CHAPTER 2

LITERATURE REVIEWS

2.1 *Histoplasma capsulatum*

2.1.1 History and Taxonomy

Samuel Taylor Darling a pathologist observed a fatal case of disseminated histoplasmosis in Panama in 1904. Darling has first thought these intracellular organisms were *Leishmania*, but on close examination, he noted that the organisms lacked kinetoplasts. Thus, he concluded that the organism was a new protozoa, which he named *Histoplasma capsulatum* (Darling, 1906). In 1912, de Rocha-Lima, a Brazilian studying in Hamburg, was the first to suggest that the microorganism of Darling was a yeast rather than a protozoa. Since then, the disease has been diagnosed with increasing frequency (Kwon-Chung and Bennett, 1992). However, the diagnosis had always been made post mortem until 1934, when Dodd and Tompkins diagnosed histoplasmosis in a living infant (Dodd and Tompkins, 1934). The fungal culture from this case was isolated and studied by De Monbreun at Vanderbilt University (DeMonbreun, 1934). De Monbreun discovered the dimorphism of *H. capsulatum* and concluded that the saprophytic form of *H. capsulatum* probably existed in nature.

In 1948, Emmons isolated *H. capsulatum* from soil sample which were collected near the entrance of a rat burrow under the edge of chicken house. After his discovery, several scientists reported the isolation of this fungus form soil and reported it in 1949 (Emmons, 1949). The first epidemic of histoplasmosis occurred in

1947 in Camp Gruber, Oklahoma (Cain et al., 1947). Since then, famous epidemics have been reported form the Midwestern and eastern states of the United States and from various countries in South America.

Duncan was the first to isolate a new form of histoplasmosis in Africa that was histopathologically different from classical histoplasmosis and described the disease in his paper (Duncan, 1958). He reported that the mycelial and yeast forms of this fungus are indistinguishable from those of the classical histolasmosis. However, some biological, physiologic, immunologic and serologic differences between the two fungi have been found. In 1952, Vanbreuseghem described the agent as a new species, *H. duboisii* (Dubois, 1952). In 1972, Kwon-Chung discovered the sexual reproduction of *H. capsulatum* and named the teleomorph *Emmonsiella capsulata* which has been transferred to the genus *Ajellomyces* by McGinnis and Katz (Kwon-Chung and Tewari, 1987; McGinnis and Katz, 1979). *Ajellomyces capsulatus is* classified in the family Onygenaceae and the order of Ascomycota.

The varietal status applies to the anamorph, including *H. capsulatum* var. *capsulatum, H. duboisii* (causative agent of histoplasmosis) and *H. capsulatum var. farciminosum* (causing epizootic lymphangitis in horses) .

2.1.2 General morphology and characteristics

Histoplasma capsulatum is a dimorphic fungus that remains in a mycelial form at environmental temperatures and grows as yeast at body temperature in mammal.

Colony and microscopic morphology of the mold form.

Histoplasma capsulatum produces moderately growing, initially white or buff-brown on agar media at 25-30 ºC (Figure 1). The white colonies tend to produce a thicker mycelial matt than the buff-brown colonies. The buff-brown colonies tend to produce sparse aerial hyphae and abundant macroconidia at first, but become white with dense aerial hyphae upon repeat transfer (Kwon-Chung and Bennette, 1992).

Histoplasma capsulatum and *H. capsulatum* var. *duboisii* generate two types of conidia on mycologic agar media at temperatures below 35ºC: tuberculate macroconidia (5-8 µm in diameter) with typical thick walled, spherical, or occasionally oblong or pear shaped and finger-like projections; and oval microconidia (2-5 µm in diameter) with smooth to finely roughened wall, and in subculture only 30% of the macroconodia show tubercles (Figure 2). Microconidia are produced abundant in fresh isolants of the fungus. Both macro- and microconidia are produced at the tips of short, narrow phores and at right angles to the vegetative hyphae. About 10% of strains of *H. capsulatum* have smooth conidia in the primary isolation. These are different from *B. dermatitidis* or other *Chrysosporium* species. However *Sepedonium* also produces tuberculate macroconidia but no microconidia production (Kwon-Chung and Bennette, 1992).

The teleomorph of *Histoplasma capsulatum* is known as *Ajellomyces capsulatus*. It is heterothallic and morphologically consistent with fungi classified in the family Arthrodermataceae of the Ascomycotina. In mated pairs appearance, tightly coiled hyphae radiate from a common source at the base of a naked young ascocarp. The morphological manifestation of *A. capsulatus* is differentiated from

other genera of Gymnoascaceae by the formation of tight coils radially arising from the ascogonium and by sinuately branched, anastomosed peridial hyphae that arise laterally from these coils (Kwon-Chung, 1973).

Colony and microscopic morphology of the yeast form

In the host or in laboratory culture at 37ºC, the fungi develop in the yeast form (parasitic form). The yeast form of the fungus does not develop directly from the conidium when transferred to an incubation at 37 ºC but originates within the mycelium itself (Howard, 1962). In vitro, the colony is cream-colored and becomes gray with age. The average size of the yeast cell is 2-3 x 3-4 µm with a narrow bud neck. In tissues, the two varieties of *H. capsulatum* differ in their appearance. *H. capsulatum* var. *capsulatum* appears as tiny 2-4 µm oval budding yeasts usually found inside macrophages or tissue histiocytes. The yeast cell of *H. capsulatum* var. *duboisii* is larger, 8-15 µm, thick-walled, may appear as short chains in tissues, and shows the scar from which its bud has been released at one end (Kauffman, 2003).

2.1.3 Sexual reproduction.

The sexual state of *H. capsulatum* was first reported to be *Gymnoascus demonbreunii*, a homothallic soil ascomycete, by Ajello and Cheng (Ajello and Cheng, 1967). *H. capsulatum* and *H. capsulatum* var. *duboisii* are heterothallic and produce the *Ajellomyces* state when two compatible (+) and (-) isolates are mated on special agar media. Organisms of (-) mating type are found more frequently in samples from patients with pulmonary histoplasmosis; however, organisms of both

mating types are represented equally in samples from patients with severe disseminated histoplasmosis and in environmental samples (Kwon-Chung et al., 1974a; Kwon-Chung et al., 1984). Mating occurs under appropriate conditions in the mycelial phase when hyphae arising from organisms of opposite mating type appose and generate a complex structure comprising of a net of short branching hyphae covered with coiled surface hyphae. Within this specialized closed structure, the cleistothecium, cytoplasmic and nuclear fusion occur followed by successive rounds of meiosis and mitosis generating sac-like asci containing 8 ascospores, the end-product of sexual reproduction (Laskowski and Smulian, 2010).

Figure 1 *Histoplasma capsulatum*. The thermal dimorphic fungus, existing in the form of mycelia at 25˚C (A) and in the form of yeast at 37˚C (B).

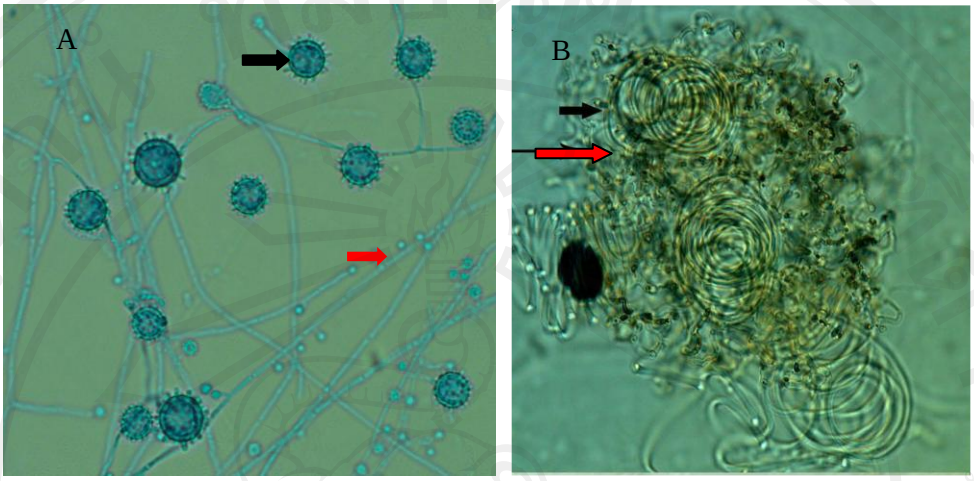

Figure 2 Asexual reproduction (A) and sexual reproduction (B) of *Histoplasma capsulatum*. Tuberculate macroconidia are indicated by black arrows and microconidia are indicated by red arrows image, 400× (A). Cleistothecia formed by *Ajellomyces capsulatus* image, 400×. The coiled surface hyphae are identified by black arrows while the net of short, branched hyphae are identified by red arrows (B).

2.1.4 Epidemiology and ecology

Histoplasmosis occurs throughout the world, but is most common in North and Central America. The most highly endemic areas of United State (Missouri, Kentucky, Tennessee, southern Illinois, Indiana and Ohio) are also the areas with the biggest concentration of starlings. In Asia, 13 culturally confirmed cases had been diagnosed from 7 countries by 1970 (Randhawa, 1970). Except for Japan, all cases were in Southeast Asia: Malaysia, Indonesia, Thailand, India, Singapore, and Vietnam.

In natural habitats, *H. capsulatum* appears to have growth requirements related to humidity, acidity, temperature, and nitrogen content, but all of the specific

conditions needed for growth in the soil have not been completely clarified (Kauffman, 2003). In avian habitats, the fungus seems to grow preferentially where the droppings are rotting and mix with soil rather than in nests or fresh deposits (Rippon, 1988). Birds themselves are not infected with *H. capsulatum*, but can transiently carry the fungus on wings and feet and contribute to its spread. Bats are different from birds, it can become infected with the organism and excrete the fungus in their feces (Schwarz, 1981). Several investigators have been reported that *H. capsulatum* may contaminate in the intestinal contents of infected bats. They often have yeast-containing ulcer within their intestinal tracts (Klite and Dierchel, 1965).

The two largest histoplasmosis outbreaks ever reported were both associated with indirect exposure to the fungus during urban construction projects in Indianapolis (Wheat et al., 1981; Wheat, 1997). In other outbreaks, workers were infected with *H. capsulatum* after cleaning birds or bat guano from bridges or heavy equipments or cleaning out old buildings, especially chicken coops (Waldman et al., 1983; Jones et al., 1999). Outside of areas with appropriate natural conditions, there also occur scattered areas with high endemicity. These are often associated with existence of caves inhabitated by bats or birds in, may be term a “closed” environments. This accounts for the so -called cave fever in areas where otherwise there is a low incidence of the disease (Rippon, 1988). Patients with AIDS are at high risk for disseminated histoplasmosis. In an endemic region such as the state of Indiana, 26.7% of AIDS patients from Indianapolis, and 2% of AIDS patients from other cities of the state were found to have disseminated histoplasmosis (Wheat et al., 1990).

H. capsulatum var. *duboisii* is more restricted in its region, and occurs only in Africa (African histoplasmosis) between the Tropic of Cancer and the Tropic of Capricorn. The accurate ecological niche of the organism has not been determined, but some cases have been described in association with bat guano (Gugnani and Muotoe-Okafor, 1997).

2.1.5 Pathogenesis

The microconidia of *H. capsulatum* are 2-4 μm, a size that allows them to be easily inhaled into the alveoli of the host. The fungus undergoes transformation to the yeast phase from the mycelial phase at 37ºC. Phagocytosis of conidia or yeast form by alveolar macrophages and neutrophils occurs through binding of the fungus to the CD_{18} family of adhesion promoting glycoproteins (Bullock and Wright, 1987). The yeast form is able to survive within the phagolysosome through several mechanisms, such as the ability to resist killing by toxic oxygen radicals and to modulate pH in phagolysosome (Newman, 2001; Wood, 2003). Surviving within macrophage, the organism is transported to the hilar and mediastinal lymphnodes and subsequently disseminated hematogenously throughout the reticuloendothelial system in most cases of histoplasmosis (Kauffman, 2003)

2.1.6 Diagnosis

2.1.6.1 Culture methods

Culturing clinical specimens is a gold standard method of microbial identification. Brain-heart infusion (BHI) agar with blood, inhibitory mold agar, yeast extract phosphate agar, or SABHI agar with antibiotics is recommended for isolating

H. capsulatum from sputum, pus, or urine. All cultures should be kept for from 4 -6 weeks. The plates should be sealed with tape, or placed in a plastic bag in which a beaker with water in placed to prevent the agar media from dryness. The initial colonies on blood agar or BHI agar are usually glabrous or winkled and cream to brown but in a few weeks aerial hyphae develop. The colony is indistinguishable from that of *B. dermatitidis* and other fungi. Thus, the macroconidia with the finger-like projections characteristic and conversion to the yeast phase are necessary for identification. Smooth macroconidia show in primary clinical isolation. The tuberculate macroconidia may not be observed in the primary isolates, they can be appeared in subculture. For the conversion to the yeast form, the addition of a drop of 1% yeast extract broth to the blood agar slants helps to prevent dehydration and also enhances conversion. In isolates that neither produce tuberculate macroconidia nor convert to yeast form at 37ºC, the exoantigen test or nucleic acid hybridization test is one of the reliable methods for identification (Kwon-Chung and Bennett, 1992). A DNA probe for *H. capsulatum* significantly shortens the time required for definitive identification in cultures thereby reducing the risk of exposure of laboratory personnel to infectious spores.

2.1.6.2 Histopathological examination

For the patient who is acutely histoplasmosis, tissue biopsy should be done as soon as possible to look for *H. capsulatum*. Finding the distinctive 2-4 µm oval budding yeast allows a presumptive diagnosis of *H. capsulatum* infection. Histopathologic examinations of bone marrow aspirate, bronchoalveolar lavage fluid, sputum, urine or skin lesions are frequently examined for the diagnosis of

histoplasmosis. Peripheral blood may show intracellular organisms in white blood cells in up to one half of the patients with the disseminated form of disease (Goodwin et al., 1980). Skin scrapings, using 10% KOH and Parker ink or calcofluor white mounts, is usually negative in histoplasmosis. Tissue biopsy should be stained by using PAS (Periodic Acid Schiff) digest, Grocott's methenamine silver (GMS) or Gram stain (Costa et al., 2000). Histopathology is especially useful and is one of the most important ways of alerting the laboratory that they may be dealing with a potential pathogen. The structure of the *H. capsulatum* yeasts in tissue is very similar to other pathogens (*Candida glabrata, Penicillium marneffei, Pneumocystis* (*carinii*) *jeroveci, Toxoplasma gondii, Leishmania donovani* and *Cryptococcus neoformans*) and these characteristics can lead to a mistake during the identification for diagnostic purposes (Guimarães et al., 2006).

2.1.6.3 Antigen detection

For diagnosing of histoplasmosis, antigen detection tests may be more effective than antibody testing (Klotz et al., 1986). During infection with the fungus, antigen can be detected in body fluids such as serum (blood), pleural fluid, bronchoalveolar lavage fluid, cerebrospinal fluid and urine (Wheat et al., 1986a; Wheat et al., 1989; Wheat et al., 1992). Antigen detection can be particularly useful in acute disease, especially in individuals infected with HIV, who have disseminated infection with a large burden of organism without detectable antibodies to the fungus (Wheat et al., 1991; Wheat et al., 1992). The first developed as a radioimmunoassay, antigen detection is now performed by enzyme immunoassay with greater facilitate and equivalent sensitivity and specificity. Patients with disseminated infection,

antigen can be detected in the urine approximately 90% and in the serum approximately 50% (Wheat, 2001). Moreover, antigen can be detected in the urine of approximately 75% of patients with high-inoculum exposure acute pulmonary histoplasmosis within the first few week of infection; however, antigen is detected in only 10% to 20% of patients with less severe and chronic pulmonary histoplasmosis.

False positive reactions have been found in majority of urine and serum samples taken from patients with blastomycosis, paracoccidioidomycosis, or penicilliosis, but not with coccidioidomycosis (Wheat et al., 1997)

2.1.6.4 Serological test

Serological tests play an important role in the diagnosis of several forms of histoplasmosis but are not terribly useful in others. The standard assays are the complement fixation (CF) test and the immunodiffusion (ID) assay. A four fold rise in CF antibody titer or $\geq$ 1:32 is considered indicative of active histoplasmosis. Antibodies persist for years after infection; thus, the presence of a single low CF titer means little other than that the patient was exposed to *H. capsulatum* at some time. The CF test appears to be less specific than the ID assay; cross-reactions occur with other fungal infections and other granulomatous processes, including tuberculosis and sarcoidosis (Picardi et al., 1976; Wheat et al., 1986b; Kauffman, 2003). The ID assay tests for the presence of M and H precipitin bands. An M antigen precipitation develops with acute infection, is often present in chronic forms of histoplasmosis, and persists for months to years after the infection has resolved. However, this technique has not been commercialized, and the standard immunodiffusion assay is the only assay available. A radioimmunoassay has been reported, but false-positive reactions

occur higher than those noted with the CF assay, and standardization has not been verified (Kauffman, 2007).

2.1.6.5 Skin test

The skin testing with histoplasmin antigen was not a very helpful diagnostic test because of problems with cross-reactions with other fungi, especially *Blastomyces dermatitidis*, interference with subsequent complement fixation antibody assays, and insensitivity in ill patients who had disseminated infection. For these reasons, the skin test reagents are no longer commercially available, but are critically essential in defining the area where *H. capsulatum* is endemic (Kauffman, 2007).

2.1.6.6 Molecular based methods

Several laboratories are working on developing PCR assays that might help with a more rapid identification of pathogenic fungi in clinical samples without the need for culturing them. However, no PCR assay for routine use is commercially available. A real-time PCR assay that correctly identified *H. capsulatum* from among a variety of fungi grown in the laboratory appears encouraging. Using real-time PCR assay, positive identification of *H. capsulatum* was shown in tissue biopsies and bronchoalveolar lavage fluid from three patients that were culture positive for this organism (Villamil et al., 2003). A newly developed nested PCR assay was applied to murine models of histoplasmosis and compared to quantitative cultures. The primers sequence used in this method were based on the small-subunit (18S) rRNA gene of *H.capsulatum* (Bialek et al., 2001). Nested PCR method was the most sensitive for detecting the fungus in tissue sections when compared with H&E staining, Grocott

staining, anti–bacilli Calmette-Guérin antibody immunostaining, Fungiqual A fluorochrome staining methods, but not significantly more sensitive than the Grocott stain (Bialek et al., 2002a). Detection of fungal DNA in paraffin-embedded biopsy specimens has been described by using nested PCR assays. A product of the *Histoplasma capsulatum* nested PCR assay targeting the gene encoding the unique fungal 100-kDa-like protein revealed a specificity of 100% without requiring sequencing. On the other hand PCR assay using primers for the 18S rRNA gene was able to amplify DNA in histopatologically negative samples (Bialek et al., 2002b). Because PCR products detected could potentially be generated by nonspecific amplification of DNA from nonpathogenic fungal species, the products had to be confirmed by sequencing (Bialek et al., 2002b). In addition, a PCR was developed for rapid identification of *H. capsulatum* isolates in culture. In this method, two oligonucleotide primers pairs, Msp1F-Msp1R and Msp2F-Msp2R, were designed based on the sequences of the M-antigen gene (Guedes et al., 2003). With this assay using respectively the primers cited, 111 and 249 bases pair products were amplified successfully from 31 *H. capsulatum* strains and the method showed 100% sensitivity (Guedes et al., 2003). Therefore, this alternative methodology seems to offer the potential for improving the current methods for identification of this pathogenic mould, mainly when applied in atypical isolates

2.1.7 Treatment

Most patients infected with *H. capsulatum* are asymptomatic or have mild illness, thus, do not need treatment with any antifungal agents. However, patients

who have severe acute pulmonary, chronic pulmonary, or disseminated histoplasmosis do require treatment (Kauffman, 2003).

Pulmonary histoplasmosis

Most acute pulmonary histoplasmosis patients do not require antifungal therapy. However, some patients do remain symptomatic for longer periods of time, should receive therapy. Oral itraconazole, 200 mg/ day, for 6-12 weeks is recommended in such cases. For severe patients, initial treatment should be treated with amphotericin B,

Treatment is indicated for all patients with chronic pulmonary histoplasmosis. Amphotericin B, at a total dosage of 35 mg/kg, was the prefer agent, but itraconazole 200-400 mg/day for one year is effective, much less toxic.

Disseminated histoplasmosis

Mild to moderate acute pulmonary histoplasmosis and mediastinal granuloma requiring antifungal therapy can be treated with itraconazole 200 mg once to twice daily for 6 to 12 weeks. Similarly, chronic cavitary histoplasmosis can be treated with itraconazole alone for a 12- to 18-month duration, sometimes longer, as chronic upper lobe disease is prone to recurrence. In brief, the sickest patients and those requiring hospitalization will require amphotericin B preparation (lipid formulation amphotericin B, 3–5 mg/kg/d intravenously or deoxycholate 0.7–1 mg/kg/d intravenously) initially as induction therapy. These patients have either severe acute pulmonary histoplasmosis or progressive disseminated histoplasmosis (PDH). Progressive disseminated disease should be treated for at least 12 months and needs secondary prophylaxis if immunosuppression persists. Maintenance itraconazole can be safely discontinued in patients with PDH after they have received at least 1 year of

itraconazole therapy, have negative results of blood cultures, have cleared their *Histoplasma* serum and urine antigen level to less than 2 ng/ml. Urine and serum antigen levels should be measured during therapy and for 12 months after therapy is ended to monitor for relapse. Patients treated with itraconazole should have itraconazole blood levels monitored to ensure adequate drug exposure (Knox and Hage, 2010)

2.1.8 Isolation of *H. capsulatum* from soil

Isolation of *H. capsulatum* directly from soil by culturing soil suspension on mycologic agar media has rarely been successful. The mouse inoculation method described by Emmons is widely used, effective method for the isolation of the organism from the natural sources (Emmons, 1949; Zeidberg et al., 1952; Emmons, 1961; Lyon et al., 2004). In brief, a soil sample is mixed with sterile saline and shaken vigorously. This suspension was allowed to settle. Portion of fluid was taken from the upper part of the liquid column after sedimentation of solid material; an antibiotic solution were added to the soil suspension, and mixture was injected intraperitoneally into each of mice. Mice were killed 1 month later, and portions of spleen, omentum, liver, and any abscesses or lesions were placed on modified Sabouraud's agar by mincing a tissue on the agar slant with the scissors by which it was taken from the organ. The tissues were spread with a stiff loop and the exertion of pressure, and the cultures were incubated at 30° C for at least 30 days.

2.2 *Cryptococcus neoformans*

2.2.1 History Taxonomy

Cryptococcus neoformans was recognized as a human pathogen in 1894 by Otto Busse and Abraham Buschke. They described the case of 31-year-old woman with a lesion on her tibia. Busse described round corpuscles in the bone lesion and isolated the organism in culture and named the organism *Saccharomyces hominis* and named the disease *saccharomycosis hominis* (Busse, 1894). On the other hand, Buschke explained the organism independently of Busse as a coccidium (Buschke, 1895). In the same year, the Italian scientist Sanfelice was isolated encapsulated yeast from fermenting peach juice and called *Saccharomyces neoformans*. He later proved its pathogenicity in laboratory animal. In 1901, Vuillemin used the name *Cryptococcus* to refer to the genus of the organism and gave the name *Cryptococcus hominis* to the organism of Busse and Buschke (Benham, 1950). In 1905 the yeast was identified as a CNS pathogen when von Hansemann described the first case of cryptococcal meningitis (Hansemann, 1905). Several different terms have been applied to the fungus currently referred to *Cryptococcus neoformans*. In 1916, two cases of meningitis were then described by Stoddard and Cutler and, on complete pathological analysis of the CNS tissue, these investigators observed yeast forms with surrounding areas of clearing in the tissue (Stoddard and Cutler, 1916). This finding was initially misinterpreted as evidence of tissue histolysis and they renamed the yeast *Torula histolytica*, and the syndrome was called torulosis. There was confusion over the fact that one yeast was now considered under three different genera (*Saccharomyces, Cryptococcus*, and *Torula*) until 1935 when Benham published a comprehensive study of yeasts that contained 22 cryptococcal strains including the

original Busse and Buschke strain (Benham 1935). She concluded that all the cryptococcal isolates should be considered as one species based on morphology, fermentation, and serological studies and finally, the name of this organism designated as *Cryptococcus hominis*.

In 1976, Kwon-Chung discovered two mating types of *C. neoformans* that produced fertile basidiospores, the organisms were subsequently separated into two varieties, var. *neoformans* (serotypes A and D) and var. *gattii* (serotypes B and C) (Kwon-Chung 1976). These two varieties were recently separated into two species, *C. neoformans* and *C. gattii*, based on their genetic background and phylogenetic diversity, as proposed by Kwon-Chung in 2002 (Kwon-Chung et al., 2002). It is possible, as more molecular information is gathered from genome sequencing, that *C. neoformans* var. *neoformans* (serotype D) and *C. neoformans* var. *grubii* (serotype A) will be divided into separate species as well.

2.2.2 General morphology and characteristics

Cryptococcus neoformans can be grown on a variety of media, and generates white, mucoid colonies that usually observed after 48 hours of incubation. The mucoid appearance of the colonies is caused by the yeast cell's production of a polysaccharide capsule (Figure 3). The optimal growth temperature of most cryptococcol strains ranges between 30 - 35ºC. *C. neoformans* var. *grubii* generally is more thermotolerant than the other two varieties (Martinez et al. 2001) whereas, *C. gattii* is even more sensitive to high temperatures, and at 40 °C most strains are killed within 24 h. The yeast forms are round to oval (2.5 and 10 µm in diameter) and hyphae are never seen with the yeast forms in nutrient media, although pseudohyphal

and hyphal forms can sometimes be found during nutrient starvation or elevated temperatures. *C. neoformans* tolerates a pH range of 4.0-7.5 but growth is inhibited at a higher pH (Haward, 1961).

2.2.3 Sexual reproduction.

Cryptococcus neoformans yeast-like forms exist in one of two mating types, 'a' or 'α', and when yeasts of opposite mating type are combined under certain conditions they can undergo conjugation to produce the perfect (sexual) state (Kwon-Chung 1975). Conjugation between the 'a' and 'α' mating strains results in the formation of the teleomorph (sexual) stage which composes of dikaryotic hyphae that bear true clamp connections. Some of these hyphae develop specialized terminal structures called basidia. Meiosis occurs at the terminal portion of the basidium and uninucleate basidiospores are formed. These basidiospores bud off at the ends of the basidia in a basipetal fashion to form four chains of spores. These spores release from the basidia onto agar and begin budding as yeasts and thus complete the sexual life cycle (Perfect and Cox, 2010).

The extent, if any, to which sexual reproduction by *C. neoformans* occurs in nature is unknown. Strains of the appropriate mating types of serotype A or D can mate with each other (A x A, A x D or D x D), and the resulting teleomorph is called *Filobasidiella neoformans* var. *neoformans* (Kwon-Chung, 1975). In addition, serotype B and C strains can mate with each other (B x B, B x C or C x C), and the teleomorphs in this case are called *Filobasidiella neoformans* var. *bacillispora* (Kwon-Chung, 1975).

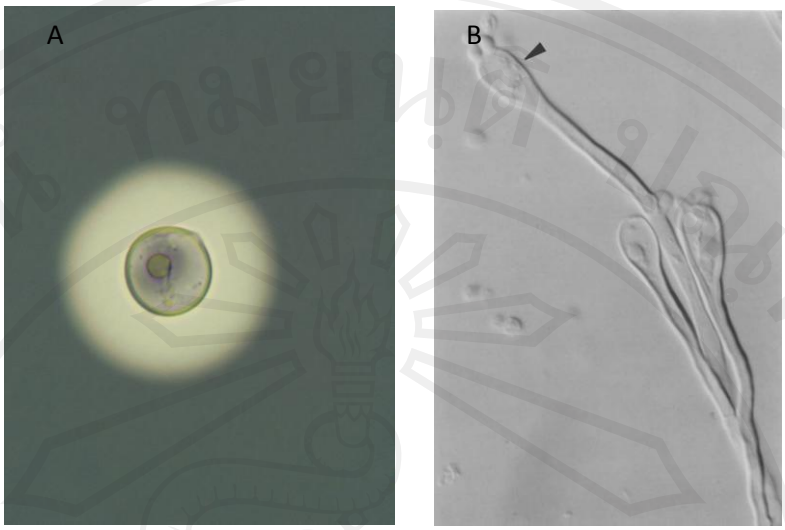

Figure 3 India ink preparation of CSF demonstrating the heavily encapsulated yeast cells of *C. neoformans* (A). The teleomorph stages of *C. neoformans* var. *neoformans* (B). This stage results from mating between two isolates of the opposite mating type, and is characterized by hyphae containing nuclei from each parent (dikaryotic hyphae). At the end of some hyphae, specialized structures called basidia (arrow) can form. Meiosis occurs in the basidia, and the resulting haploid basidiospores bud off at the apex (B).

2.2.4 Epidemiology and ecology

Cryptococcosis is a worldwide infection which only rarely causes disseminated disease in healthy individuals. Most patients with cryptococcosis have evidence of some underlying immunocompromising conditions. The most common of these underlying conditions worldwide is AIDS, followed by prolonged treatment with immunosuppressive drugs, organ transplantation, malignancies, and sarcoidosis (Pappas et al. 2001; Perfect and Casadevall, 2002).

Cryptococcus neoformans var. *neoformans* has been isolated from various sources in nature and is noted for its association with accumulations of avian guano, especially pigeon (*Columba livia*) excreta. It has also been isolated from a variety of other avian excreta including canaries (Hasenclever and Emmons, 1963; Swinne, 1979), parrots (Lopez-Martinez and Castanon-Olivares, 1995), cuckoos (Taylor and Duangmani, 1968), budgerigars (Bauwens et al., 1986), chickens (McDonough et al., 1961; Kohl et al., 1985), and other species (Gustin and Kelley, 1971; Keerativasee et al., 2008). Other environmental isolations have been reported from rotting vegetables, wood, dairy products and soil. The pigeon is unlikely the major source of cryptococci in nature, since only low concentrations of organisms are found in samples from the beak, crop, feet and rectal swabs (Littman and Borok, 1968). Pigeons are resistant to *C. neformans* infection by gastrointestinal and intramuscular inoculation, but infection can be induced by intracerebral or ocular inoculation. Resistance to infection in pigeon may be the result of combination of high body temperature (41°C-43°C) and the presence of endogenous gut bacterial flora that are inhibitory to cryptococcal multiplication. The available evidence suggests that, although pigeons may be infected with the fungus in their crops, they are resistant to disseminated infection. Pigeon may contribute to the propagation of the fungus by providing a rich culture medium in the form of pigeon excreta and could disseminate *C. neoformans* in the environment by carrying the fungus in their beaks and feet (Casadevall and Perfect, 1998).

Cryptococcus gattii has a more restricted geographical distribution than *C. neoformans* var. *neoformans*, causing human disease in climates ranging from temperate to tropical in Australia, Papua New Guinea, parts of Africa, India, South-

East Asia, Mexico, Brasil, Paraguay and Southern California (Kwon-Chung and Bennett , 1984b). The first environmental isolation of pathogen *C. neoformans gattii* from the environment was made by Ellis and Pfeiffer, in the Barossa Valley of South Australia (Ellis and Pfeiffer, 1990). These investigators established its specific ecological association with *Eucalyptus camaldulensis*, a species of red gum widely distributed in mainland Australia. Subsequently, another species of red gum, *Eucalyptus tereticornis*, has been confirmed as a natural habitat. Morover, Pfeiffer and Ellis (Pfeiffer and Ellis, 1997) showed that three additional eucalypts, namely, *E. blakelyi* (Blakely's red gum), *E. gomphocephala* (tuart), and *E. rudis* (flooded gum), also serve as abodes for *C. gattii*.

Cryptococcus neoformans var. *grubii* can also be found in soil contaminated by bird guano. Its ability to survive within scavenging predators of the soil has led to studies of *C. neoformans* growth in amoeba (Steenbergen et al. 2001), *Dictyostelium discoideum* (Steenbergen et al. 2003), and nematodes such as *Caenorhabditis elegans* (Mylonakis et al. 2002).

2.2.5 Pathogenesis

Cryptococcosis occurs primarily by inhalation of the infectious particles, either dehydrated (poorly encapsulated) yeasts or basidiospores (which are ideally sized at 1-2μm for lung deposition), into the alveoli within the lungs. After the yeasts are inhaled into the lungs of a susceptible host, the earliest innate immune response elicited is composed of alveolar macrophages, which is then followed by the arrival of polymorphonuclear cells, activated macrophages, and lymphocytes. Several studies have shown that CD4+ and CD8+ cells are especially important in containing the

spread of cryptococci from the site of initial infection in the lung to the central nervous system (Hill and Harmsen, 1991). Most primary infections of the lungs are asymptomatic in immunocompetent host because they are readily contained by the immune system. Disseminated cryptococcosis in the immunocompromised population may represent either reactivation of latent infection or in some cases a primary infection with immediate disease due to an initial ineffective immune response.

Cryptococcus neoformans appears to have some type of neurotropism in humans host because of its common propensity to cause meningoencephalitis. The reasons for this unique presentation are unclear. There is some presumption that because of its ability to use catecholamines as substrates for melanin production, *C. neoformans* has a survival advantage in the CNS where these products occur in large amounts. It is also possible that this yeast has special surface properties or has the ability to reside within host cells and traverse across the blood–brain barrier within them. Once the yeast is in the CNS compartment, the relatively immunologically sequestered environment of the CNS allows it to grow easily.

Recent advances in molecular biologic research have declared several virulence factors in *C. neoformans*. The three classical virulence factors of *C. neoformans* include capsule formation, melaninogenesis, and the ability to grow at 37 ºC (Perfect, 2005.). The polysaccharide capsule, which is composed of glucuronoxylomannan (GXM), is unique to *Cryptococcus* species and is considered an essential virulence factor that has multiple effects on host immunity. Furthermore, *C. neoformans* have an enzyme that catalyzes the conversion of diphenolic compounds to form melanin, which may have a biological role in protecting the yeasts

from host oxidative stresses. Finally, its ability to grow at 37ºC is a basic part of the virulence composite for most of the human pathogenic fungi. Other virulence factors include phospholipase and urease production and enzymes associated with protection against oxidative stresses.

2.2.6 Diagnosis

2.2.6.1 Culture methods

Cryptococcus can be found from biologic samples including CSF, sputum, and skin biopsy specimens on routine culture media. Colonies can usually be observed on fungal culture media within 48 to 72 hours. Antibacterial agents, preferably chloramphenicol, can be added. *C. neoformans* colonies appear as opaque, white, creamy colonies that may turn orange-tan or brown after extended incubation. The mucoid appearance of the colony is related to the polysaccharide capsule. *Cryptococcus* does not routinely produce hyphae or pseudohyphae, or ferment sugars, but is able to assimilate inositol and hydrolyze urea .*C. neoformans* and *C. gattii* do not assimilate nitrate but have the ability to use galactose, maltose, galactitol, and sucrose (Viviani et al., 2003). There are special media such as canavanine–glycine–bromthymol blue (CGB) agar which can be used to differentiate *C. gattii* strains from *C. neoformans* strains .

2.1.6.2 Histopathological examination

Histopathological staining of CSF sediment has proven to be more sensitive for rapid diagnosis of cryptococcal meningitis than the India ink method (Sato et al., 1999). A variety of positive staining methods have been described to exhibit the

organism in tissue or fluids; ranging from the nonspecific Papanicolaou or hematoxylin and eosin stains, to the more specific fungal stains such as Calcofluor, which binds fungal chitin, or Gomori methenamine silver (GMS), which stains the fungal cell wall (Casadevall and Perfect, 1998). Several stains can identify the polysaccharide capsular material surrounding the yeasts including Mayer's mucicarmine, periodic acid-Schiff (PAS), and alcian blue stains (Casadevall and Perfect, 1998). The Fontana-Masson stain appears to identify melanin in the yeast cell wall. The fungus is observed as a yeast that reproduces by formation of narrow-based budding with a prominent capsule.

2.2.6.3 Serological test

Using serum cryptococcal antibodies as the only diagnostic instrument for cryptococcosis has not been approved for early diagnosis of cryptococcosis. However, detection of cryptococcal capsular polysaccharide antigen in serum or body fluids via a latex agglutination technique has been effective in the fungus detection and is the most useful diagnostic serological test available for cryptococcosis. This test uses latex particles coated with polyclonal cryptococcal capsular antibodies or antiglucuronoxylomannan monoclonal antibodies and has overall sensitivities and specificities of 93% to 100%and 93% to 98%, respectively (Tanner et al., 1994).

Enzyme immunoassay (EIA) for detection and quantification of cryptococcal polysaccharide antigen of all four serotypes of *C. neoformans* in sera and CSF has been developed to detect the major component of the polysaccharide capsule, glucuronoxylomannan (GXM), with sensitivities and specificities of 85.2% to 99% (Tanner et al., 1994).

2.2.6.4 Molecular based methods

Several DNA-based methods of identification have recently been developed (Mitchell et al., 1994). Although conventional mycological methods are sufficient to identify *C. neoformans* at the species level, molecular methods have been used in epidemiological studies to identify the variety, serotype, or individual strain of *C. neoformans*. Several probes have been studied to distinguish the variety, serotype, or strain of *C. neoformans*. PCR amplification of specific DNA from clinical material has proven successful with other microorganisms and can be expected to become a standard procedure in clinical microbiology laboratories (Persing et al., 1993). Recently, the occurrence of *C. neoformans* and *Cryptococcus gattii* in bird excreta in the state of Parana´ in Brazil was determined by Lugarini et al. A total of 141 samples of Passerine and Psittacine excreta from captive birds were collected. Additionally, 25 clinical samples from Hospital de Cl´nicas, in the state of Parana´ were also analyzed. The determination of molecular and mating type of the isolates was performed by multiplex PCR with primers CNa-70S and CNa-70A for *C. neoformans*; and CNb-49S and CNb-49A for *C. gattii*. *Cryptococcus neoformans* var. *grubii* was isolated from 36 (25.53%) of Passerine and Psittacine excreta samples. Almost all clinical samples were classified as *C. neoformans* var. *grubii*. All environmental and clinical isolates were mating type a. Two nested-primer pairs specific for internal transcribed spacer regions of ribosomal DNA of *C. neoformans* (Mitchell et al., 1994) were sequentially used. In addition, there have been reported that the development of a nested PCR based assay for the detection of *Cryptococcus neoformans* in cerebrospinal fluid in 1998 (Rappelli et al., 1998). The technique was

then applied to 40 cerebrospinal fluid samples , it showed positive reactions for 21 clinical samples from patients who had been previously diagnosed as having cryptococcal meningitis by conventional techniques and negative reactions for all 19 negative controls. Nested PCR is here compared with other diagnostic methods currently used in patients' follow-up exams during anticryptococcal therapy. The results demonstrate that the developed nested PCR is a sensitive, specific, and reproducible technique and it represents a promising method to be used in the analysis of CSF samples from patients suspected of having cryptococcal meningitis.

In 2002, two PCR protocols targeting the 18S rRNA gene of *Cryptococcus neoformans* were established, compared, and evaluated in murine cryptococcal meningitis. One protocol was designed as a nested PCR performed in conventional block thermal cyclers. The other protocol was designed as a quantitative single-round PCR adapted to LightCycler technology. One hundred brain homogenates and dilutions originating from 20 ICR mice treated with different azoles were examined. A fungal burden of 3 $x10^1$ to 2.9 $x10^4$ CFU per mg of brain tissue was determined by quantitative culture. Specific PCR products were amplified by the conventional and the LightCycler methods in 86 and 87 samples, respectively, with products identified by DNA sequencing and real-time fluorescence detection. An analytical sensitivity of 1 CFU of *C. neoformans* per mg of brain tissue and less than 10 CFU per volume used for extraction was observed for both PCR protocols, while homogenates of 70 organs from mice infected with other fungi were PCR negative. Specificity testing was performed with genomic DNA from 31 hymenomycetous fungal species and from the ustilaginomycetous yeast *Malassezia furfur*, which are phylogenetically related to *C. neoformans*. Twenty-four strains, including species of human skin flora like *M. furfur*

and *Trichosporon* spp., were PCR negative. Amplification was observed with *Cryptococcus amylolentus*, *Filobasidiella depauperata*, *Cryptococcus laurentii*, and five species unrelated to clinical specimens. They concluded that the sensitive and specific nested PCR assay as well as the rapid and quantitative LightCycler PCR assay might be useful for the diagnosis and monitoring of human cryptococcal infections (Bialek et al., 2002c).

2.2.7 Treatment

Cryptococcosis treatment depend on the site and severity of infection. An important distinction is made in the guidelines to differentiate treatment of CNS versus non-CNS disease, therefore, a lumbar puncture should be performed among immunocompromised patients with cryptococcal disease to rule out meningitis.

The recommended initial management of cryptococcal meningitis in patients with HIV consists of the rapidly fungicidal regimen of amphotericin B (0.7 to 1 mg/kg/day) plus flucytosine (100 mg/kg/day)

For patients who develop pulmonary cryptococcosis with mild to moderate illness (no diffuse pulmonary infiltrates or dissemination,) fluconazole 400 mg for 6–12 months is recommended. Patients with severe pulmonary illness should be initially managed with amphotericin B (0.7 to 1 mg/kg/d) plus flucytosine, as in meningitis. Therapy for non-CNS disease can generally be discontinued after one year of therapy if the CD4 count is >100 cells/mm^3 and the titer is ≤1:512 and/or is stable.Treatments for *C. neoformans* and *C. gattii* are similar (Warkentien and Crum-Cianflone, 2010).