CHAPTER III

LITERATURE REVIEW

Cryptococcosis is an infection caused by the encapsulated fungus *Cryptococcus neoformans*. *C. neoformans* was first isolated from peach juice in Italy by F. Sanfelice in 1894. Sanfelice called cryptogamic yeast *Saccharomyces neoformans*. The disease was then called saccharomycosis. Almost at the same time, Brusse, a German pathologist observed the round-to-oval "corpuscle" in a sarcoma-line lesion of the tibia of 31-year-old-woman. In 1985, Sanfelice proved the pathogenesis of *S. neoformans* by inoculating the yeast into experimental animals. In 1901, the French mycologist Jean-Paul Vuillemin, reclassified by Brusse in the Genus *Cryptococcus* (18, 4). The first published case of cryptococcal meningitis diagnosed ante mortem was a 29-year-old woman with leptomeningeal involvement described by Verse in 1914. Cryptococcosis was considered a rarity in early 1900s. The co-incidence of the increasing number of cryptococcosis case and HIV-case are reported (19).

Mycologic features

Cryptococcus neoformans is an encapsulated ubiquitous environment basidiomycetous yeast. It is the causative agent of cryptococcosis, a potentially serious disease that affects healthy and immunocompromised individuals, especially patients with AIDS (3). The life cycle of *C. neoformans* involves 2 different forms; asexual and sexual. The asexual form exists a yeast cell and reproduces by budding. These haploid, unicellular yeasts are the only forms of *C. neoformans* that have been recovered from the environment and human infection. These yeast-like forms exist in one of 2 mating type: α- or a- and when yeasts of each mating type are combined on certain agars. They can undergo conjugation to produce the perfect (sexual) state. Conjugation between the α- and a-mating types results in the formation of the teleomorph which consists of dikaryotic hyphae that bear clamp or hypha outgrowth connections. Some of the hyphae develop into specialized structures called basidia.

Meiosis occurs at the terminal portion of the basidium and uninucleate basidiospores are formed. The spores are initially unencapsulated but can quickly develop capsules when released from the basidia and begin budding, thus completing the life cycle. The perfect state of *C. neoformans* has been observed only in the laboratory and never conclusively in nature. When the mating types of *C. neoformans* strain recovered from the either environment or patient samples have been determined, there has always been a large excess of α-mating types over a-mating type (19). α-Mating type and a-mating type are determined by a single locus. The α-mating type strain has been shown to be more virulent than the a-mating type. PCR analysis with primers specific for genes in the MAT α or MAT a mating type loci revealed that serotype AD strains are heterozygous for the diploid strain that result from mating between serotype A and D strains. MAT α strains have been shown to develop extensive hyphae, without mating, and to produce viable basidiospores under appropriate condition. Mating type and ploidy are also important for understanding the ecology and virulence of this fungus (20).

C. neoformans has 5 serotypes: A, B, C, D, and AD, and is subdivided into 3 varieties, i.e. var. *grubii* (serotype A), var. *neoformans* (serotype D), and var. *gattii* (serotype B and C) (20, 21). The 3 varieties differ in some biochemical properties such as the ability of var. *gattii* strain to use glycine or D-proline as sole source of nitrogen whereas var. *neoformans* strain can not. The var. *gattii* strain is also resistant to the chemical canavanine whereas var. *grubii / neoformans* strain is usually sensitive to utilize glycine as the sole carbon and nitrogen source, and sensitive to canavanine. *C. neoformans* var. *grubii / neoformans* can be separated from *C. neoformans* var. *gattii. C. neoformans* var. *gattii* can be grown on CGB agar but var. *neoformans* strain usually rare isolates on CGB agar because CGB agar contains glycine and canavanine. When var. *gattii* strain is grown on CGB agar, glycine is metabolized and results in the formation of ammonia which alkalinizes the medium and turn it blue (19). Serotyping was performed by a slide agglutination procedure using polyclonal antibodies obtained from the immunization of male New Zealand white rabbits with heat-killed cells from the reference strain of serotype A, B, C, and D. Based on the antigenic determinants of the polysaccharide capsule serotype A, D, and AD and serotype B and C have been identified (17). Recent study, Aoki *et al* (1999) (11) developed new useful PCR primer pairs which should be specific for *C.*

neoformans serotype A or B on the basis of RAPD fingerprinting pattern analysis of *C. neoformans*.

Although each variety can produce similar clinical syndromes, there are some differences among var. *grubii*, var. *neoformans*, and var *gattii*. Infections with varieties *neoformans* and *grubii* frequently present with disseminated infection and meningoencephalitis. Disease from *C. neoformans* var. *neoformans* and var. *grubii* is associated with immunocompromised individuals, specifically with AIDS patients. In contrast, disease due to *C. neoformans* var. *gattii* almost always affects individuals with no identifiable immune impairment (3, 22, 23).

Ecology and epidemiology

A better understanding of the ecology and the epidemiology of *C. neoformans* are important for the development of strategies of primary prevention in immunocompromized people. Destroying chain of transmission is also important and necessary (24). *C. neoformans* var. *neoformans* has been isolated from various sources in nature and is noted for its association of avian guano, especially pigeon droppings. It has also been isolated from droppings of caged birds including canaries, parrots, and budgerigars. Other environmental isolations have been made from rotting vegetables, wood, dairy products, and soil. The pigeon is the obscure host of cryptococcus in nature. Beak, crop, feet, and rectum are the site that can found low concentration of *C. neoformans*, because the bird has high body temperature which is inhibitory to cryptococcal multiplication. Dry pigeon droppings are the potential source of cryptococcus in the nature. Very high concentration of yeast (asexual form) of these organisms is found in dry pigeon droppings, an environment which is unfavorable to the growth of mast other organism. In contrast, fresh pigeon droppings have been high concentration of ammonia that can inhibit cryptococcus growth (13).

Mating type is also important for understanding the ecology of *C. neoformans*. Mating type α is more frequent than MAT a, among clinical as well as environmental isolates. Wild-type haploid *C. neoformans* could develop a hyphal phase under appropriate conditions, producing basidia with viable basidiospores. This finding possibly suggested the hypothesis that the association of the hyphal phase with the α-

mating type may explain the role of this mating type in the environment (3). The forming ability of basidiospores was independent of serotype (19).

Several infectious particles spread by air represent the most important source of pulmonary contamination after inhalation, and there is no consensus about its origin whether it is asexual form or sexual form. Although, it is believed that dissemination to the brain occurs from the lungs. Evidence from animal studies showed that it may be possible for the fungus to gain access to the brain by transiting directly through the sinus cavity. Molecular studies with Brazilian samples showed that the majority of infections are caused by heterothallic strains classified as MAT α that are also prevalent in environmental samples of *C. neoformans* (21). The ratio of MAT α : MAT a for environmental isolates was ~ 40:1 and for clinical isolates was ~ 30:1. The highly number of MAT α in clinical and environmental isolates suggest that MAT α cells are more infective than MAT a cells. However, the MAT α to MAT a bias is conserved in environmental isolates as well (3).

C. neoformans var. *grubii* / *neoformans* (serotype A, D, and AD) has been isolated from various sources in nature and it is noted for its association with accumulation of avian guano, especially pigeon droppings. It has also been isolated from droppings of caged birds, including canaries, parrots,. and budgerigars. *C. neoformans* var. *gattii* (serotype B and C) has a more restricted geographical distribution than *C. neoformans* var. *grubii* /var. *neoformans* (serotype A, D, and AD), and causes human disease in climates ranging from temperature to tropical in Australia, India, South-East Asia, Mexico, Brazil, Paraguay, and Southern California. *C. neoformans* var. *gattii* has been isolated from various *Eucalyptus* species such as *E. camadulensis*, *E. tereticornis*, *and E. gomphocephala.* The life cycle of *C. neoformans* var. *gattii* in association with the trees is unknown (13, 24). Studies of Lazera *et al* (1998) (21) gave new dimensions to the ecology of the cryptococosis agent in Brazil demonstrating that yeast is associated not only to *Eucalyptus* sp. , but also to other species of tree.

The recent availability of DNA fingerprinting technique has greatly extended our knowledge of the *C. neformans* epidemiology over the last few years. Indeed, RAPD analysis, RFLP, karyotyping, allele sequencing, and multilocus enzyme electrophoresis have helped answer several important questions (15). RAPD

fingerprinting has been demonstrated to have the ability to discriminate between closely related isolates within a give population (11). Meyer *et al* (2003) (16) reported that RAPD fingerprinting technique generated with M13 primer was useful for distinguishing among the varieties, serotypes, and molecular type (VNI-VNIV, VGI-VGIV) of *C. neoformans*.

RAPD are DNA fragments, which amplified by the polymerase chain reaction (PCR) using short synthetic primers (generally 10 bp) of random sequence. The number and location of these random primer sites vary for different strains, thus creating a characteristic pattern of bands for particular strains. RAPD analysis is technically simple, less time consuming, and often detects variation among isolates that are invariant with RFLP analysis (25). Casali A.K. (2003) (15) used microsatellite-specific primer M13 and the minisatellite-specific primer $((GACA)_4)$ grouped the majority of isolates into the molecular type VNI (89.5 of the clinical and 52.6% of the environmental isolates). Meyer *et al* (1999) (26) had shown that more than 1,000 global cryptococcal isolates were grouped into eight molecular types (VNI-VNIV, VGI-VGIV). In all studies, the molecular type VNI and VGI were the most common genotypes worldwide. Horta, J.A. (2002) (6) described the isolation and characterization of 17 clinical and 10 environmental (pigeon droppings) isolates from the Brazilian state Rio Grande do Sul, using RAPD analysis. His results are also consistent with reports that *C. neoformans* var. *grubii* serotype A is represented among AIDS patients not only in Brazil but also all over the world. This variety has long been known to be associated with pigeon droppings, and the majority of pigeon dung isolates examined in most countries has also been serotype A.

Molecular typing and serotyping of *C. neoformans* isolated from clinical and environmental sources have been studies in worldwide. The differently geographical countries have been found the different serotypes and molecular types. Serotype A and D have been isolated from various sources in nature and associated with pigeon droppings. For *C. neoformans* var. *gattii* serotype B, a specific ecological association with *E. calmadulensis* and *E. tereticornis* has been established (27). In France, Hermosa *et al* (1997) (28) could isolate *C. neoformans* from 26 clinical and 29 environmental isolates from the same area. They suggested that pigeon droppingd are a potential source of pathogenic strain of *C. neoformans* serotype D. In Spain, Baro *et*

al (1999) (17, 29) determined serotypes of 154 isolates of *C. neoformans* from clinical and environmental sources from different areas of Spain. All clinical isolates belonged to *C. neoformans* var. *grubii* / *neoformans*. Serotype A was the major serotype (62%) in clinical specimens, but serotype D (29%) and AD (9%) are frequent and predominant in some areas. Environmental sources were serotype A (79%), serotype D (15%), and serotype AD (16%). They suggested that there were some relative prevalence of serotype D in some areas of France and Italy. Data from American isolates also showed a higher prevalence of serotype A than serotype D in Brazil and Cuba. Meyer *et al* (2003) (16) studied molecular typing of *C. neoformans* in IberoAmerican countries. The result showed that the 226 clinical isolates, 177 (66.5%) were obtained from HIV-positive patients, with 139 (78.5%) being VNI, 14 (7.9%) VNII, 13 (7.4%) VNIII, 6 (3.4%) VNIV, 3 (1.7%) VGII, and 2 (1.1%) VGIII. Most of the environmental isolates belonged to *C. neoformans* var. *grubii* with 73.1% being VNI (n=49) and 1.5% VNIII (n=1) AD hybrid. The remaining 17 (25.3%) isolates were *C. neoformans* var. *gattti*. In the past, Li *et al* (1993) (30) studied *C. neoformans* in China. They found that 78% were serotype A and 22% were serotype AD. Creseo and Gallo (1997) (31, 32) studied *C. neoformans* from 97 environmental and 4 clinical isolates in southern Italy. All proved to be *C. neoformans* var. *grubii* (serotype A). They suggested that geographical climatologically conditions may play a role in *C. neoformans* serotype diffusion. It was furthermore revealed that, unlike the data reported for the serotypes of *C. neoformans* in mainland Italy, serotype D was not presented in the southern Italy. In Bangkok, Thailand, Imwidthaya *et al* (1989) (33) isolated *C. neoformans* from 13 patients. Also, 13 isolates of *C. neoformans* were obtained from feces of 30 pet birds. All 26 isolates of *C. neoformans* were found to be of serotype A and D. Sukroongreung *et al* (1996) (34) isolated *C. neoformans* from 187 patients prior to the AIDS epidemic in Thailand. They found that 55% were serotype B, 28% (serotype A), 5% (serotype D), and 11% (serotype C). In contrast, among the 169 clinical isolates obtained between January 1993-March 1995 (AIDS epidemic), serotype A was outstandingly predominant ~ 93%, serotype B was relatively low (3.6%) and both serotypes D and AD were 1.8%. In 1997, Poomwan *et al* (35) isolated *C. neoformans* from 139 AIDS patients. 133 clinical isolates were serotype A.

In 1999, Imwidthaya *et al* (5) studied *C. neoformans* in 87 Thai patients with cryptococcal meningitis. These were 86 patients with serotype A and one patient with serotype B. In 2002, Krelstein *et al* (36) used Fourier Transform Infrared (FTIR) spectroscopy that could be examined the genetic heterogeneity of *C. neoformans*. They found that isolated *C. neoformans* strains from pigeons (serotype D) could be divided into 3 clusters and from pet birds (serotype A) into 2 different clusters by FTIR spectroscopy. It is important to take into account heterogeneity of strains within serotypes for determination of infection chains of human disease.

Biochemistry, Molecular Biology and Virulence

C. neoformans can be grown on a variety of agars, and develops white, mucoid colonies that usually become visible to the naked eye within 48 hours of incubation. The mucoid appearance of the colonies is caused by the cell's production of a polysaccharide capsule. The optimal growth temperature of most strains is between 30 °C and 35 °C, and the maximally tolerated temperature is 40-^C. The yeast forms are round to oval with diameter between 2.5-10 μm. Hyphae are never seen with the yeast forms, although pseudohyphael form can sometimes be found when the yeast cells are stressed by environmental conditions such as elevated temperature. Mucoid colony, encapsulated budding yeast on an India ink preparation, and rapid urease are the important properties of *C. neoformans*. Among the cryptococci, *C. neoformans* is the only one that can produce enzyme phenol oxidase when they was cultured on special agar such as niger seed agar, bird seed agar, and caffeic acid agar after that *C. neoformans* will be changed in its color from white to black colony(phenol oxidase positive) (19). Culture of *C. neoformans* grown in the presence of precursors (L-DOPA, dopamine, norepinephrine, and epinephrine), are brown to black in color. The appearing color is the melanin pigment. Melanin is a negatively charged pigment that is produced by laccace through the oxidative polymerization of phenolic compounds (22).

Virulence factors in *C. neoformans* such as polysaccharide capsule, phospholipase, urease, and proteinases. Mating types are essential survival factors in the human host. At least 4 genes, CAP59, CAP60, CAP64, and CAP10 are essential for capsule formation and confer virulence. The capsule is composed of at least 3 components; mannoprotein, galactoxylomannan, and glucuronoxylomannan (GXM).

The main component of the polysaccharide capsule is GXM. The capsule inhibits ingestion of yeast cells by phagocytes in the absence of opsonin. Phagocytosis of cryptococcal cells requires either complement or antibody opsonins (22, 24). The virulence-enhancing properties of the cryptococcus laccase, involved in the formation of melanin from catecholamine precursors. The mechanism of action of laccase in pathogenesis is not entirely clear. Melanin appears to be laid down on the inner aspect of the cell wall and is known to have antioxidant effects. Alternatively, laccase through its iron oxidase activity may protect *C. neoformans* from phagocyte attacking by reducing iron-catalyzed formation of hydroxyl radicals. Substrates for laccase include dopa, dopamine, adrenalin, and noradrenalin found in abundance in the central nervous system (24). The neurotropism of *C. neoformans* may be explained in part by the fungus ability to use such neurotransmitters as epinephrine and dopamine as substrates for melanin production (22). Infection with *C. neoformans* is thought to occur by inhalation of aerosolized organisms from environmental sources, and the sizes of cryptococcal cells collected from pigeon droppings are compatible with alveolar deposition. Furthermore, Nosanchuk *et al* (1999) (37) showed structures similar to the melanin "ghosts" of melanized cryptococcal cells that were isolated from pigeon droppings contaminated with *C. neoformans*. They suggested that environmental *C. neoformans* cells are melanized. Melanin synthesis in pigeon droppings may potentially protect cryptococcal cells against environmental stresses such as UV light and extremes in temperature. They implied that initial infection may involve exposure to melanized cells. In addition to capsule and melanin pigment, *C. neoformans* can produce protease and phospholipase. These enzymes can be promoted *C. neoformans* to penetrate into host tissue (7).

Cryptococcosis

Cryptococcosis is an opportunistic important disease of the advanced stage of HIV infection, occurring when the concentration of CD4-positive thymic-derived lymphocytes is below 200 cells/mm^3. Cryptococcosis has already disseminated to the brain at the time of diagnosis in almost all AIDS patients. The remainder has fever and positive culture from blood, bronchoalveolar lavage (BAL) fluid, urine or some other sites (18). The clinical presentation of cryptococcosis is dependent upon the immunocompetence of the infected patient. Infection may be superficial or deep,

localized or diffuse, and can selectively involve specific organ or disseminate to multiple organ systems. The portal of entry for the organism is the lung; however, pulmonary infection is usually asymptomatic. Central nervous system involvement is the most frequently diagnosed form of cryptococcosis (38). Cryptococcal meningoencephalitis is the common form of cryptococcosis patient. The clinical presentation of the infection in AIDS versus non-AIDS patient is slightly different. The onset of symptoms was usually over a period of 1-2 weeks, the 3 most common symptoms were headache, fever, and malaise. Symptoms such as stiff neck, photophobia, and vomiting were seen only in a minority of patients. Pulmonary cryptococcosis is less common than meningitis as a presenting complaint in patients with AIDS. The symptoms of pulmonary cryptococcosis may be shown fever, cough, dyspnea, pleuritic chest pain, and haemoptysis. *C. neoformans* can cause almost any type of skin lesion. It was noted that skin lesion could appear a acneiform lesions, purpura, vesicles, nodules, abscesses, superficial granuloma, etc. (19). Among AIDS patients in Thailand, cryptococcosis is the second most common opportunistic infection after tuberculosis (5). Husain *et al* (2001) (39) reviewed the published reported. They found that *C. neoformans* infection was documented in 2.8% of the organ transplant recipients, with an overate death rate of 42%. The type of primary immunosuppressive agent used in transplantation influenced the predominant clinical manifestation of cryptococcsis.

Infection due to *C. neoformans* var. *gattii* was significantly more common in rural or semi rural areas on the Australian mainland, broadly corresponding to the distribution of the environmental reservoir of this variety, *E. camalsulensis* and *E. tereticonis* (40). In Singapore, the first case report of *C. neoformans* var. *gattii* from a patient was presented by Taylor and colleagues in 2002 (41). A patient had visited Bangkok in 2000 and Kuala Lumpur, Malaysia, in June 2000. Capsulated yeasts were seen in a bronchial biopsy and *C. neoformans* var. *gattii* was subsequently cultured from a stereotaxic brain biopsy. *C. neoformans* can be infected the various animals such as cat, dog, fox, monkey, cattle, etc. Cryptococcosis is the most common systemic fungal infection in cats. Nasal and pulmonary disease manifestations are common. Infection of the nasal cavity is reported most frequently (56.3-83.0% of cases). Central nervous system signs of disease result from diffuse or focal miningoencephalitis (42). Malik et al (2003) (43) reviewed the avian cryptococcosis.

They found 15 unreported cases of avian cryptococcosis from Australia. Cryptococcus species produced localized invasive disease of the upper respiratory tract of captive parrots living in Australia. The appearance of mycotic rhinitis can invade to other sites nearby the upper respiratory tract such as beak, sinus, and palate. *C. neoformans* var. *gattii* was isolated from infective sites. They suggested that exposure to eucalyptus material may be a predisposing factor.

Behavior of the pigeon

Columbia livia is the scientific name of the Rock Dove (Rock pigeon, feral pigeon, and common pigeon). The pigeons develop populations in nearly all cities around the world. They can rear up to 11 young each year. The great pigeon populations are responsible for many problems in the cities. The feeding sites of pigeons may be several miles away. When pigeons are not feeding or mating, most of their day is spent cooing, preening, and sunbathing. Pigeons prefer flat and smooth surfaces on which to rest and feed. Typical feeding sites are parks, squares, food-loading docks, garbage areas, railroad sidings, food plants, and wherever people eat outdoors(44, 45). The rock dove has a dark bluish-gray head, neck, and chest with glossy yellowish, greenish, and reddish-purple iridescence along its neck and wing feathers. The bill is dark grayish-pink. Two dark bands across the wings are seen in most pigeons and one bluish-gray band across the tail (46). Haag-Wackernagel (2004) (47) studied the daily activities rhythms and the home range of feral pigeons by Global Positioning System (GPS)-based. He found that the pigeon flew the distance between the two places at a speed of 50 km/h on the first way and 46 km/h on the flight back to the loft. The two phases had 0.8 km between the first and second places.