโตคอกคัสนีโอฟอร์แมนส์เป็นโรคติดเชื้อแบบฉวยโอกาสในผู้ป่วยที่ติดเชื้อเอชไอวีระยะสุดท้ายและพบได้ทั่วโลก อวัยวะที่พบการติดเชื้อได้มากที่สุดคือปอดและระบบประสาทส่วนกลาง การติดเชื้อเริ่มจากการหายใจเอาเชื้อเข้าสู่ปอดเกิดการติดเชื้อที่ปอดเป็นการติดเชื้อแบบ pulmonary cryptococcosis การวินิจฉัยทางห้องปฏิบัติการร่วมกับการใช้อาการทางคลินิกมีความจำเป็นในการใช้วินิจฉัยโรคและให้การรักษาในระยะแรกของการติดเชื้อก่อนการแพร่กระจายของเชื้อสู่อวัยวะอื่นที่มีความรุนแรงของโรคมักขึ้น ผู้ป่วยที่ติดเชื้อราคริปโตคอกคัสนีโอฟอร์แมนส์ที่ปอดเชื้อจะถูกจำกัดบริเวณไว้ที่ปอดและเหนียวทำให้ร่างกายสร้างแอนติบอดีที่จำเพาะต่อเชื้อและสามารถตรวจพบได้ในซีรัม การศึกษาครั้งนี้ใช้วิธีอิมมูโนฟลูออเรสเซนซ์เพื่อการตรวจหาแอนติบอดีต่อเชื้อราคริปโตคอกคัสนีโอฟอร์แมนส์ในผู้ป่วยที่เป็นโรคติดเชื้อที่ปอด, ระบบประสาทส่วนกลาง และการติดเชื้อที่อวัยวะอื่นนอกปอดและระบบประสาทส่วนกลาง ผู้ป่วย 8 ราย ที่มีการติดเชื้อที่ปอดตรวจพบระดับของแอนติบอดีต่อเชื้อโดยวิธีอิมมูโนฟลูออเรสเซนซ์ โดยมีไโตเตอร์ 1:4 ถึง >1:1280 จากซีรัมของผู้ป่วย ผู้ป่วยที่มีการติดเชื้อในระบบประสาทส่วนกลาง 10 ราย ตรวจไม่พบแอนติบอดีต่อเชื้อ ผู้ป่วย 2 รายจาก 8 รายที่มีการติดเชื้อที่อวัยวะอื่นนอกปอดและระบบประสาทส่วนกลางตรวจพบระดับของแอนติบอดีไโตเตอร์ 1:40 และ 1:80 การตรวจหาระดับแอนติบอดีต่อเชื้อในกลุ่มผู้ป่วยจากเลือด 50 ราย พบ 5 รายมีระดับของแอนติบอดีไโตเตอร์ 1:80 ถึง 1:320 ตัวอย่าง 5 รายดังกล่าวให้ผลบวกของการตรวจหาแอนติบอดีโดยวิธี tube agglutination โดยมีระดับของแอนติบอดีที่ไโตเตอร์ 1:2 และ 1:4 กลุ่มผู้ป่วยที่ไม่ได้เป็นโรคจากการติดเชื้อราคริปโตคอกคัสนีโอฟอร์แมนส์ตรวจไม่พบแอนติบอดีต่อเชื้อด้วยวิธีอิมมูโนฟลูออเรสเซนซ์ การทดสอบด้วยวิธีอิมมูโนฟลูออเรสเซนซ์มีความไวและความจำเพาะเพียงพอสำหรับการนำมาใช้ในการตรวจทางห้องปฏิบัติการเพื่อการวินิจฉัยระยะแรกของการติดเชื้อราคริปโตคอกคัสนีโอฟอร์แมนส์ที่ปอดก่อนที่จะมีการแพร่กระจายเข้าสู่ระบบประสาทส่วนกลางและอวัยวะอื่นๆ

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CHAPTER I

INTRODUCTION

1. Background of the problem.

The incidence of infections caused by the encapsulated yeast *Cryptococcus neoformans* has raised markedly over the past 20 years as results of the HIV epidemic and increasing use of immunosuppressive therapies.¹ Cryptococcosis is a common opportunistic infection and AIDS-defining illness in patients with late-stage HIV infection, particularly in Southeast Asia and Southern and East Africa.^{2,3} Cryptococcal infections occur worldwide without any defined endemic areas. The clinical presentations of cryptococcosis vary depending on the hosts and site(s) of infection. The most commonly involved organs are lungs and central nervous system.⁴ Mortality from HIV-associated cryptococcal meningitis remains high (10-30%), even in developed countries, because of the inadequacy of current antifungal drugs in combination with the complication of raised intracranial pressure.⁵ The cryptococcosis has been accounted for 13-44% of all deaths in Cohort studies of HIV-infected patients from sub-Saharan Africa.⁶⁻⁸

This fungus is ubiquitous, being commonly found in aged bird droppings but also in soil and decayed woods.⁹ *Cryptococcus neoformans* is a encapsulated round to oval yeast like fungus measuring 4-6 microns characteristic with polysaccharide capsule of variable thickness of 1-30 microns surrounding. In its natural environment the capsule is thinner and the yeast is smaller, while thicker capsule tends to be found from infected tissue. The major capsular polysaccharide, glucuronoxylomannan, the phenoloxidase enzyme and the organisms' ability to grow at 37°C have been considered to be the virulence factors of *Cryptococcus neoformans*.⁹ Not being part of

the normal microbial flora of human, *Cryptococcus neoformans* is only transiently isolated from persons with no pathologic feature.¹⁰ It is generally accepted that the organisms enter into the hosts by the respiratory route in the form of dehydrated haploid yeasts or basidiospores. The infection is commonly initiated by inhalation of the organisms into lung which leads to pulmonary cryptococcosis. Most primary infections have no clinical symptoms and are usually discovered only by routine chest X-ray. When present, symptoms include chronic cough, low grade fever and pleuritic pain. The primary infections may remain localize in the lung or may be disseminated, especially in immunocompromised or immunosuppressive hosts, to central nervous system, leading to central nervous system cryptococcosis.¹¹ This type of life-threatening cryptococcosis has three subtypes consisting of meningitis, meningoencephalitis, and cryptococcomas of which meningitis is the most common and diagnosed cases. In some patients with central nervous system cryptococcosis, infections may be extensively disseminated throughout the body involving visceral organs as well as lymph nodes. While cryptococcal meningitis is the most common entity of cryptococcosis, primary pulmonary cryptococcosis, which is the initial stage in the pathogenesis of central nervous system, is mostly left undiagnosed until the disease progress to life threatening type.¹² In addition, patients with chronic lung disease, such as bronchitis and bronchiectasis, may also have asymptomatic colonization, with *Cryptococcus neoformans* being isolated from their sputum over years. In some cases, subclinical cryptococcus infections may be a result of environmental exposure. Normal individuals may experience a self-limiting pneumonia with accompanying sensitization. In some patients, especially in immunocompromised and immunodeficient patients, invasive pulmonary cryptococcosis might occur when primary infections may not readily resolved leading to a more chronic pneumonia progressing slowly over several years.

Diagnosis of pulmonary cryptococcosis by clinical may be difficult to discriminate from other types of pneumonitis. Laboratory diagnosis specific to *Cryptococcus neoformans* infection in combination with clinical symptom seems to be important for the early diagnosis and treatment of pulmonary cryptococcosis before dissemination of the disease. In case of pulmonary infection, *Cryptococcus neoformans* is localized in the lung and it could induce the host to produce specific

antibody which can be detected in the serum.¹³ In contrast to the detection of antigen in circulation, this is mostly failed in patient with primary pulmonary cryptococcosis since the host would form the granulomatous tissue surrounding the pathogens.¹³ Although the granulomas can be detected by chest X-ray but the differential diagnosis from the other types of pulmonary infection is impossible. Nowadays, there are three methods for detection of *Cryptococcus neoformans* specific antibodies, the tube agglutination test, indirect immunofluorescence test¹⁴ and enzyme linked immunosorbant assay.¹⁵ The tube agglutination method is less sensitive and the agglutination reaction may be interpreted after three or even five days of the assay. The indirect immunofluorescence technique is specific and superior to the tube agglutination in sensitivity. Furthermore it needs only two or three hours for semi-quantitative test which is important for early diagnosis and treatment including monitoring during and after treatment. Enzyme linked immunosorbant assay is highly sensitive and specific, but it is time consuming, laborious and not a conventional method for semi-quantitative test for antibody detection.

Other localized *Cryptococcus neoformans* infections including osseous cryptococcosis could be diagnosed by the sensitive and specific assay for *Cryptococcus neoformans* antibody such as indirect immunofluorescence which gave positive results earlier than culture. *Cryptococcus neoformans* specific antibody in the patients may be the indicator of better prognosis comparing to patients with no antibody detection since many biological effects of the antibodies including the ability to fix complements, promote opsonization, facility of antibody-dependent cellular cytotoxicity as well as increase cryptococcal killing by macrophages and relative avidity.¹⁶ In this study, indirect immunofluorescence for *Cryptococcus neoformans* antibody detection will be developed and evaluated in patients with pulmonary cryptococcosis, cryptococcal meningitis and disseminated cryptococcosis

CHAPTER II

OBJECTIVES

1. To develop immunofluorescent test for the detection of *Cryptococcus neoformans* antibody in serum.
2. To detect *Cryptococcus neoformans* antibody by immunofluorescence in three types of cryptococcosis (pulmonary cryptococcosis, central nervous system cryptococcosis, and extrapulmonary-nonmeningeal cryptococcosis).

CHAPTER III

LITERATURE REVIEW

3.1 *Cryptococcus neoformans*

Cryptococcus neoformans is an environmental saprophyte and an encapsulated fungal organism (figure3-1) that can cause disease in apparently immunocompetent, as well as immunocompromised, host.^{17, 18} The rarity of its isolation as a human commensal and of human to human transmission suggests that human infection is an accidental dead-end event in its life cycle. *C. neoformans* is uniquely amongst the thousand of basidiomycete fungi. Under selective pressure in the environment, it has evolved a number of characteristics that enable them to survive within human and other mammalian and avian host.²⁰ Sexual reproduction between a- and α -mating type isolates of *C. neoformans* has been described in the laboratory, with the formation of hyphae and following meiosis, basidiospores, but in the environment and in clinical specimens *C. neoformans* is found as a budding yeast. The yeasts are spherical to oval cells, 5-10 μm in diameter, with polysaccharide capsule which is a major virulence factor and the substrate detected by cryptococcal antigen tests.

Two varieties and five serotypes of *C. neoformans* were recognized but the varieties have now been accorded species status: *var. neoformans*, now *C. neoformans* (serotype A, D and AD; based on capsular polysaccharide antigen), and *var. gattii*, now *C. gattii*, (serotype B and C). Most clinical isolates are serotype A. *C. neoformans* is found worldwide in association with soil contaminated with pigeon or chicken excreta and usually causes infections in immunosuppressed individuals.^{17, 19} *C. gattii*, on the other hand, is found primarily in tropical and subtropical regions, has been associated with several species of eucalyptus tree and causes infection predominantly

in apparently immunocompetent individuals. In addition, studies from South America have reported both species found in the decaying heartwood of a number of trees.²¹ This may be an important ecological niche suggested by the presence of a laccase enzyme in both species that could be involved in the breakdown of lignin and the fact that *C. neoformans* and *C. gattii* are closely related to other wood-rotting fungi.²² Serological surveys suggest that most individuals are exposed to the organism, starting after the first 2 years of life.²³ However, the precise circumstances and frequency of exposure like the ecology of the organism, are still not completely understood.

Before the AIDS epidemic, the incidence of cryptococcosis in the USA was less than one case per million persons per year. In the 1980s, cryptococcosis emerged as an important opportunistic infection among persons with AIDS, occurring in 5-10% of AIDS patients in the USA, Europe and Australia. In Southeast Asia and Africa, cryptococcosis appears to be relatively more common as an AIDS-related infection than it ever was in Europe or North America. In Thailand, cryptococcosis was accounted for 19% of AIDS-defining illness between 1994 and 1998.³ In Uganda, the incidence of cryptococcal disease in patients with CD4 counts < 200 cells/ μ l was estimated at 10.3 cases per 100 persons per years of follow-up cases.⁶ It seems most likely that the high incidence of cryptococcosis in parts of Africa and Asia reflects differences in exposure rather than host susceptibility or cryptococcal strain virulence, although no studies have addressed this issue. As a consequence of the epidemiology of meningitis; cryptococcal meningitis is now the leading cause of community acquired meningitis, ahead tuberculous- and bacterial meningitis in South Africa.²⁵⁻²⁶

Other groups at risk of cryptococcosis are patients with sarcoidosis, lymphoproliferative disorders and those on immunosuppressive therapy. In a series of 306 HIV-seronegative patients with cryptococcosis, the predisposing condition were steroid use (28%), organ transplant (18%), chronic organ failure (liver, lung, kidney) (18%), malignancy (18%) and rheumatological disease (13%).²⁷ Twenty-two percent of patients had no identified predisposing factor. Infection in apparently immunocompetent patients is also a feature of *C. gattii* cryptococcosis in Australia and Southeast Asia. Increasing use of organ transplantation and developments in immunosuppressive therapies for cancers and other systemic diseases since the 1970s

has led to an increase in the incidence in non-HIV-associated cryptococcosis in the USA to up to 1 case per 100,000 persons per years in the 1990s.²⁸

Most susceptible to infection are patients with T-cell deficiencies.^{17, 18} If untreated, cryptococcal meningoencephalitis is 100% fatal, and even when treated with the most effective antifungal drugs, cryptococcal infections can be fatal if the host does not have adequate T-cell-dependent immune function¹⁸.

To be classified as a pathogen, an organism must be able to cause infection under certain conditions. By this definition, *C. neoformans* can certainly be classified as a pathogen. Because the immunodeficient are more susceptible to infection with this yeast-like organism than the immunocompetent, *C. neoformans* is frequently referred to as an opportunistic pathogen. The factors that make *C. neoformans* a pathogen can be divided into two major groups. The first comprises the basic characteristics needed to establish an infection and survive in the human host; the second comprises the virulence factors that affect the degree of pathogenicity.

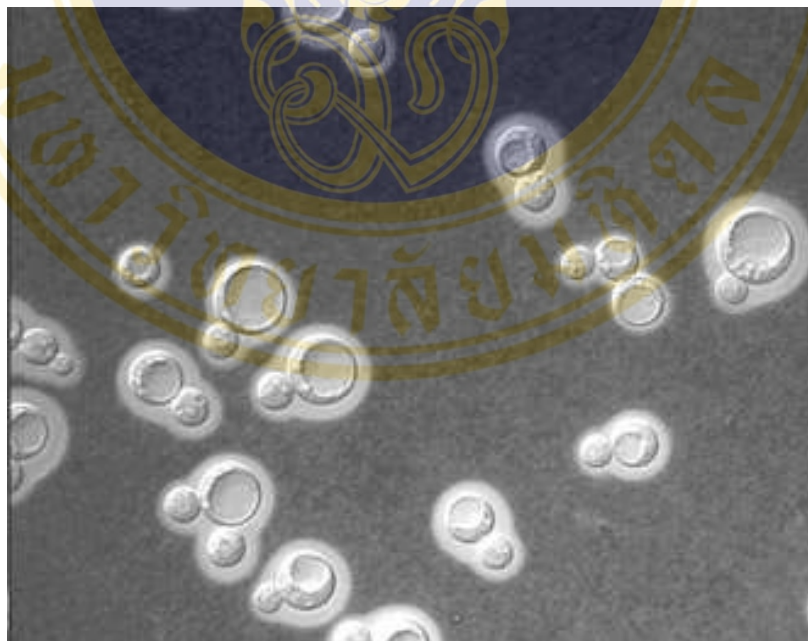


Figure 3-1 India ink preparation of *C. neoformans* showing the characteristic of polysaccharide capsule and budding.

3.2 Pathogenesis

It is generally accepted that the organism enters the host by the respiratory route in the form of dehydrated haploid yeasts or as basidiospores. After sometime in the lungs, the organism hematogenously spreads to extrapulmonary tissues; since it has a predilection for the brain, infected persons usually contract meningoencephalitis¹⁷. The primary pulmonary infection is frequently asymptomatic and may be eradicated or contained within granulomata, depending on host factors, inoculum and possibly isolate virulence. The severe pulmonary cryptococcosis can resemble PCP, with symptom such as coughing, fatigue, fever and shortness of breath. After dissemination of the cryptococcal organisms either acutely or after a period of latency to extrapulmonary sites, other symptom can occur. The commonest manifestation (seen in two-thirds of cases of cryptococcosis) is a particular predilection for the brain, inflammation of the membranes surrounding the brain and spinal cord – cryptococcal meningitis.

Goldman et al.³⁰ have described the pathogenesis of pulmonary *C. neoformans* infection in a murine model that examined the lungs at various times after intratracheal inoculation. Infected rat did not appear ill, and none manifested signs or respiratory distress. *C. neoformans* were in the lung parenchyma 2 hours postinoculation, indicative of rapid penetration at the level of alveoli, and glucurunoxylomannan (GXM) antigen was present in surrounding tissue shortly thereafter. Serum GXM levels, despite extensive pulmonary infection, were low. Despite initial deposition of yeasts in the alveoli, organisms and inflammation were subsequently localized to the peribronchiolar regions such that about day 7 the histologic picture was that of a bronchiolar pneumonia. Extrapulmonary dissemination occurred early in the course of infection. However, the extrapulmonary burden was low despite extensive pulmonary involvement. Containment and reduction of *C. neoformans* infection in the lungs appear to depend largely on an effective cellular immune response. Two phases of host cellular inflammatory response were discernible: early local macrophage recruitment at 2 to 4 days followed by granulomatous inflammation, which reached maximum intensity 14 days after infection. The granulomatous phase was preceded by lymphocyte influx with macrophage proliferation and maturation into epithelioid

histiocytes; this was paralleled by a shift of yeast from extracellular to intracellular spaces. Serum antibodies response to GXM included IgM and IgG. IgM was transient and no significant IgM was detected subsequently. *C. neoformans* infection elicited IgG to GXM in most rats, such that some rats had a greater than threefold increase in IgG titer on day 7. In contrast to IgM, serum IgG persisted and there was an upward trend in IgG titer, with three of five rats having ninefold increase in IgG to GXM at day 25 relative day 2. Tissue IgG deposits, serum IgG to GXM and localization of tissue GXM immunoreactivity to epithelioid cells were noted at 2 to 4 weeks. A 10-fold decrease in lung fungal burden occurred 25 days postinfection and was associated with resolving granulomas, fewer proliferating cells, and decreased tissue GXM.

Blackstock et al.¹² studied immune responses induced by two *C. neoformans* isolates in mice that while the virulent isolate induced CMI response as detected by delayed type hypersensitivity (DTH) reactivity and a T helper 1 (Th1) cytokine profile, these responses appeared early after intratracheal infection and were rapidly down regulated. In addition, the increased expression of capsule on the highly virulent isolate is responsible, in part, for the induction of increased secretion of the third component of complement. The amount of capsule expressed by cryptococcal isolates might contribute to differences in the early inflammatory responses elicited and subsequently to the type of immune response that develop during infection. The weakly virulent isolate was cleared rapidly, whereas the highly virulent isolate remained in the lungs in stable numbers over the first 24 hours after infection. At the time of infection, the capsules of 2 isolates were similar in size.

Direct evidence for latent infection was provided by autopsy studies demonstrating cryptococcal cells within pulmonary granulomata in individuals dying of unrelated causes.²⁹ Goldman et al.³⁰ have described a rat model that may reflect latent infection in an inherently resistant host such as man. In this model, pulmonary infection is controlled without dissemination, but viable cryptococcal cells remain for at least 18 months in interstitial granulomata within macrophages and epithelioid cells. Abrogation of immune control by corticosteroid administration results in extracellular yeast and disseminated extrapulmonary infection. Garcia-Hermoso et. al.³¹ showed that the isolates from patients were diagnosed with cryptococcosis in France, some of whom were from Africa but had lived in France for a median of >9 years, suggested

that reactivation of such latent infection may be important in HIV-associated cryptococcosis. There was a significant clustering of isolates from African patients compared with the isolates from European patients had acquired their isolate long before the development of that patients.

3.3 Basic characteristics for pathogenicity

In order to enter the alveolar spaces of the lungs and establish pulmonary infection, an organism must produce viable forms smaller than 4 μm in diameter. The typical vegetative form of *C. neoformans* is yeast with a cell diameter ranges between 2.5 μm and 10 μm . The organism can also undergo sexual reproduction, and since it is a basidiomycete (*Faloblasidiella neoformans*), it forms basidiospores. The basidiospores are approximately 1.8 μm to 3 μm in diameter. Although the exact nature of the infectious particles of *C. neoformans* is not known, they are presumed to be the dehydrated yeast cells or basidiospores of the appropriate size range to get into the lungs. Once inside the lungs, the yeast cells become rehydrated and acquired the characteristic polysaccharide capsule (figure 3-2). In case of basidiospores, they would convert to encapsulated blastoconidia.

To cause infection in humans, a *C. neoformans* isolate must be able to grow at 37°C in an atmosphere of approximately 5% CO₂ and at a pH of 7.3-7.4. To survive at 37°C, the organism must have an intact gene that encodes the *C. neoformans* calcineurin A catalytic subunit.³² Calcineurin is a serine-threonine specific phosphatase that is activated by Ca²⁺-calmodulin and is involved in stress responses in yeast.³² Although calcineurin A mutant strain of *C. neoformans* can growth at 24°C, they cannot survive in vitro at 37 °C, in 5% CO₂ or at alkaline pH.³² Since these are similar to conditions in the host, one would predict that the calcineurin A mutant would not survive in the human host. In support of that prediction, Odom et al. have shown that such mutants are not pathogenic for immunosuppressed rabbits.³² Calcineurin A appears to be a basic requirement for *C. neoformans* survival in the host and consequently is a necessary factor for the pathogenicity of the organism.

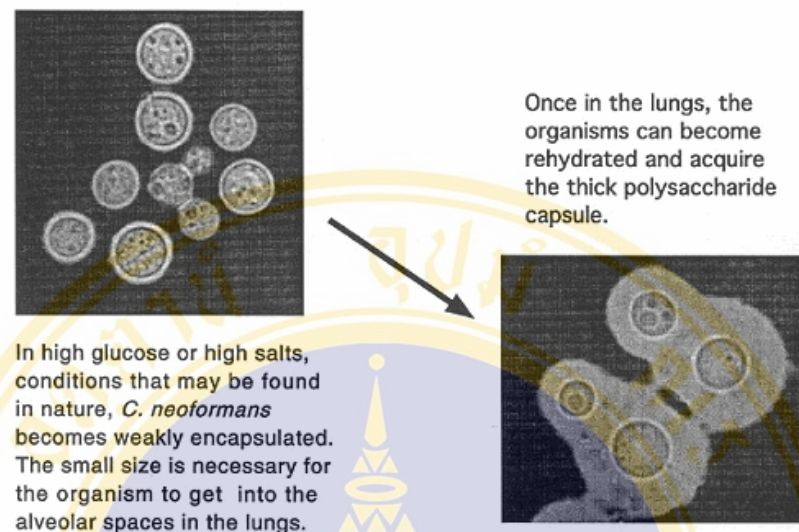


Figure 3-2 Showing of the yeast cells with weakly encapsulated capsules which become rehydrated and acquired the characteristic polysaccharide capsules in the lungs.

Virulence factors increase the degree of pathogenicity of a microbe. *C. neoformans* has a number of virulence factors. Without the virulence factors, *C. neoformans* would be considered low-grade pathogens. The severity of the host's disease results from a combination of several virulence factors superimposed on the host's innate and immune resistance status. The virulence factors are capsule, cryptococcal products polysaccharide capsule and the soluble extracellular constituents of *C. neoformans*, melanin production, mannitol production and potential factors such as superoxide dismutase, proteases, phospholipase B and lysophospholipase. Cryptococcal products are probably the dominant virulence factors.

C. neoformans has a capsule composed primarily of a high molecular weight polysaccharide that has a backbone consisting of α -1,3-D-mannopyranose units with single residues of β -D-xylopyranosyl and β -D-glucurunopyranosyl attached.³²⁻³⁶ This polysaccharide is referred to as glucurunoxylomannan (GXM)³² and has four serotypes: A and D are produced by *C. neoformans* var. *neoformans*, and B and C are produced by *C. neoformans* var. *gattii*. The evidence indicates that the capsule is a key

virulence factor for *C. neoformans*; acapsular mutants are typically avirulent, whereas encapsulated isolates have varying degrees of virulence.³⁷⁻⁴⁰ Two capsular genes *CAP59* and *CAP64*, have been described.⁴¹⁻⁴³ Complementation of an acapsular, avirulent mutant with *CAP59* resulted in a virulent transformant with a capsular phenotype, and deletion of *CAP59* by homologous integration caused the encapsulated organism to become acapsular and avirulent.⁴¹ Similar observations have been made with the *CAP64* gene.^{42,43} Although their biochemical functions have not been defined, it appears that the two genes are essential for capsule formation and virulence.

In disseminated cryptococcosis, measurable level of cryptococcal product are present in the body fluids of the patients.⁴⁴ Although GXM is the major cryptococcal component in the body fluids, it is highly probable that the organism also sheds galactoxylomannan (GalXM) and mannoproteins (MP) in vivo. This speculation is based on indirect evidence from in vivo studies and on the fact that GalXM and MP are in culture medium when the organism is grown in vitro.^{36,45} Furthermore, if patients with disseminated cryptococcosis have high cryptococcal antigen titers at the onset of therapy, they are less likely to respond to therapy or more likely to die before therapy is completed than patients with low cryptococcal antigen titers.⁴⁶ In the other word, it seems that the “free” cryptococcal antigen level in host’s circulatory system, spinal fluid and body fluid is affected to host defense mechanism because high “free” antigen can neutralize the antibody in host serum.

3.4 The immune response to fungal infection

Of the over 100,000 fungal species in existence, only a small percentage is known to cause human infection. Cryptococcosis predominantly affects persons with advanced AIDS, lymphoid and hematological malignancies and transplant recipients. The immune response varies with respect to the fungal species encountered. The relative importance of specific innate- and adaptive defense mechanism differs, depending upon the organism and anatomical site of infection (Table 3-1)

Within a species, the fungal morphotype (e.g. yeast, pseudohyphae and hyphae of *Candida albicans*) may be an important determinant of the host response. Whereas yeast and spores are often effectively phagocytosed, the larger size of hyphae

precludes effective ingestion. Pathogenic fungi have developed mechanisms to elude and subvert host defenses. Some fungi have evolved as intracellular parasites and can survive within phagocytes by using them to evade fungal killing and to disseminate throughout the host.

Airway is directly connected to the outer environment and always exposed to infectious agents, such as bacteria, fungi, and viruses. To protect the host from these agents, respiratory organs develop a highly sophisticated defense system. Initially, anatomical and mechanical systems, which include nasal hairs, nasopharyngeal channels, glottis, and highly divided branches of bronchi trap large size particles in the inhaled air. These particles are caught by the mucous blanket lining the bronchial surface and cleared by ciliary movement into the upper airway. In contrast, particles of less than 5 μm in diameter reach the alveolar spaces. In order to keep the sterility in lung, additional mechanisms are equipped in these areas, which are largely divided into the two categories: innate and acquired host defense systems. The former consists of humoral components including antibodies and complements, phagocytic cells like neutrophils and macrophages, dendritic cells, and innate immune lymphocytes, while the latter is associated with antigen-specific responses mediated by antibody and cellular immunity.

Initial exposure to *C. neoformans* is thought to most often occur with inhalation of aerosolized fungus. Airway turbulence and proper ciliary function prevent most of the yeast from reaching the distal lung.⁴⁷ Normally, fungus that reaches the lung parenchyma is controlled by the host response without extra-pulmonary dissemination. *C. neoformans* is either eliminated completely or restricted to the lung within granulomas, where it may persist in a latent state indefinitely. When infection is not sequestered, as in cases of defective cell-mediated immunity, pneumonitis and/or dissemination may ensure. The rationale for predilection for *C. neoformans* to infect the central nervous system is not well understood. Impaired opsonophagocytosis probably plays a role as has been suggested with other encapsulated pathogens.

Fungal infection is believed to relate with dysfunction of innate immunity. Infection of *C. neoformans*, a yeast-like fungal pathogen with a thick polysaccharide capsule takes place by inhaling the desiccated yeast cells into the lungs. The organism

reaches the subpleural area to establish the primary lesions. In normal hosts, the infection is usually self-limiting, since host defense mechanisms can eliminate the infection. In contrast, in immunocompromised patients with impaired cell-mediated immunity, the infection is not limited to the primary site of infection; it frequently disseminates to the central nervous system, which is often associated with a high mortality in these patients. Disseminated infection of this fungal pathogen to the brain has attracted clinical attention as a serious problem, particularly with the increased number of patients with AIDS.^{48, 49}

Table 3-1 Defects in host defenses that predispose patients to infections with specific fungi.

Fungal pathogen	Host factor
Candida (mucosal)	Impaired cell mediated immunity
Candida (disseminated)	Impaired mucosa and integument, Neutropenia
Aspergillus	Neutropenia, high dose corticosteroid
Cryptococcus	Impaired cell mediated immunity, corticosteroid
Zygomycetes	Neutropenia, deferoximine treatment, corticosteroid, diabetes ketoacidosis
Fusarium	Neutropenia, impaired integument, corticosteroid
Pseudosporium	Neutropenia
Trichosporon	Neutropenia, impaired integument

Several shared defense mechanisms are operative in response to a range of fungi. Neutrophils, macrophages and monocytes are fundamentally important antifungal effector cells. Phagocytes already residing in target organs at the time of infections attempt to kill or damage fungi. Additional effector cells, including neutrophils and monocytes, are recruited to sites of infection by the action of

inflammatory signals, such as cytokines, chemokines and complements. Fungi are killed or damaged by production and/or release of reactive oxygen intermediates and antimicrobial peptides.⁵⁰⁻⁵¹ Whether the cells use intracellular or extracellular antifungal mechanism depends upon the infecting species, morphotypes, and route of exposure.⁵²⁻⁵⁴

In cryptococcosis, any organ system may be involved; major sites of infection are the central nervous system and lungs. *C. neoformans* is the only medically important fungus to possess a capsule is the organism's major virulence factor. The vast majority of cases occur in immune compromised hosts. In a recent survey of two cities in the USA, over 90% of cases were associated with AIDS.⁵⁵ In the USA and other Western countries; the availability of highly effective antiretroviral therapy has resulted in a decline in the rate of cryptococcosis in the HIV-infected population. However, persons in resource-poor settings and those with limited access to health care continue to be at substantial risk.⁵⁵ Also at risk are patients with diabetes mellitus, and recipients of medications (e.g. corticosteroids) that depress cell-mediated immunity. The latter group includes those receiving immunosuppressive therapy for organ transplants. There is a predilection for cryptococcosis among patients with lymphoproliferative diseases including chronic lymphocytic leukemia (CLL), multiple myeloma, Hodgkin's disease, chronic myeloid leukemia (CML), and lymphosarcoma.⁵⁶ The prognosis for disseminated cryptococcosis in patients with neoplastic diseases may be worse than in patients with AIDS.⁵⁷

Humoral factors contribute to antifungal defense. Human serum and saliva inhibit *C. neoformans* growth. Encapsulated *C. neoformans* are potent activators of the alternative complement system with resultant deposition of C3 breakdown product on the capsular surface. Unopsonized *C. neoformans* is not well recognized by phagocytes. The presence of C3 on the cryptococcal capsule facilitates binding by multiple complement receptors.⁵⁸ Complement activation also generates C5a and C3a, which assist in opsonization and leucocyte trafficking. As with other fungi, C5a-deficient mice are particularly susceptible to disseminated infection.⁵⁹

The role of antibodies in cryptococcosis has been the subject of intense study but their role remains controversial. Anticapsular antibodies are found in most human sera, but they are generally non-opsonic or only weakly opsonic. In mouse models of

cryptococcosis, administration of anticapsular monoclonal antibodies may be protective, non-protective, or deleterious, depending upon the type and concentration of immunoglobulin used and the immune status of the host. For example, the same monoclonal antibody can be protective in an immune competent mouse but deleterious in a CD4⁺ T cell-deficient mouse. The effect of an individual antibody can vary depending on its concentration relative to challenge inocula.⁶⁰ Moreover, isotype switching can convert a protective antibody into a deleterious one. Naturally formed opsonic capsule-binding antibody is not consistently present during infection. A candidate monoclonal antibody has been developed for testing in human with cryptococcosis.⁴⁷ *In vitro*, monoclonal antibodies enhance internalization and killing of *C. neoformans* growth by an antibody-dependent cellular cytotoxicity mechanism.⁶¹ Moreover, by promoting phagocytosis, anticapsular antibody enhances antigen presentation to T cells. *In vivo*, antibody reduces the level of circulating capsule by inhibiting its release from the fungus and by enhancing the clearance of capsular material from the circulation.⁶²

3.5 The biological activities of antibodies against *C. neoformans*

C. neoformans is an opportunistic yeast that is surrounded by a polysaccharide capsule. Anticapsular and/or anti-GXM antibodies occurring in cryptococcosis patients, raised in experimental animals or produced as monoclonal antibodies, have a variety of biological activities including complement fixation^{63,64}, opsonization for phagocytosis by mouse resident peritoneal macrophage and human neutrophil⁶⁵⁻⁶⁷, enhancement of anticryptococcal activity of human natural killer cells¹⁶³, and *in vivo* clearance of cryptococci via passive immunity^{68, 69}.

Several studies indicate that anticapsular antibody is found in the sera of normal subjects who have not been specifically immunized with *C. neoformans* antigens. Diamond et al. found that pooled sera from normal human donors are opsonic for phagocytosis of a weakly encapsulated strain of *C. neoformans*.⁷⁰ The ability of the serum to opsonize the yeast was diminished if the serum was adsorbed at 0°C with encapsulated cryptococci. The opsonic activity was restored by the addition of purified human immunoglobulin G (IgG) to the adsorbed serum, suggesting the presence of

anticryptococcal antibody in normal serum. Direct evidence for production of antibody to cryptococcal polysaccharide by normal subjects was provided by Henderson et al.⁷¹ Dromer et al. studied anticapsular antibodies in normal subjects and in patients with AIDS-related complex or AIDS without a known history of cryptococcosis.⁷² Serum samples from most normal subjects contained anticapsular IgM (60%), and a lesser number (27%) contained anticapsular IgG. Anticapsular antibody occurred less frequently in patients with AIDS.

Some patients with cryptococcosis develop anticryptococcal antibody during a cryptococcal infection. Diamond and Bennette found approximately 40% of 81 cryptococcosis patients studied in the pre-AIDS era had detectable levels of antibody at the start of therapy.⁴⁶ At least two studies showed that antibodies in sera from patients with cryptococcosis are predominately of the IgG class.^{73, 74} In contrast, antibody produced in response to immunization of volunteers with cryptococcal polysaccharide is predominantly IgM.⁷¹ In Houpt et al.⁷⁵, studies of HIV-infected patients suggest those portions of the synthesis of antibodies reactive against GXM are lost through HIV infection and the progression to AIDS. Although not statistically significant, there was a trend toward a lower frequency of anti-GXM IgG when CD4 levels fell below 200 cells per μ l. Anti-GXM IgG was found in 28% of the normal and 10% of AIDS group. A similar reduction was reported by Dromer et al.⁷² and in Houpt et al.⁷⁵ studies, two possible functions of anti-GXM IgG could be of important in resistance to cryptococcosis, ie., initiation of the classical complement pathway and Fc-mediated opsonization of encapsulated cryptococci. All normal subjects have relative high levels of IgG antibodies directed against cryptococcal cell wall, glucan⁷⁶. When nonencapsulated cryptococci are incubated in normal human serum, these anti-cell wall antibodies initiate the classical complement pathway, leading to deposition of C3 fragments at the cell wall.^{74, 77} This classical pathway initiation is characterized by an immediate accumulation of C3 fragments on the yeast cell. Incubation of encapsulated cryptococci in normal human serum leads to activation of the alternative pathway. Alternative pathway initiation is characterized by a 4 to 7 min lag before appreciable amounts of C3 can be detected on the yeast cells. The anticapsular IgG found in sera from normal donors with high levels of antibody is unable to alter the lag that characterizes alternative pathway initiation by encapsulated cryptococci. The

amount of anti-GXM antibody needed to initiate the classical pathway is not known, but it is likely that levels of naturally occurring anti-GXM antibodies are not sufficient to significantly alter what is normally an alternative pathway process.

Despite the fact that naturally occurring anti-GXM IgG does not initiate the classical pathway, the possibility remained that this antibody could facilitate phagocytosis via direct interaction with phagocyte *Fc* receptors. In particular, this antibody could act synergistically with C3 fragments that were deposited via the action of the alternative pathway.⁷⁸ This question was addressed in several ways. First, the opsonic activities of normal sera with high and low levels of anti-GXM IgG were compared. Second, anti-GXM antibody was blocked by incorporation of purified GXM into the buffer system. Finally, anti-GXM antibody was removed by adsorption at 0°C with encapsulated cryptococci. Results from all three approaches indicated no contribution to phagocytosis of encapsulated cryptococci by naturally occurring IgG. The low density of anti-GXM IgG could bind at the capsular surface. The capsule is permeable to potentially opsonic IgG and this IgG binds to sites beneath the capsule surface such that it cannot interact with the macrophage *Fc* receptors while binds C3 heavily at the capsular surface. Cells opsonized with only IgG were engulfed poorly by macrophage and neutrophil. Yeast cells opsonized with IgG and C3 were engulfed in large number by neutrophils and to a lesser extent by macrophages. The failure to achieve antibody-mediated opsonization is most likely due to the concentration of anti-GXM IgG and C3 in the serum.

Eckert et al.⁷⁹ produced monoclonal antibodies (MAbs) that used to study the opsonic activity for phagocytosis by macrophages. The antibodies reactive with epitopes on the polysaccharide were equally opsonic for the yeast cells. The potent opsonic activity of MAbs confirms the conclusion that antibodies binding to any epitope on the capsular polysaccharide are opsonic if appropriate phagocyte receptors are available. Dromer et al.⁸⁰ have reported the production of two MAbs specific for cryptococcal polysaccharide. These antibodies were produced by immunization with type A polysaccharide. The antibodies were of the IgG1 isotype. These antibodies agglutinated and precipitated cells and polysaccharide, of serotype A respectively. More sensitive assays such as immunofluorescence and competitive binding studies indicated very weak reactivity with cells and polysaccharide of serotype B and D.

Cleare et al.⁸¹ studied the MAbs 2H1, a murine immunoglobulin G1_K antibody which binds *C. neoformans* capsular glucurunoxylomannan. These MAbs can modify the course of lethal *C. neoformans* infection in mice and potentiates amphotericin B against *C. neoformans*. Maitta et al.⁸² studied immunogenicity and efficacy of *C. neoformans* capsular GXM. An antibody titer and isotype switching depend strongly on the nature of the immunogen. The IgG subclass expression has been shown to influence binding to GXM and other polysaccharide antigens. For example, variable-region-identical mouse-human chimeric MAbs to GXM manifested IgG subclass-dependent differences in GXM specificity and opsonic activity. IgG2 is the predominant subclass of human antibodies to capsular polysaccharides, including GXM and it has been proposed that the preferential use of IgG2 antibodies to capsular polysaccharides due to a function of its avidity and ability to polymerize. Since human serum IgG4 is functionally monovalent, it might not be able to bind the relevant GXM epitope for which GXM mimotope is a surrogate antigen when monovalency eliminates avidity, whereas the avidity of IgG2 might enhance binding. Along the same lines, lower levels IgM against GXM might reflect epitope blocking by monomeric IgG4. However, further investigation is required to identify the mechanisms responsible for differences in the GXM reactivities. For *C. neoformans*, antibody-mediated immunity might involve regulation of the cellular immune response. Pirofski et al.⁸² studied about human antibodies elicited by vaccination with a *C. neoformans* GXM capsular polysaccharide vaccine and they reported that antibodies against the GXM capsular polysaccharide (CPS) of *C. neoformans* were detectable in the serum of both HIV-positive and HIV-negative individuals. The existence of GXM antibodies, like murine GXM antibodies, may be either protective or nonprotective. Murine antibodies that bind the *C. neoformans* GXM enhance the survival of mice with lethal cryptococcosis and enhance phagocytosis of *C. neoformans* by macrophages in vitro. They reported, both MAbs and polyclonal Abs produced similar fluorescence labeling patterns. Therefore, they were suggesting that both antibodies could bind a similar epitope.

Kozel et al.⁸³ reported the positive test for antibody appear to be inversely related to the quantity of antigen in the patient. For example, 22 of 29 patients with cryptococcal meningoencephalitis and a negative India ink smear of cerebrospinal

fluid had detectable antibody in their serum, whereas only 4 of 40 patients with positive India ink smears had positive tests for antibody⁸⁴. Conversely, the incidence of positive antibody tests in patients increases when the patients exhibit solitary lung lesions, no central nervous system involvement, or early central nervous system involvement^{85, 86, 87}. These observations of cryptococcosis in human being as well as several studies of immune response to cryptococcal polysaccharide in animal⁸⁸ have led to the speculation that antibody is neutralized by excess antigen or some form of supraoptimal doses of cryptococcal polysaccharide. Murphy and Cozad⁸⁹ demonstrated that immunological unresponsiveness can occur. And that unresponsiveness appears to be the result of a decline in the number of cells capable of producing antibody rather than neutralization of antibody by the excess of antigen. These findings suggest that immune unresponsiveness may play some role in the poor immune response observed in human cryptococcosis. Diamond et al.⁹⁰ suggested that cryptococcal antibody is not directly protective. Instead, antibody production may be simply a concomitant of an active host response to infection. The recent description of an antibody-dependent cell-mediated mechanism for killing of *C. neoformans* suggests a more active role for antibody in resolution of the disease.⁹¹⁻⁹²

3.6 The serodiagnosis for cryptococcosis

Presumptive diagnosis of cryptococcosis can sometime be made using signs, symptoms, and epidemiologic data, however, such diagnosis should be confirmed by standard culture and histologic laboratory procedures. Unfortunately, diagnosis of a mycotic infection cannot always be obtained by culture or histology, despite repeated efforts. In such situations, immunologic procedures can be used to provide rapid and presumptive evidence of infection, and immunologic reactions often give the first clues to the existence of a fungal infection.¹⁴ Serological tests can also yield information on the effects of chemotherapy and in many cases, lead to increased efforts to isolate and identify the etiologic agent.

A positive serologic reaction even though on a single specimen, can be diagnostically significant. The low titers for *C. neoformans* antibody may reflect early infection, a cross-reaction or residual antibody from a previous infection. Many of the

diagnostic antigens used in medical mycology are unpurified mixtures of multiple antigenic factors, some of which are shared by fungi of different genera and by other microorganism. When a serum titer is low or when cross reactions are encountered, a prudent diagnosis rest on (i) the results of a variety of serologic tests performed with a battery of antigens (including those representing antigenically related species), (ii) the examination of serial serum specimens for titer changes, (iii) tests for specific precipitins, and (iv) information on the acquisition of hypersensitivity. Some persons suffering from a systemic mycosis are immunologically unresponsive. Others may not demonstrate antibody against a fungus because serum is taken in the latent period of the primary immune response or because the patient is immunologically impaired. Consequently, negative immunological results do not exclude a diagnosis of mycotic infection. Knowledge of the clinical history and therapy of the patient is helpful in the interpretation of serologic data.

Antibody responses are useful indices in the determination of the cause and the prognosis of a mycosis. Dependable diagnosis can frequently be made from one serologic test. Low titers or cross-reactions in the complement fixation test are, however, difficult to interpret. In such cases, serum samples taken 3 weeks apart should be studied. Ideally, when the course of an infection is monitored, serum samples should be taken early in the course of illness, at its height, during convalescence, and several weeks after recovery. Four-fold or greater rises in titer are usually acceptable diagnostic signs of disease, although cross-reactions with heterologous antigens may appear early in an illness and cause confusion. Usually, as the disease progresses, cross-reacting antibody titers remain stable or increase at a lower rate than the homologous titer. False serodiagnosis may be avoided by the use of reference precipitates in immunodiffusion tests.

The conventional method for the diagnosis of cryptococcosis is time consuming and, in many cases, inadequate. Until recently, persons suffering from cryptococcosis were considered to be essentially immunologically inert. Previous immunologic tests had only limited applications¹⁴, and results were difficult to interpret. During the last two decades, work on serologic procedures for cryptococcosis has resulted in the development of diagnostically and prognostically useful tests. These procedures are an indirect immunofluorescence (IFA) technique, an

EIA, and a tube agglutination (TA) test for cryptococcal antibodies and a LA test and an EIA for cryptococcal antigen.^{14, 93} The antibody tests are of value in the detection of early or localized cryptococcosis and in the determination of a prognosis.

Serologic tests for *C. neoformans* antigens or antibodies or both should be considered in patients who have symptoms of pulmonary or meningeal infection. The IFA and TA antibody tests are reactive with about 50% of the sera from patients with active cases of extrameningeal cryptococcosis. The IFA test has a specificity of about 77%, whereas the TA test has a specificity of about 89%¹⁴. Although the IFA test is not entirely specific, it is valuable in the detection of those cases of cryptococcosis that are negative for *C. neoformans* agglutinins and antigens. The TA test for cryptococcal antibodies has been found to be diagnostically reliable. Agglutinins were detected in the early stages of central nervous system infection and in infections with no central nervous system involvement¹⁴.

Casadevall A. et al.⁶¹ and Martinez L.R. et al.⁹⁴ studied about characterization of a murine monoclonal antibody to *C. neoformans* polysaccharide by immunofluorescence and spot capture ELISA. Reactivity of the monoclonal antibody to 13 *C. neoformans* strains was shown by indirect immunofluorescence. All protective monoclonal antibody bounded with annular immunofluorescence pattern on *C. neoformans*, whereas nonprotective monoclonal antibody often showed punctate pattern. They also reported technique of the spot capture ELISA used to detect *C. neoformans*. They also employed spot capture ELISA using GXM Mab to detect *C. neoformans* in solution. An ELISA spot assay that use one monoclonal antibody to capture and hold yeast cells on polystyrene plate and a second biotin labeled antibody to promote deposition of an insoluble dye on the yeast cell capsule.

Martinez L.R. et al.⁹⁴ determined the antibody to *C. neoformans* glucuronoxylomannan (GXM) that inhibits the release of capsular antigen. Antibody concentration was determined by ELISA relative to isotype matched standards. *C. neoformans* capsular GXM was measured by capture ELISA. The antibodies to *C. neoformans* GXM significantly reduced serum GXM levels in rodents. The antibody promotes ingestion of soluble GXM by reticuloendothelial cells and interferes with this process by cross-linking GXM molecules in the capsule and thus preventing their release from encapsulated yeast. Zaragoza O. et al.⁹⁵ determined the antibodies

produced in response to *C. neoformans* pulmonary infection in mice by indirect immunofluorescence and analyzed the titer of serum GXM binding antibody and demonstrated both specific IgM and IgG by ELISA. Murine cryptococcal pulmonary infection elicited serum immunoglobulin M (IgM) and IgG to the capsular polysaccharide, but only IgG stained yeast cells in alveoli. Both isotypes produced punctate immunofluorescence pattern on yeast cells by indirect immunofluorescence. Goldman D. et al.⁹⁶ measured serum antibody to GXM, in rat after intratracheal inoculation with *C. neoformans*, by a double sandwich ELISA. The IgM response was transient and no significant IgM was detected subsequently. *C. neoformans* infection elicited IgG to GXM in most rats. In contrast to IgM, serum IgG persisted and there was an upward trend in IgG titer. Mukherjee J. et al.⁹⁷ studied the importance of antibody against diverse strains of *C. neoformans*. Differences in strain susceptibility to antibody immunity could have contributed to the contradictory observations that antibody prolonged survival in mice. Titers of antibody and level of GXM binding antibody in serum were determined by ELISA. There were significant differences in levels in serum of IgG1 to GXM. Murphy J.W. et al.⁹⁸ studied the protective and nonprotective CMI component against *C. neoformans* induced by vaccination. The titer of anticryptococcal antibody was determined by ELISA. The immunization could not induce protective CMI responses

Cryptococcosis is best diagnosed serologically through the concurrent use of three tests: the LA test for antigen and the IFA and TA tests for *C. neoformans* antibodies¹⁴. The positive antibody test is suggestive of infection by *C. neoformans* and could also reflect a past infection or a cross-reactivity. Antibodies may be detected in the early course of the disease and in localized infections. As the disease progresses, abundant antigens may be produced and detected, with concurrent exclusion of antibody. The antibody test may have prognostic value. With effective chemotherapy, the antigen titer declines and antibody may become demonstrable by one or both tests.

CHAPTER IV

MATERIALS AND METHODS

4.1 Materials

4.1.1 *Cryptococcus neoformans* culture

Cryptococcus neoformans isolates were obtained from 3 patients proved to be central nervous system cryptococcosis. Serotype of the *C. neoformans* isolates was performed and identified as serotype A by agglutination technic from Meiji Pharmaceutical University. The *C. neoformans* isolates were subcultured onto SDA, harvested and used as sources of antigen for tube agglutination- and indirect immunofluorescence assays for *C. neoformans* antibody detection in sera of patients with cryptococcosis.

4.1.2 Specimens

1. Specimens to be used in *C. neoformans* antibody detection were sera from the patients submitted to Immunology Laboratory for the detection of *C. neoformans* antigens.
2. *C. neoformans* antibody positive sera were obtained by tube agglutination test screening. The sera were used as positive sera for determination the optimal conditions of indirect immunofluorescence and positive control sera in subsequent assays for *C. neoformans* antibody detection by indirect immunofluorescence.
3. Normal human sera were obtained from blood donors and used to determine optimal conditions of indirect immunofluorescence and

negative control sera in subsequent assays for *C. neoformans* antibody detection by indirect immunofluorescence.

4.2 Methods

4.2.1 Preparation of *C. neoformans* suspension for tube agglutination and indirect immunofluorescence.

1. Subculture *C. neoformans*, isolated from 3 patients with central nervous system cryptococcosis onto SDA slants.
2. Incubated at 37°C in incubator for 3-4 days to obtain sufficient amount of *C. neoformans*. (figure 4-1)
3. Added 2 mL. of 0.9% sodium chloride solution containing 1% formalin to each *C. neoformans* culture tube.
4. Harvested the *C. neoformans* from SDA tubes by scraping and gentle mixing the culture to suspend *C. neoformans*.
5. Pipette and pooled the suspension of *C. neoformans* of the three isolates into the test tubes. (figure 4-2)
6. Incubated at 4°C overnight.
7. Checked sterility of *C. neoformans* suspension by subculture onto SDA slant tube and incubate at 37°C overnight.
8. Washed the *C. neoformans* suspension with sterile 0.9% sodium chloride solution 2-3 times.
9. Pooled *C. neoformans* suspension in a flask and keep at 4°C in refrigerator.
10. Counted the *C. neoformans* suspension with hemocytometer to make dilution of *C. neoformans* suspension in 0.9% sodium chloride solution containing 1 % formalin to the concentration of 7.5×10^6 cells/mL.
11. Kept *C. neoformans* suspensions in 0.9% sodium chloride solution containing 1% formalin in flask at 4°C.

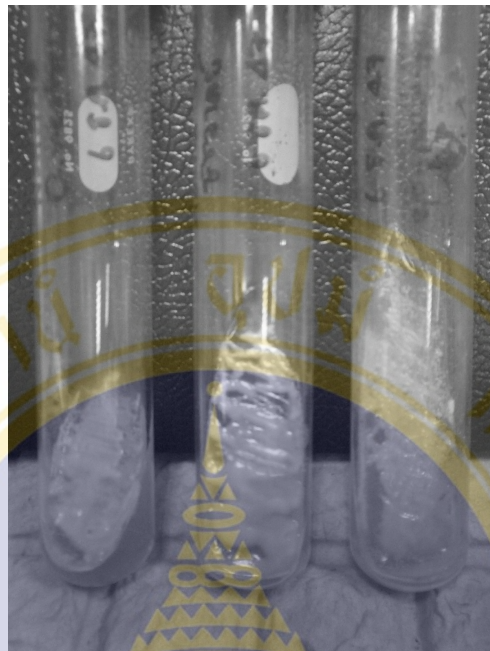


Figure 4-1 Sabouraud Dextrose Agar with sufficient amount of *Cryptococcus neoformans* subcultured from 3 patients with CNS cryptococcosis

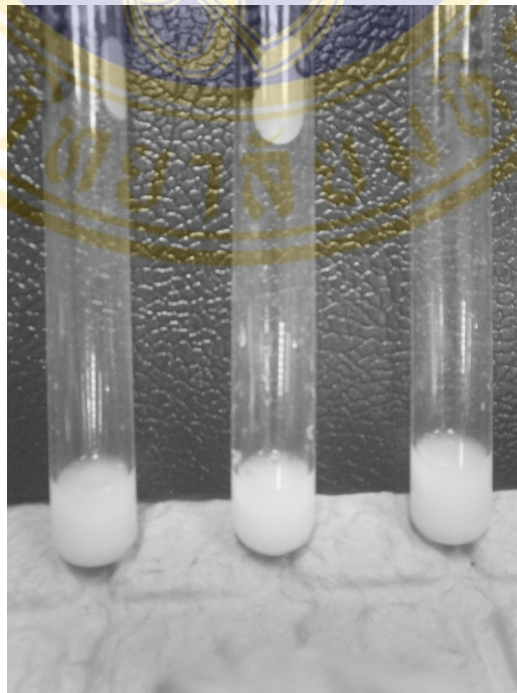


Figure 4-2 Suspensions of the 3 isolates of *Cryptococcus neoformans* after killing with 0.9% sodium chloride containing 1% formalin.

4.2.2 Manual *Cryptococcus neoformans* cells count

1. Mixed the suspension of *Cryptococcus neoformans* with vortex mixer
2. Pipette the suspension with red blood cell diluting pipette to level 0.5
3. Then pipette sterile normal saline solution to level 101
4. Mixed the suspension in red blood cell diluting pipette for 3 minutes
5. Let mixture stand for 1 minute before charging to hemocytometer
6. Cleaned the hemocytometer and its cover slip with an alcohol pad and then dry with a wipe
7. Discarded the first 3 to 4 drops of mixture
8. Carefully charged hemocytometer with the diluted suspension by gently expel contents until chamber is properly filled
9. Placed hemocytometer for 1 minute to allow cells to settle
10. Mounted the hemocytometer on the microscope and lower its condenser.
11. Procedure for counting
 - 11.1. Cells are scanned under a 10x objective to ensure even distribution.
 - 11.2. Used a 40x objective (high dry) to count the cells in the 5 squares. (figure 4-3a) This counting procedure is repeated on the opposite side of the hemocytometer
 - 11.3. To count the cells in the tertiary squares, use the following pattern: (figure 4-3b)
 - 11.3.1. Counted cells starting in the upper left corner square, continue counting to the right hand square, drop down to the next row; continue counting from the right hand square to the left square. Continue counting in this fashion until the total area in that secondary square has been counted.
 - 11.3.2. Counted all cells that touch any of the upper and left lines, do not count any cell that touches a lower or right line.
 - 11.4. The difference between the highest and lowest number of cells of the 10 squares should be no greater than 25.
12. calculations:

$$\text{No. of cells/mm}^3 = \text{No. of cells} \times 10,000$$

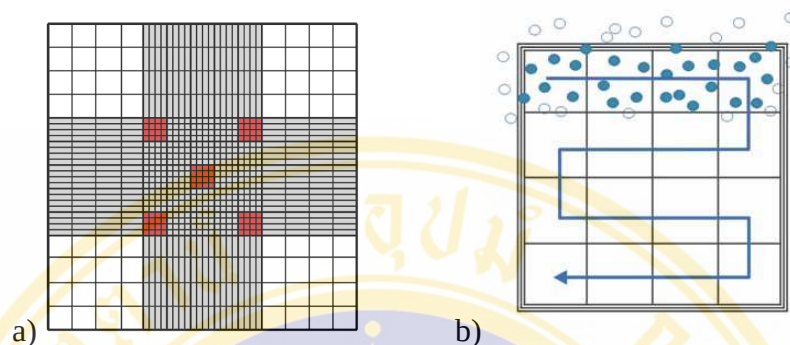


Figure 4-3 Hemocytometer a) The red areas for counting *C. neoformans* cells on hemocytometer b) The pattern of the counting *C. neoformans* in the red area on hemocytometer

4.2.3 Preparation of *C. neoformans* substrate antigen for indirect immunofluorescence.

The protocol of preparation of *C. neoformans* substrate antigen for indirect immunofluorescence was as the followings.

1. Dropped the *C. neoformans* suspension of 7.5×10^6 cells/mL. onto each ring of the clean microscope slides (50 μ L). The excess from the drop was removed with the same pipette. (figure 4-4)
2. The film of Cryptococcal cells was left for air dry at room temperature.
3. Fixed slides containing *C. neoformans* by dipping in sterile distilled water containing 10 % methanol for 10 minutes.
4. Left the slides to dry in room temperature.
5. Kept the slides in -40°C deep freezer until use.

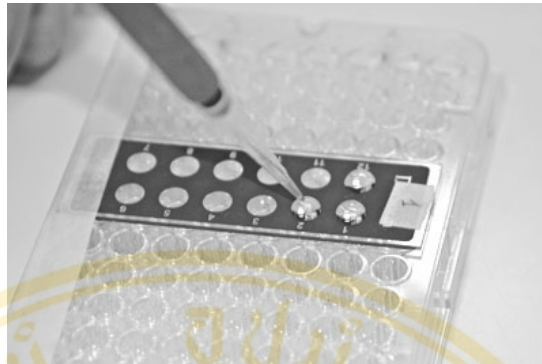


Figure 4-4 Antigen slide preparation.

4.2.4 Tube agglutination test for screening of positive and negative sera for *Cryptococcus neoformans* antibody.

The protocol of tube agglutination test for screening of positive and negative sera for *C. neoformans* antibody was as the followings.

1. Heated the *C. neoformans* suspension adjusted to a concentration of 7.5×10^6 cells per ml. at 56°C for 30 minutes.
2. Inactivated sera to be screened for positive and negative *C. neoformans* antibody at 56°C for 30 minutes.
3. Made serial two fold dilutions of the sera starting from 1:2 dilution to 1:8 dilution.
4. Mixed the diluted sera with equal volume of *C. neoformans* cells suspension.
5. The mixtures are placed on the rotary shaker for 2 minutes.
6. Incubated at 37°C for 2 hours, then examine for the result of agglutination.
7. If no any agglutination, the mixtures were kept at 4°C for 72 hours, during which time reading are taken at 24 hour interval.
8. The serum showing any degree of agglutination is determined to be positive for *Cryptococcus neoformans* antibody.

4.2.5 Determination of optimum conditions for indirect immunofluorescence.

1. Placed the prepared slides containing *C. neoformans* at room temperature until reaching to room temperature.
2. Serum positive and negative for *C. neoformans* antibody by tube agglutination and normal human sera were diluted in FA buffer to the dilution of 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640 and 1:1280.
3. Overlaid the diluted positive and negative sera onto each ring of slides containing *C. neoformans*.
4. Incubated the slides at 37°C for 15 minutes in moist chamber.
5. Washed the slides in FA buffer 2 times for 10 minutes each.
6. Washed the slides in distilled water for 1 minute.
7. Overlaid FITC-antihuman globulin, diluted in FA buffer containing 2% Tween80 to the dilution of 1:100, 1:200, and 1:1000, 1:2000, 1:4000, 1:6000, and 1:8000, onto each dilution of positive serum and negative serum.
8. Incubated at 37°C for 15 minutes in moist chamber.
9. Washed the slides in FA buffer 2 times for 10 minutes each.
10. Washed the slides in distilled water for 1 minute.
11. Dropped mounting medium and cover the reaction rings of slides with coverglasses.
12. Examined the slides under fluorescent microscope for the thick wall-round cells showing fluorescence of the capsules of the yeast cells as positive results for *C. neoformans* antibody. (Figure 4-5)
13. The highest dilution of FITC-antihuman globulin giving the positive result with positive serum for *C. neoformans* antibody and negative result with negative serum was used as the optimal dilution of FITC-antihuman globulin for the detection of *C. neoformans* antibody in patient sera.



Figure 4-5 The thick wall-round cells showing fluorescence of the capsules of the *C. neoformans* cells under fluorescent microscope.

4.2.6 Indirect immunofluorescence in patient sera with cryptococcosis

Sera from patients with pulmonary cryptococcosis, cryptococcal meningitis and disseminated cryptococcosis were tested for *C. neoformans* antibody as follow.

1. Placed the prepared slides containing *C. neoformans* at room temperature until the temperature of slides reached the room temperature.
2. Serum positive and negative for *C. Neoformans* antibody by tube agglutination and the patient sera were diluted in FA buffer to be the dilution of 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280.
3. Overlaid the positive, negative and the diluted patient sera onto each ring of slides containing *C. neoformans*.
4. Incubated the slides at 37°C for 15 minutes in a moist chamber.
5. Washed slides in FA buffer 2 times for 10 minutes each.
6. Washed slides in distilled water for 1 minute.
7. Overlaid FITC-antihuman globulin, diluted in FA buffer containing 2% Tween80 at the optimal dilutions onto each ring of slides.
8. Incubated at 37°C for 15 minutes in a moist chamber.
9. Washed slides in FA buffer 2 times for 10 minutes each.

10. Washed slides in distilled water for 1 minute.
11. Dropped mounting medium and cover the reaction rings of slide with coverglasses.
12. Examined the slides under fluorescent microscope for the thick wall-round cells showing fluorescence of the capsules of the yeast cells as positive result for *C. neoformans* antibody.
13. The patient sera giving fluorescence under fluorescent microscope were positive results for *C. neoformans* antibody. The highest dilution of patient serum giving 1+ fluorescence was a positive titer of *C. neoformans* antibody detected by indirect immunofluorescence.

4.2.7 Stability test of the indirect immunofluorescence.

In order to assess the stability of the prepared *C. neoformans* substrate antigen for indirect immunofluorescence, a number of slides containing *C. neoformans* as previously described were made and stored in deep freezing at -40°C. The slides were divided into 6 sets (2 slides per set) and were tested at different times, i.e., 0, 1, 2, 3, 4 and 5 months with a panel of 2 positive and 2 negative control sera. The control sera were aliquoted (approximately 0.5 ml. amount) and stored in deep freezing at -40 °C until used for stability testing. The results obtained at different times of the assays were compared with those results of the first time.

CHAPTER V

RESULTS

5.1 Screening test for *C. neoformans* antibody positive sera by tube agglutination

One hundred patient sera submitted to Immunology Laboratory for the detection of *C. neoformans* antigens were screened by tube agglutination to obtain sera positive for *C. neoformans* antibody. The sera positive for *C. neoformans* antibody by the screening were used as positive controls in subsequent detection of *C. neoformans* antibody by tube agglutination and indirect immunofluorescence in normal sera and sera of the patients with cryptococcosis. Fifteen of the one hundred sera gave positive results for *C. neoformans* antibody.

5.2 Detection of *C. neoformans* antibody in normal sera by tube agglutination

A total of fifty normal human sera from blood donors were used for *C. neoformans* antibody detection by tube agglutination and the results obtained were used in the comparison with the results obtained by indirect immunofluorescence to assess the validity of immunofluorescence in the detection of *C. neoformans* antibody. Of the 50 sera tested, 45 sera were negative for *C. neoformans* antibody by tube agglutination. Four sera gave positive results of 1:4 dilution and one serum gave positive result of 1:2 dilution. (Table 5-1)

5.3 The optimal conditions of indirect immunofluorescence

The sera positive for *C. neoformans* antibody and the sera negative for *C. neoformans* antibody by tube agglutination were used to determine the optimal dilution of FITC labeled polyclonal rabbit anti-human globulin in conjunction with the *C. neoformans* substrate prepared for indirect immunofluorescence as well as the suitable initial dilution of the sera to be tested by indirect immunofluorescence. The sera which were diluted to 1:10, 1:20, 1:40 were tested, and the sera of 1:40 gave lower background comparing with 1:10 and 1:20 of which the differentiation between positive and negative was unclear. The FITC labeled polyclonal rabbit anti-human globulins which were diluted to the dilution of 1:100, 1:200 were tested for the optimum dilution of indirect immunofluorescence. The FITC labeled polyclonal rabbit anti-human globulin that was diluted to 1:100 and 1:200 gave high background which interfered the fluorescence of *C. neoformans*. The FITC labeled polyclonal rabbit anti-human globulin which was diluted to 1:1000, 1:2000, 1:4000 also gave background. At the dilution of 1:8000 the background disappeared but some positive sera became negative. The dilution of 1:6000 was tested and gave the optimum result by the yeast cells showing fluorescence with positive serum and no fluorescence reactivity with the negative serum. The dilution of 1:6000 of FITC labeled polyclonal rabbit anti-human globulin was chosen for optimal dilution of FITC labeled polyclonal rabbit anti-human globulin and the 1:40 dilution was used as initial dilution of the sera to be tested.

5.4 Detection of *C. neoformans* antibody in normal sera by indirect immunofluorescence

Fifty normal human sera from blood donors were used for *C. neoformans* antibody detection by indirect immunofluorescence with 1:6000 dilution of FITC labeled polyclonal rabbit anti-human globulin. Five of the sera from blood donors gave positive results of *C. neoformans* antibody by indirect immunofluorescence. Two sera gave positive results of 1:320 dilution, two sera gave positive results of 1:160

dilution and one serum gave positive result of 1:80 dilution. The five sera positive by immunofluorescence also gave positive results by tube agglutination with the dilution of 1:4, 1:4, and 1:2 respectively. Two sera with positive results of 1:4 dilution by tube agglutination gave positive results of 1:320 dilution by indirect immunofluorescence and the other two sera with positive results of 1:4 dilution gave positive results of 1:160 dilution by immunofluorescence. One serum with positive result of 1:2 dilution by tube agglutination gave positive result of 1:80 dilution by indirect immunofluorescence. (Table 5-1)



Figure 5-1 The thick wall-round cells showing fluorescence of the capsules of the yeast cells under fluorescent microscope.

5.5 Indirect immunofluorescence in sera of patients with cryptococcosis

The patients were retrospectively analyzed using the results of laboratory investigations, chest X-rays and clinical manifestations around the time the specimens obtained to be tested for *C. neoformans* antibody in order to classify cryptococcosis into pulmonary cryptococcosis, cryptococcal meningitis and extrapulmonary nonmeningeal cryptococcosis. Pulmonary cryptococcosis was defined as the patients with lung lesions determined by chest X-ray and positive results for *C. neoformans*

culture from sputum or bronchoalveolar lavages and no central nervous system involvement. Eight patient sera with pulmonary cryptococcosis were assayed for *C. neoformans* antibody by indirect immunofluorescence with the optimal dilution of FITC labeled polyclonal rabbit anti-human globulin. All of the patient sera gave positive results with *C. neoformans* antibody by indirect immunofluorescence. **(Table 5-2)** Two patients gave positive results of >1:1280 dilution. Three patients gave positive results of 1:160 dilution and three patients gave positive results of 1:40 dilution.

The patients with CNS cryptococcosis were defined as those with clinical manifestations involved central nervous system and gave positive results with *C. neoformans* culture from cerebrospinal fluids and/or positive results for *C. neoformans* antigen by latex agglutination in cerebrospinal fluids. All of the patients also gave positive results of *C. neoformans* antigen by latex agglutination from sera. Ten patient sera with CNS cryptococcosis were assayed for *C. neoformans* antibody by indirect immunofluorescence with optimal dilution of FITC labeled polyclonal rabbit anti-human globulin. All of the ten patient sera gave negative results of *C. neoformans* antibody by indirect immunofluorescence. **(Table 5-3)**

The patients with extrapulmonary nonmeningeal disseminated cryptococcosis were defined as clinical manifestations. The patients who have *C. neoformans* grows from blood culture or those with the presence of lymphadenopathy with lymph node biopsies showing *C. neoformans* by H&E staining. Some of the patients gave positive results for *C. neoformans* antigen by latex agglutination in serum. Eight patient sera with extrapulmonary nonmeningeal disseminated cryptococcosis were assayed for *C. neoformans* antibody by indirect immunofluorescence with optimal dilution of FITC labeled polyclonal rabbit anti-human globulin. Six of the patient sera gave negative results for *C. neoformans* antibody detection by indirect immunofluorescence. Two sera of the patients gave positive results for *C. neoformans* antibody detection by indirect immunofluorescence. **(Table 5-4)** One patient serum gave positive result of 1:80 dilution and one patient serum gave positive result of 1:40 dilution.

The patient's sera with diseases other than cryptococcosis including tuberculosis, *Pneumocystis carinii* pneumonia, histoplasmosis and pure red cell aplasia were used for *C. neoformans* antibody detection by indirect immunofluorescence. All

of the patient's sera gave negative results for *C. neoformans* antigen by latex agglutination in serum and cerebrospinal fluid. The total of thirteen patient sera were used for *C. neoformans* antibody detection by indirect immunofluorescence. All thirteen sera gave negative results for *C. neoformans* antibody detection by indirect immunofluorescence. (Table 5-5)

5.6 Stability test of indirect immunofluorescence

The stability test of slides containing *C. neoformans* was performed by immunofluorescent test for *C. neoformans* antibody of the slides, stored at -40°C and were tested at 0, 1, 2, 3, 4, and 5 months with a panel of 2 sera positive for *C. neoformans* antibody and 2 sera negative for *C. neoformans* antibody. The test results obtained at 1, 2, 3, 4, 5 months of storage gave the same results as the sera detected for *C. neoformans* antibody at the first time of assays. The fluorescence of the 2 positive sera could be clearly discriminated from the 2 negative sera, because the positive reactions showed the distinct fluorescence on the capsule of *C. neoformans*, whereas the negative reactions demonstrated no fluorescence. (Table 5-6) The stability of indirect immunofluorescence was validated for the long period of the storage of *C. neoformans* substrate at -40°C without changing the antibody titer of the pooled of the sera positive for *C. neoformans* antibody and the sera negative for *C. neoformans* antibody which were aliquoted and stored at -40°C.

Table 5-1 Results of *C. neoformans* antibody in sera of donated blood tested by tube agglutination and indirect immunofluorescence

No.	<i>C. neoformans</i> Ab		No.	<i>C. neoformans</i> Ab		No.	<i>C. neoformans</i> Ab	
	TA	IFA		TA	IFA		TA	IFA
1	<u>1:4</u>	<u>1:320</u>	21	N	N	41	N	N
2	<u>1:4</u>	<u>1:320</u>	22	N	N	42	N	N
3	<u>1:4</u>	<u>1:160</u>	23	N	N	43	N	N
4	<u>1:4</u>	<u>1:160</u>	24	N	N	44	N	N
5	<u>1:2</u>	<u>1:80</u>	25	N	N	45	N	N
6	N	N	26	N	N	46	N	N
7	N	N	27	N	N	47	N	N
8	N	N	28	N	N	48	N	N
9	N	N	29	N	N	49	N	N
10	N	N	30	N	N	50	N	N
11	N	N	31	N	N			
12	N	N	32	N	N			
13	N	N	33	N	N			
14	N	N	34	N	N			
15	N	N	35	N	N			
16	N	N	36	N	N			
17	N	N	37	N	N			
18	N	N	38	N	N			
19	N	N	39	N	N			
20	N	N	40	N	N			

C. neoformans Ab, *Cryptococcus neoformans* antibody; TA, tube agglutination; IFA, indirect immunofluorescence; N, negative

Table 5-2 Results of *C. neoformans* antibody in sera determined by indirect immunofluorescence, chest x-ray and *C. neoformans* culture in patients with pulmonary cryptococcosis

No.	<i>C. neoformans</i> Ab*	Chest X-ray result	Anti-HIV status	<i>C. neoformans</i> Culture (source, result)
1	>1:1280	Reticulonodular infiltration in both lung	Negative	Sputum/BAL, positive
2	>1:1280	Reticulonodular infiltration in both lung	Negative	sputum/BAL, positive
3	1:160	Bilateral interstitial infiltration	Positive	Sputum, positive
4	1:40	Interstitial infiltration in left lower lung	Positive	BAL, positive
5	1:160	Bilateral interstitial infiltration	Positive	Sputum, positive
6	1:160	Bilateral interstitial infiltration	Positive	Sputum, positive
7	1:40	Patchy Infiltration in upper left lung	Positive	Sputum, positive
8	1:40	Reticulonodular infiltration in both lung	Positive	Sputum, positive

C. neoformans Ab*, titer of *Cryptococcus neoformans* antibody; BAL, bronchoalveolar lavage

Table 5-3 Results of *C. neoformans* antibody in sera determined by indirect immunofluorescence, clinical manifestations and other laboratory results in patients with CNS cryptococcosis

No.	<i>C. neoformans</i> Ab	Serum Ag ^a	CSF Ag ^b	CSF Culture	Anti-HIV status	Clinical manifestations
1	Negative	Positive	ND	Positive	Positive	Seizure
2	Negative	1:512	1:32	ND	Positive	Headache, stiffness neck, fever
3	Negative	Positive	>1:1024	ND	Positive	Seizure
4	Negative	>1:1024	1:256	ND	Positive	Seizure
5	Negative	1:4	1:16	ND	Positive	Fever, headache
6	Negative	>1:1024	1:256	ND	Positive	Seizure
7	Negative	1:1024	ND	Positive	Positive	Seizure
8	Negative	1:512	1:32	Positive	Positive	Headache, fever
9	Negative	>1:1024	>1:1024	ND	Positive	Seizure
10	Negative	Positive	Positive	ND	Positive	Headache, stiffness neck

C. neoformans Ab, titer of *Cryptococcus neoformans* antibody; ND, not determined; serum Ag^a, titer of *C. neoformans* antigen in serum; CSF Ag^b, titer of *C. neoformans* antigen in cerebrospinal fluid.

Table 5-4 Results of *C. neoformans* antibody in sera determined by indirect immunofluorescence, clinical manifestation and other laboratory results in patients with disseminated cryptococcosis

No.	<i>C. neoformans</i> Ab	Serum Ag ^a	CSF Ag ^b	Anti-HIV status	Clinical manifestations/ <i>C. neoformans</i> culture
1	Negative	1:512	Negative	Positive	Fever, abdominal pain
2	Negative	1:256	1:32	Positive	Headache, fever, Lymph node enlargement
3	Negative	1:1024	1:128	Positive	Hemoculture positive
4	1:80	1:1024	1:128	Positive	Hemoculture positive
5	1:40	Negative	ND	Positive	Necrotizing granulomatous lymphadenopathy and AFB not seen in lymph node
6	Negative	1:256	ND	Positive	Disseminated cryptococcosis
7	Negative	>1:1024	ND	Positive	Disseminated cryptococcosis
8	Negative	>1:1024	ND	Positive	Disseminated cryptococcosis

C. neoformans Ab, titer of *Cryptococcus neoformans* antibody; ND, not determined;
serum Ag^a, titer of *C. neoformans* antigen in serum;
CSF Ag^b, titer of *C. neoformans* antigen in cerebrospinal fluid.

Table 5-5 Results of *C. neoformans* antibody in sera of non - *C. neoformans* infections determined by indirect immunofluorescence, clinical conditions, other laboratory results and evidence of infection in other clinical conditions.

No.	<i>C. neoformans</i> Ab	Serum Ag ^a	CSF Ag ^b	Anti-HIV status	Clinical conditions/ Evidence of infection
1	Negative	Negative	ND	positive	Pulmonary TB with PCP
2	Negative	Negative	ND	Positive	Disseminated & pulmonary TB
3	Negative	Negative	ND	Positive	Pulmonary TB
4	Negative	Negative	ND	Positive	Pulmonary TB
5	Negative	Negative	ND	Positive	Prolonged cough with pulmonary TB
6	Negative	Negative	ND	Positive	Disseminated <i>Histoplasma capsulatum</i> & pulmonary TB
7	Negative	Negative	ND	Positive	Disseminated TB
8	Negative	Negative	ND	Positive	Disseminated TB
9	Negative	Negative	ND	Positive	Disseminated TB with PCP
10	Negative	Negative	Negative	Positive	CNS tuberculosis
11	Negative	Negative	ND	Positive	Disseminated <i>Acinetobacter</i> sp.
12	Negative	Negative	ND	Positive	Pulmonary TB
13	Negative	Negative	ND	Positive	Pure red cell aplasia

C. neoformans Ab, titer of *Cryptococcus neoformans* antibody; ND, not determined; serum Ag^a, titer of *C. neoformans* antigen in serum; CSF Ag^b, titer of *C. neoformans* antigen in cerebrospinal fluid; PCP, *Pneumocystis carinii* pneumonia; TB, tuberculosis.

Table 5-6 The results of stability test of immunofluorescence assay for detection of *C. neoformans* antibody in sera

	Titer of <i>C. neoformans</i> antibody					
	1 st	2 nd	3 rd	4 th	5 th	6 th
	months					
Positive control #1	1:80	1:80	1:80	1:80	1:80	1:80
Positive control #2	1:80	1:80	1:80	1:80	1:80	1:80
Negative control #1	Neg	Neg	Neg	Neg	Neg	Neg
Negative control #2	Neg	Neg	Neg	Neg	Neg	Neg

Neg, negative; 1:80 the titer of *C. neoformans* antibody in serum

CHAPTER VI

DISCUSSION

Cryptococcus neoformans is an encapsulated yeast that commonly enters hosts through the respiratory route. The lung is, therefore, the initial site of infection. Early diagnosis for pulmonary cryptococcosis is needed before dissemination and progression of the disease to be more severe type concerned with central nervous system. Most of the fifty sera from donors gave negative results for *C. neoformans* antibody by indirect immunofluorescence and tube agglutination. However, five donor sera (10%) gave positive results of low levels of *C. neoformans* antibody. The indirect immunofluorescent assay for *C. neoformans* antibody detection gave positive results in the same donors as those obtained by tube agglutination. Unfortunately, no clinical information of these donors is available. The donors may have exposed to *C. neoformans*, developed self-limited infection, had clinically silent infection or never exposed nor been infected at all. However, indirect immunofluorescence gave higher titer than the results obtained from tube agglutination. Moreover, indirect immunofluorescence needs only two or three hours for semi-quantitative evaluation compared to three days duration is needed by tube agglutination.

All of the eight patients with pulmonary cryptococcosis gave positive results for *C. neoformans* antibody detection by indirect immunofluorescence with supporting evidences by chest x-rays results and cultures. Patient sera with CNS cryptococcosis gave negative results for *C. neoformans* antibody detection by indirect immunofluorescence. In some patients with central nervous system cryptococcosis, infections may be extensively disseminated throughout the body involving visceral organs. In these patients with systemic or extrapulmonary nonmeningeal disseminated cryptococcosis, the free circulating antigen disseminated hematogenously can neutralize the specific antibody in the patient sera. In disseminated cryptococcosis patients, two of eight sera gave positive results with low dilution for *C. neoformans*

antibody. The presence of *C. neoformans* antibody may be used for assessment the severity of the disease or for better prognosis.

The results of *C. neoformans* antibody in the sera of patients with the diseases other than cryptococcosis (pulmonary tuberculosis, disseminated histoplasmosis) tested by indirect immunofluorescence gave negative results. These results indicated that the test has no cross-reactions with some common pathogens other than *C. neoformans*.

Vogel RA et al.⁹⁹ has reported the results of *C. neoformans* antibody in sera assayed by indirect immunofluorescence from patients with proven cryptococcal disease which gave positive results in 80 % of the 45 cases and negative reaction were noted in 12 patients. The sera with the negative indirect immunofluorescence were subsequently shown to have low concentrations of gamma globulins. The positive results for antibody are suggestive of infections by *C. neoformans* especially in the early course of the disease and in localized infections, and could also reflect a past infection. The incidence of positive antibody results in the patients increases when the patients exhibit solitary lung lesions, no central nervous system involvement, or early central nervous system involvement. Casadevall A et al.⁶¹ and Martinez LR⁹⁴ demonstrated that protective monoclonal antibodies bound on *C. neoformans* with an annular immunofluorescence pattern. All of the eight sera from patients with pulmonary cryptococcosis in this study gave positive results for *C. neoformans* antibody by indirect immunofluorescence with annular immunofluorescence pattern on *C. neoformans* which could be the confirmation for the protective antibody against the pathogens.^{61, 94} This is the evidence that the humoral response does occur in early stage of cryptococcosis of the pulmonary cryptococcosis patients and this antibody is applicable for early diagnosis of cryptococcosis. However, no specific antibody could be detected in patient sera with CNS cryptococcosis and low levels of specific antibody detected in extrapulmonary nonmeningeal disseminated cryptococcosis patients. It might be caused by the decreasing of antibody production in HIV patients or the neutralization of specific antibody by circulating *C. neoformans* antigen which is enormous in the patient serum.

Henderson et al., 1982 showed that the immune response of cryptococcosis in experimental animals is similar to that in immunocompetent individuals where there is

an excess antigen in blood circulation, the antibody cannot completely neutralize the antigen and leads to disease progression. Those abundant antigen could be then detected in blood circulation. In contrast to in the immunocompromise patients, the antibody may not be produced and undetectable which might be the result of reduction of the cells that capable to produce antibody rather than the neutralization of antibody by excess antigen in blood circulation.⁷¹ The negative results for serum antibody correlated significantly with high risk of death from cryptococcal meningitis.⁶⁴ Unfortunately, the pattern of immune response to cryptococcal polysaccharide is erratic, in addition, when circulating antibody is found, titers are usually low, regardless of antibody assay. On occasion, humoral antibody has been detected early in the infection, throughout the infection or concurrent with circulating polysaccharide. The positive result for antibody appears to be inversely related to the quantity of antigen in the patient.

The indirect immunofluorescence assay for *C. neoformans* antibody detection is considered valuable particularly in early diagnosis pulmonary cryptococcosis. The reproducibility and stability of the indirect immunofluorescence tests were assessed by assaying two positive sera and two negative sera repeatedly for six times in six months. All tested sera showed identical results. This indicates that the indirect immunofluorescence tests are reliable and reproducible since the stability of indirect immunofluorescence was validated by using the long period of the storage of *C. neoformans* substrate at -40°C without changing the antibody titers of the pooled sera positive for *C. neoformans* antibody and the sera negative for *C. neoformans* antibody which were aliquoted and stored at -40°C.

CHAPTER VII

CONCLUSION

Pulmonary cryptococcal infection is relatively rare. Several types of yeasts can be isolated from the respiratory tract of immunocompetent hosts. *C. neoformans* is not considered a normal respiratory flora in human or animals. As a consequence, its isolation from respiratory specimens warrants attention for physicians. In some cases, *C. neoformans* can represent a pulmonary infection, either alone or in association with a disseminated disease, in other cases, it can represent an asymptomatic carriage.¹ The distinction between disseminated cryptococcal disease, pulmonary cryptococcosis and airway colonization with *C. neoformans* has important diagnostic and therapeutic implications. However, this distinction may be difficult to ascertain.

Cryptococcosis is usually a systemic invasive fungal infection that typically presents as meningitis. It is assumed that *C. neoformans* generally gains access to the host by inhalation and that the respiratory system is the usual portal of entry. The evaluation of the significance of respiratory cryptococcal isolates becomes an important part of the decision-making process.

In case of pulmonary infection, *C. neoformans* is localized in the lung and could induce the host to produce specific antibody which can be detected in the serum. The detection of antigen in circulation is mostly failed in patients with primary pulmonary cryptococcosis since the hosts would form granulomatous tissues surrounding the pathogen.¹³ The detection of *C. neoformans* antigen in sera can give positive results only in the cases with extensive pulmonary infections or those with initially hematogenous spread of the disease. Although the granulomas can be detected by chest X-ray but discrimination from other types of pulmonary infections is difficult.

The indirect immunofluorescence for *C. neoformans* antibody offers the sensitivity and specificity and the test is reproducible yielding the results comparable to the gold standard test by tube agglutination test. The assay is simple, rapid, inexpensive and easy to perform. The slides containing *C. neoformans* are stable in

deep freezing at -40°C for at least up to 6 months. The performance of the assays could be completed in approximately 3 hours. Thus, it could be used as an alternative and definitive test for diagnosis of cryptococcosis especially pulmonary cryptococcosis.

The indirect immunofluorescence is considered to be valuable particularly in the purpose of early diagnosis of primary pulmonary cryptococcosis before the disease disseminated to the central nervous system.



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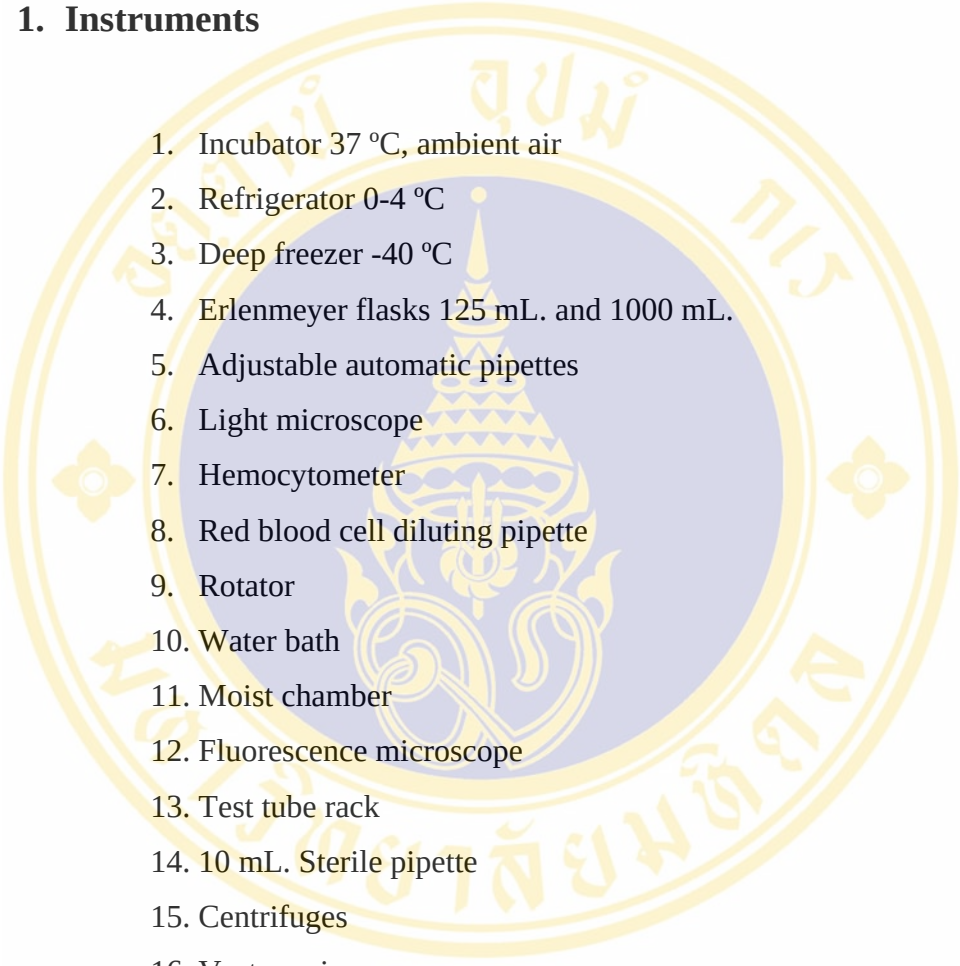
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APPENDIX

1. Instruments

- 
1. Incubator 37 °C, ambient air
 2. Refrigerator 0-4 °C
 3. Deep freezer -40 °C
 4. Erlenmeyer flasks 125 mL. and 1000 mL.
 5. Adjustable automatic pipettes
 6. Light microscope
 7. Hemocytometer
 8. Red blood cell diluting pipette
 9. Rotator
 10. Water bath
 11. Moist chamber
 12. Fluorescence microscope
 13. Test tube rack
 14. 10 mL. Sterile pipette
 15. Centrifuges
 16. Vortex mixer

2. Supplies

1. Coverglass and oil immersion
2. Microscopic slides
3. Automatic pipette tips
4. Test tubes 13 x 100 mm.

3. Reagents

1. Sabouraud dextrose agar (SDA) slant

Casein peptone	5.0	g.
Meat peptone	5.0	g.
Dextrose	40.0	g.
Agar	15.0	g.
Demineralized water	1,000	mL.

pH 5.6±0.2 at 25°C

Heat boiling with agitation to complete dissolve.

Sterilize by autoclaving at 121 °C for 15 minutes

2. 0.9% sodium chloride solution

9 g. of NaCl per litre of water

- 154 mmol/L sodium
- 154 mmol/L chloride
- Osmolality 308 mosm/L

3. 1% Formalin in 0.9% sodium chloride solution

40% formalin	1	mL.
0.9% sodium chloride solution	99	mL.

Mix well on magnetic stirrer

4. 10 % Methanol in Sterile Distilled Water

Methanol	100	mL.
Distilled water	900	mL.

Mix well on magnetic stirrer

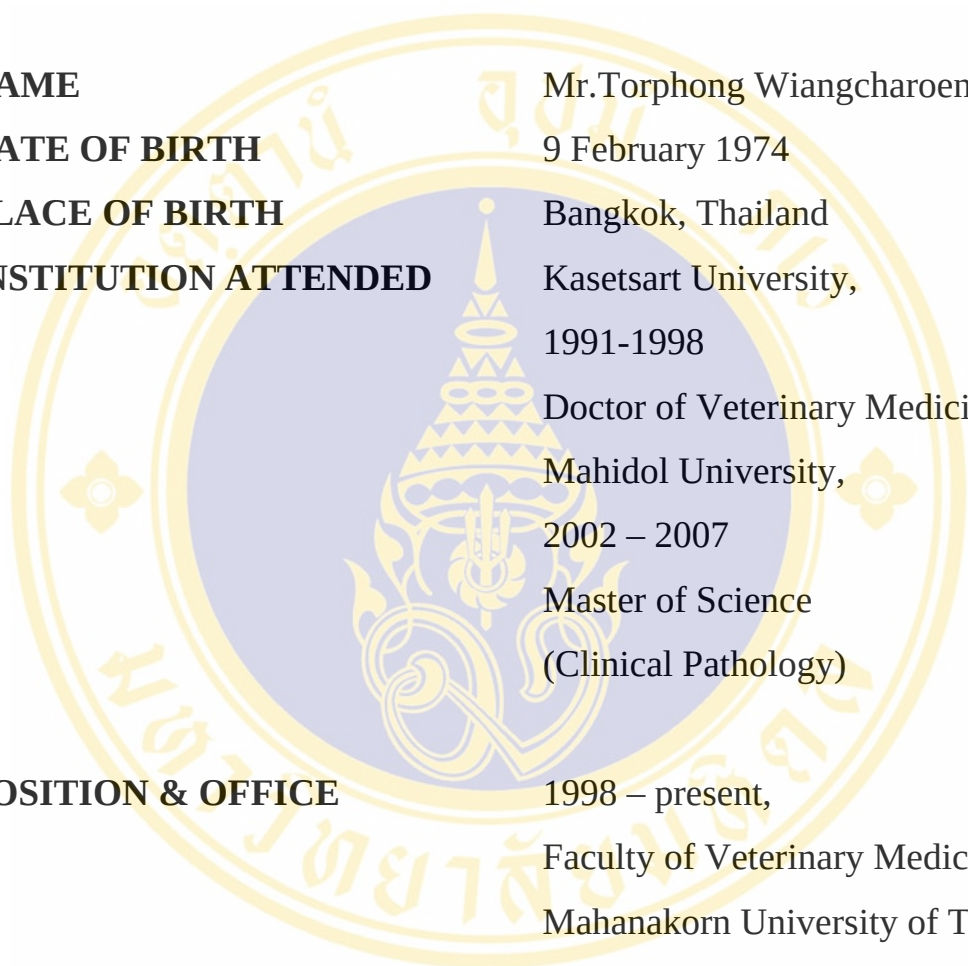
5. 0.1 M Phosphate Buffer Saline pH 7.2±0.1 Containing 2%Tween80
(FA buffer)

NaCl	15.30	g
Na ₂ HPO ₄	1.45	g
KH ₂ PO ₄	0.42	g
Add distilled water	2000	mL
Adjust pH to 7.2 with NaOH or HCl		
Add NaN ₃ , 0.1 % w/v		
Add Tween 80, 2% w/v		

6. Sterile Distilled Water

7. FITC labeled polyclonal rabbit anti-human globulin (IgA, IgG, IgM,
kappa, Lamda/FITC; DAKO)

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