II. LITERATURE REVIEWS

A. History and Taxonomy

C. neoformans was identified as a human pathogen in 1894. The first description of Cryptococcosis is generally attributed to two German physicians, Otto Busse and Abraham Buschke. In 1894 , Busse, a pathologist, observed the fungus in tissue from the original clinical case of sarcoma-like tibial lesions in a 31-year-old woman. He believed the round, encapsulated yeast to be *Saccharomyces* and named the disease *Saccharomycosis hominis* (Busse, 1894). The German surgeon Buschke reviewed the histopathological features of this same case and concluded that the etiologic agent was a coccidium (Buschke, 1895). During this same period, the Italian scientist Sanfelice isolated a yeast from peach juice and in 1895 named it *Saccharomyces neoformans*, believing that it could produce cancerous tumors, thus giving taxonomic priority to this specific epithet. In 1896, Curtis described a case similar to that of Busse and Buschke. The patient had a yeast in a myxomatous tumor of the hip. He called the organism *Saccharomyces subcutaneous tumefaciens.* However, when the eminent French mycologist P. Vuillemin examined the several cultures, he did not find ascospores, which are characteristic of the genus *Saccharomyces*; therefore, in 1901 he transferred the yeasts to the genus *Cryptococcus* as *C. hominis* for the isolants of Busse and Curtis and *C. neoformans* for the isolants of Sanfelice (Rippon, 1988). The genus *Cryptococcus*, from the Greek word *kryptos* meaning "hidden", was created by Kutzing in 1833 for a group of yeasts that lacked the ability to produce endospores. After several years, controversies regarding the nomenclature of *C. neoformans* were settled (Benham, 1950), as it had been given a variety of names, including *Saccharomyces neoformans*, *Blastomyces neoformans*, *Cryptococcus hominis*, and *Torula histolytica.* Benham's proposal widened the classification for both pathogenic and nonpathogenic yeasts and ushered in the use of "cryptococcosis", rather than synonym such as torulosis, European blastomycosis, and Busse-Buschke disease, as the standard designation for this infection. Finally, following a proposal by Fell et al. in 1989, the genus *Cryptococcus*

was conserved and *C. neoformans* was designated as the type species. As Benham noted, there are different antigen types of *C. neoformans*, arising from antigen differences in the polysaccharide capsule. *C. neoformans* var. *neoformans* contains those strains that possess serotype A and D capsular types, while *C. neoformans* var. *gattii* contains the serotype B and C (Benham, 1935).

The name *C. neoformans var. gattii* was proposed in 1970 by Vanbreuseghem and Takashio (Vanbreuseghem and Takashio, 1970). This nomenclature was based on the atypical morphology of an isolate of *C. neoformans* from the cerebrospinal fluid (CSF) of an African boy with meningitis. In 1975, the teleomorph (sexual state) of *C. neoformans* was identified by Kwon-Chung and placed in the genus *Filobasidiella* (Kwon-Chung, 1975). Mating was shown to be controlled by two alleles (a and α) at a single mating locus (Kwon-Chung, 1976a). Initial mating studies suggested that serotypes A, D and B, C constituted two separate species (Kwon-Chung, 1976b). *Filobasidiella bacillispora*, named for the morphologically distinct smooth-walled, bacilliform basidiospores produced by mating within serotypes B and C, was subsequently reduced to a variety of *F. neoformans*. According to the International Code of Botanical Nomenclature, the correct nomenclature for *C. neoformans*, serotype B and C, is *F. neoformans* var. *bacillispora* Kwon-Chung (anamorph, *C. neoformans* var. *gattii* Vanbreuseghem et Takashio). However, *C. neoformans* var. *gattii* is the term in common use (Sorrell, 2001). Thus, present understanding of the life cycle of this species is that it has an asexual and a sexual stage. The yeast anamorph, *C. neoformans*, is composed of the two varieties *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii* (Casadevall and Perfect,1998).

B. General morphology and characteristics

Although there are 19 known species of the genus Cryptococcus, the etiologic agent in virtually all cases of human cryptococcosis is *C. neoformans*. First described in 1894, *C. neoformans* is an encapsulated basidiomycetous, usually spherical to oval budding yeast. *C. neoformans* is a yeast containing a polysaccharide capsule that greatly varies in size, according to the strain and the culture conditions.

Most *C. neoformans* isolates have medium-sized polysaccharide capsules that

result in cell diameters ranging from 4 to 10 μm. Some poorly encapsulated strains have diameters of only 2 to 5 μm. In heavily encapsulated strains recovered from infected tissues, the diameter of the cell can be as large as 80 μm (Levitz, 1991; Segal, 1994 ; Casadevall and Perfect, 1998). *C. neoformans* consists of two varieties, *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii*, which are the anamorph states of the basidiomycetes *Filobasidiella neoformans* var. *neoformans* and *F. neoformans* var. *bacillispora*, respectively, as discovered by Kwon-Chung (Segal, 1994). The teleomorph state is characterized by the production of basidiospores, which are the product of sexual reproduction. The two varieties of *C. neoformans* are similar in the yeast form, or anamorph state, but distinct phenotypic differences are evident in the teleomorph state: the basidiospores of var. *neoformans* are spherical, oblong, elliptical, or cylindrical with finely roughened walls, whereas those of var. *gattii* are bacilliform and smooth-walled (Ellis and Pfeiffer, 1992 ; Kwon-Chung et al., 1982a). Sexual reproduction appears to occur much less frequently in nature than asexual or vegetative reproduction (Buchanan and Murphy, 1998). By using cross-absorbed rabbit polyclonal antisera, the capsular serotypes of *C. neoformans* were defined as serotypes A through D and AD (Vogel, 1966). *C. neoformans* var. *neoformans* may possess capsular serotypes A, D, or AD, and *C. neoformans* var. *gattii* are serotype B or C. In addition to their serotypes, the two varieties of *C. neoformans* also differ in certain biochemical properties, ecology, and epidemiology (Mitchell and Perfect, 1995). Gueho et al. found a relatively large phylogenetic distance between serotypes A and D by analysis of partial 26S rRNA sequences (Gueho et al., 1993). Meyer et al. used PCR fingerpringting analysis to demonstrate that strains of serotypes A and D could be distinguished from each other (Meyer et al., 1993). Similarly, Varma and Kwon-Chung reported the isolation of a DNA probe (UT-4p) that was able to discriminate between these serotypes (Varma and Kwon-Chung, 1992). Brandt et al. demonstrated that serotypes A and D could be distinguished by their multilocus enzyme electrophoresis profile (Brandt et al., 1993).

Recently, it has been proposed that serotypes A and D be given separate variety status, *C. neoformans* var. *neoformans* (serotype D) and *C. neoformans* var. *grubii* (serotype A) The structure of the capsular polysaccharide responsible for the

antigenic reaction has also been investigated and was found chemically different in the five serotypes.

C. neoformans has minimal growth requirements of simple carbon and nitrogen sources, and even vitamin supplementation with thiamine may not be required for growth (Bruatto et al., 1992). The growth rate of *C. neoformans* depends on a variety of conditions. However, in most complete media at 37°C, the exponential doubling time varies among different strains from 2.5 to 6 h (Miller et al., 1990). Temperature is an important environmental signal for *C. neoformans* growth. Unlike *C. neoformans*, most other species of *Cryptococcus* are unable to grow at 37° C and are nonpathogenic. Indeed, the growth of *C. neoformans* is tightly regulated by temperature, since in vitro temperatures of 39 to 40 °C will significantly slow its growth rate, and at this temperature, the yeasts undergo intracellular vacuolization and produce aberrant budding patterns and pseudohyphal structures. *C. neoformans* var. *gattii* is even more sensitive to high temperatures, and at 40 °C most strains are killed within 24 h. The importance of temperature to pathobiology is further emphasized by temperature-sensitive mutants of *C. neoformans*, which do not grow at 37 °C and are avirulent in animals regardless of their ability to form capsules or produce melanin (Kwon-Chung et al., 1982c). *C. neoformans* tolerates a pH range of 4 to 7.5, but growth is significantly inhibited at a higher pH (Gordon and Devine, 1970 ; Littman, 1958). Alkalinization can be used to sterilize contaminated environmental sites (Walter and Coffee, 1968). Sunlight can sterilize sites contaminated with *C. neoformans* (Ishaq et al., 1968) UV radiation has been shown to kill *C. neoformans* (Wang and Casadevall, 1994).

In general, it is not difficult to isolate *C. neoformans* from clinical or environmental specimens, as the fungus grows well on various culture media at 25 °C and 37 °C. However, for specimens that may be heavily contaminated, such as bird droppings or sputum from patients with AIDS, differentiation of *C. neoformans* from other yeasts and bacteria may be time consuming if routine fungal isolation media, such as Sabouraud dextrose agar, are used. Fortunately, selective media and rapid identification tests have been described that take advantage of the organism's distinct biochemical profile including its production of urease, resistance to antibacterial

agents such as chloramphenicol, creatinine assimilation, and phenoloxidase activity (Rippon, 1988 ; Cooper and Silva-Hutner, 1985 ; Staib et al., 1989). Phenoloxidase enables *C. neoformans* to synthesize melanin from certain catecholamine precursors. Thus, pigmented colonies result when the organism is grown on selective media enriched in catecholamines, such as birdseed agar.

C. Virulence factors

Virulence factors increase the degree of pathogenicity of a microbe. *C. neoformans* has a number of virulence factors; generally, the virulence of an isolate cannot be attributed to any single factor, but rather it is attributed to many working in unison to cause progressive disease. The virulence factors of *C. neoformans* are its ability to grow at 37 °C, requiring the factor calcineurin (Odom et al.,1997), production of a polysaccharide capsule (Chang and Kwon-Chung, 1994), expression of the enzyme laccase (Ikeda et al., 1993), which forms a melanin-like pigment when grown on substrates containing polyphenolic or polyaminobenzene compounds (Chasakes and Tyndall, 1975), mannitol production, and potential factors such as superoxide dismutase, proteases, phospholipase B, and lysophospholipase. The polysaccharide capsule and the soluble extracellular constituents of *C. neoformans* are probably the dominant virulence factors (Buchanan and Murphy, 1998).

1. Growth at 37 °C

To initiate a systemic infection, any microorganism must be capable of growing within the host. To cause infection in human, a *C. neoformans* isolate must grow at 37 °C in an atmosphere of approximately 5 % CO_2 and at a pH of 7.3 to 7.4. To survive at 37 °C, the organism must have an intact gene that encodes the *C. neoformans* calcineurin A catalytic subunit (Odom et al., 1997). Calcineurin is a serine-threonine specific phosphatase that is activated by Ca^{2+}-calmodulin and is involved in stress responses in yeasts. Although calcineurin A mutant strains of *C. neoformans* can grow at 24 °C, they cannot survive in vitro at 37 °C, in 5 % CO_2, or at alkaline pH. 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 (Odom et al., 1997). 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.

2. Capsule

The capsule of *C. neoformans* is clearly a virulence factor. *C. neoformans* has a capsule composed primarily of a high molecular weight polysaccharide that has a backbone of α-1,3-D-mannopyranose units with single residues of β-D-xylopyranosyl and β-D-glucuronopyranosyl attached (Bhattacharjee et al., 1984 ; Cherniak and Sundstrom, 1994). This polysaccharide is referred to as glucuronoxylomannan (GXM) (Cherniak et al., 1980) and has four serotypes : A and D, produced by *C. neoformans* var. *neoformans*, and B and C, 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 (Fromtling et al., 1982 ; Kozel and Cazin, 1971 ; Kwon-Chung and Rhodes, 1986). To complement the acapsular mutations, libraries of genomic DNA in a telomere-based vector which dramatically increases the transformation efficiency were constructed. A two-polymer partition system was adopted to enrich the Cap^+ transformants. With these approaches, four genes, *CAP10*, *CAP59*, *CAP60*, and *CAP64* were cloned, which complement four different groups of these acapsular mutants (Chang and Kwon-Chung, 1994 ; Chang and Kwon-Chung,1998 ; Chang et al., 1996). Although these genes are important for capsule formation, the biochemical function of these genes in the pathway of capsule synthesis remains unclear (Perfect et al., 1998).

3. Melanin Synthesis

One characteristic that differentiates pathogenic isolates of *C. neoformans* from nonpathogenic isolates and other *Cryptococcus* species is the organism's ability to form a brown to black pigment on a medium (such as birdseed or caffeic acid agar) that contains diphenolic compounds (Kwon-Chung, 1992). This pigment, first

described by Staib, is a melanin-like compound produced by *C. neoformans* isolates with phenoloxidase activity (Shaw and Kapica, 1972).

Biochemical analyses suggest that melanogenesis in *C. neoformans* is accomplished by conversion of dihydroxyphenols such as 3,4-dihydroxyphenylalanine (DOPA) to dopaquinone (Fig. 1). This conversion is catalyzed by a phenoloxidase and is the rate-limiting step, presumably because subsequent steps in the pathway, such as dopaquinone rearranging to dopachrome and ultimately autoxidation to melanin, are spontaneous (Polacheck and Kwon-Chung, 1988). *C. neoformans* lacks a tyrosinase enzyme required for endogenous production of dihydroxyphenols (Torres-Guerrero and Edman, 1994) ; thus to produce melanin, a *C. neoformans* isolate must be able to acquire diphenolic compounds from its growth environment, and it must have the enzyme phenoloxidase to catalyze conversion of these compounds into the subsequent melanin intermediated. The brain is a tissue rich in catecholamines such as DOPA and is a favorite target for infection by *C. neoformans*. Melanin has been hypothesized to protect the fungus against introgen- and oxygen-based oxidative attack by host cells (Wang et al., 1995).

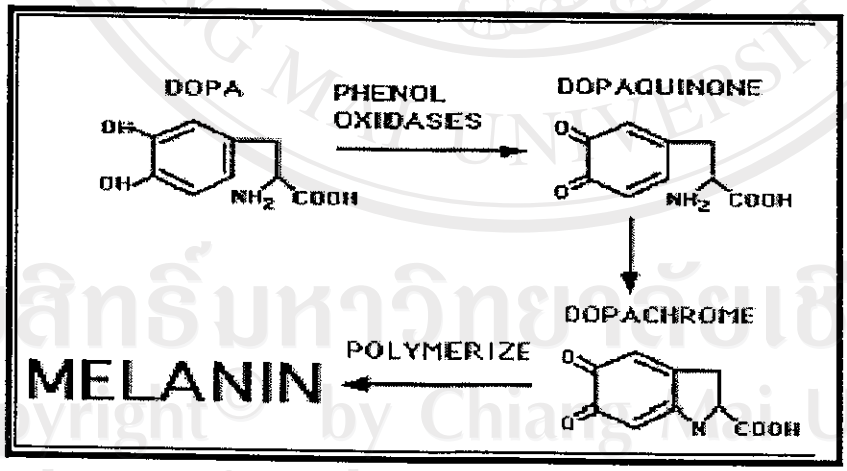

Figure 1. Pathway for melanin synthesis by *C. neoformans* (Polacheck and Kwon-Chung, 1988).

4. Mannitol Production

In 1990, Wong et al. showed that *C. neoformans*, as a species, produces large amounts of D-mannitol in vitro (Wong et al., 1990). The early observations lead the authors to hypothesize two roles for mannitol in pathogenesis. First, high concentrations of D-mannitol in the CNS may contribute to brain edema, a major factor in the neurological damage caused by cryptococcal meningitis. Second, mannitol is a potent scavenger of hydroxyl radicals, and cryptococcal-reduced D-mannitol may help protect the organism from oxidative damage (Wong et al., 1990).

D. Ecology and Epidemiology

C. neoformans is a cosmopolitan fungus, and cases of cyptococcosis have been reported from all regions of the world. The fungus is a free-living organism that can survive in a variety of environmental niches. The saprophytic nature of *C. neoformans* has been known since 1894 when Sanfelice cultured it from peach juice (Sanfelice, 1894). The sporadic nature of human cryptococcosis, the extreme rarity of documented human-to-human transmission events, and the high prevalence of *C. neoformans* in the environmental sources. Thus, knowledge of the ecology and the life cycle of this fungus is important for understanding of the epidemiology of human infection. Among the most significant observations, *C. neoformans* strains are divided into the varieties *neoformans* and *gattii* on the basis of phenotypic, biochemical, and genetic differences; and there are the association of *C. neoformans* var. *neoformans* with avian excreta, and the association of *C. neoformans* var. *gattii* with eucalyptus trees (Casadevall and Perfect, 1998).

1. Distribution of *C. neoformans* var. *neoformans* in Nature

C. neoformans was isolated in nature first from peach juice in 1895 and then from milk in 1901 (Rippon, 1988 ; Sanfelice, 1895). It was not isolated again from a saprophytic source until Emmons (Emmons, 1951, 1955) isolated the organism from soil in 1951 and from pigeon excreta in 1955. Although pigeon droppings commonly are colonized with *C. neoformans*, pigeons do not appear to become sick due to cryptococcosis, perhaps because their high body temperature is detrimental to growth of the organism (Emmons, 1955). Since Emmons's original reports, *C. neoformans*

has been isolated from soil, pigeon excrement, and sites contaminated by pigeon excrement in various parts of the world. In avian nesting areas, the yeast cells possess minimal capsules, and hence the cells are dry and easily aerosolized (Bulmer, 1990 ; Neilson et al., 1977). In this state, the yeast cells are smaller and capable of being inhaled to the level of the alveolus (Neilson et al., 1977 ; Powell et al., 1972).

C. neoformans has been isolated from the excrement of a variety of avian species in addition to pigeons, including chickens, parrots, sparrows, starlings, turtledoves, canaries, and skylarks (Muchmore et al., 1963 ; Littman and Walter, 1968 ; Bauwens et al., 1986 ; DeVroey and Swinne, 1986). The reason for the high frequency of *C. neoformans* in avian excreta is not clear but may be related to the ability of the fungi to assimilate xanthine, urea, uric acid, and creatinine, all of which are abundant in the dropping (Littman and Walter, 1968). As alluded to earlier, *C. neoformans* is occasionally isolated from various nonavian sources, including fruits, vegetables, dairy products, and the digestive tract of the cockroach (Sanfelice, 1895 ; Klein, 1901 ; Pal and Mehrotra,1984 ; Swinne et al., 1986). In Brazil, in 1993, *C. neoformans* var. *neoformans* was isolated from decaying wood within a hollow of *Syzygium jambolana* tree (Lazera et al., 1993), and in 1996, from hollows of three other species of living trees (*Cassia grandis*, *Senna multijuga* and *Ficus microcarpa*) (Lazera et al., 1996).

2. Distribution of *C. neoformans* var. *gattii* in Nature

In 1990, Ellis and Pfeiffer reported the first isolate of the yeast pathogen *C. neoformans* var. *gattii* from the environment. The fungus was cultured from leaf litter from beneath *Eucalyptus camaldulensis* (river red gum), a species of gum tree found throughout many areas of Australia (Ellis and Pfeiffer, 1990). Further environmental work has now established an association between *C. neoformans* var. *gattii* and *E. tereticornis*, the forest red gum. *C. neoformans* var. *gattii* was isolated from the hollow of an *E. tereticornis* tree at Currumbin on the Gold Coast, Queensland, in January 1991 (Pfeiffer and Ellis, 1992). Recently, 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. neoformans* var. *gattii*.

Outside Australia it has been more difficult to culture *C. neoformans* var. *gattii* from environmental sources. Positive samples have been obtained from eucalyptus trees (generally of the red gum group) in California (Ellis and Pfeiffer, 1994), India (Chakrabarti et al., 1997), Italy (Montagna et al., 1997), and Brazil (Montenegro and Paula, 2000). Despite extensive environmental sampling, *C. neoformans* var. *gattii* has not been recovered from central Africa (Swinne et al., 1994), South Africa or Papua New Guinea (Laurenson et al., 1997). While the majority of environmental isolates of *C. neoformans* var. *gattii* have been of eucalypt origin, in South America, serotype B has been isolated from other environmental sources. These included a wasp nest in Uruguay (Gezuele et al., 1993), bat guano contaminated with animal and plant material in an old house in Brazil (Lazera et al., 1993) and decaying wood of *Moquilea tomentosa* (the pink shower tree) in Brazil (Lazera et al., 2000). A few samples from hollow specimens of the trees, *Guettarda acreana* (in the Brazilian rainforest) and *Erythrina jambolanum* and a 'tento' tree (in an urban area of occidental Amazonia), were also positive (Fortes et al., 1999). In a single report from Colombia, the less common serotype C was isolated from the detritus of two of 68 almond tree (*Terminalia catappa*) sampled in Cucuta, a city in the northeast of the country (Callejas et al., 1998).

3. Epidemiology of *C. neoformans*

C. neoformans var. *neoformans* (serotype D) and *C. neoformans* var. *grubii* (serotype A) are primarily the etiologic agent of cryptococcosis in patients with AIDS, and serotype A comprises the overwhelming majority of clinical isolates. Infections due to strains belonging to serotype D are more prevalent in certain geographic areas, including France, Italy, and Denmark (Bennett et al., 1984 ; Dromer et al., 1996a ; Mitchell and Perfect, 1995). In France, serotype D causes 21% of cases of cryptococcosis (Dromer et al., 1994, 1996b). Serotype D infections are more likely than serotype A infections to occur in older patients, to result in skin involvement, and to be associated with corticosteroid therapy. In a seminal paper on the epidemiology of *C. neoformans*, Kwon-Chung and Bennett (Kwon-Chung and Bennett, 1984) reported that the prevalence of *C. neoformans* var. *gattii* is high in geographically restricted locations in tropical and subtropical areas, including Australia, Brazil,

Cambodia, Hawaii, Southern California, Mexico, Paraguay, Thailand, Vietnam, Nepal and Central Africa (Fig.2) In these areas this variety comprised more than 37% of isolates. All isolates were serotype B, except in Southern California, where 11 of 30 were serotype C. In Brazil, *C. neoformans* var. *gattii* infection is endemic, especially in the northeast of the country, where 75% of all *C. neoformans* isolates have been serotype B. Prior to 1993 and the acquired immune deficiency syndrome (AIDS) era in Thailand, 10 of 18 (55%) of *C. neoformans* isolates were serotype B and two were serotype C, compared with 4% serotype B between 1993 and 1995 (Sukroongreung et al., 1996 ; Poonwan et al., 1997). The apparent rarity of *C. neoformans* var. *gattii* infections in AIDS patients is another unexplained observation ; only three cases have been reported, in a Zairean patient, a Canadian patient who had been to Mexico before his infection was acquired (Kwon-Chung and Varma, 1989, and a patient living in Los Angeles, USA (Clancy et al., 1990).

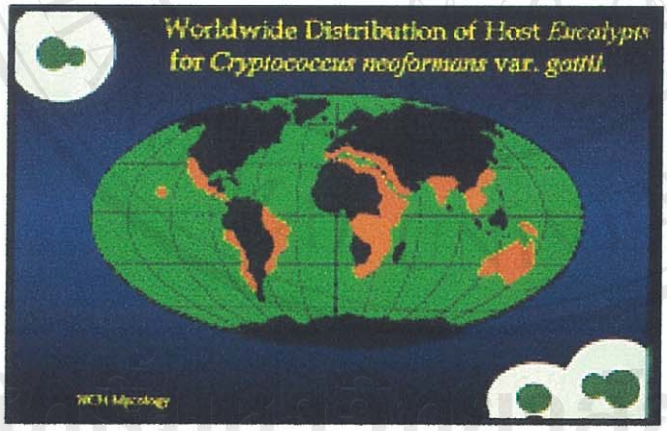

Figure 2. Worldwide Distribution of Host *Eucalypts* for *C. neoformans* var. *gattii.*

E. Clinical Manifestation

Cryptococcosis has been considered a sporadic infection with a worldwide distribution. The major variety, *C. neoformans* var. *neoformans* and var. *grubii*, are ubiquitous in the soil and in avian guano. *C. neoformans* is rarely isolated from healthy individuals (Randhawa and Paliwal, 1977) and does not appear to be a common human commensal, the extreme rarity of documented human-to-human transmission events (Beyt and Waltman, 1978), and the high prevalence of *C. neoformans* in the environment indicate that human infection is acquired from environmental sources.

Infection of man by *C. neoformans* occurs by inhalation of the organism and its arrival in the lung periphery where an initial inflammatory response occurs (Fig. 3)

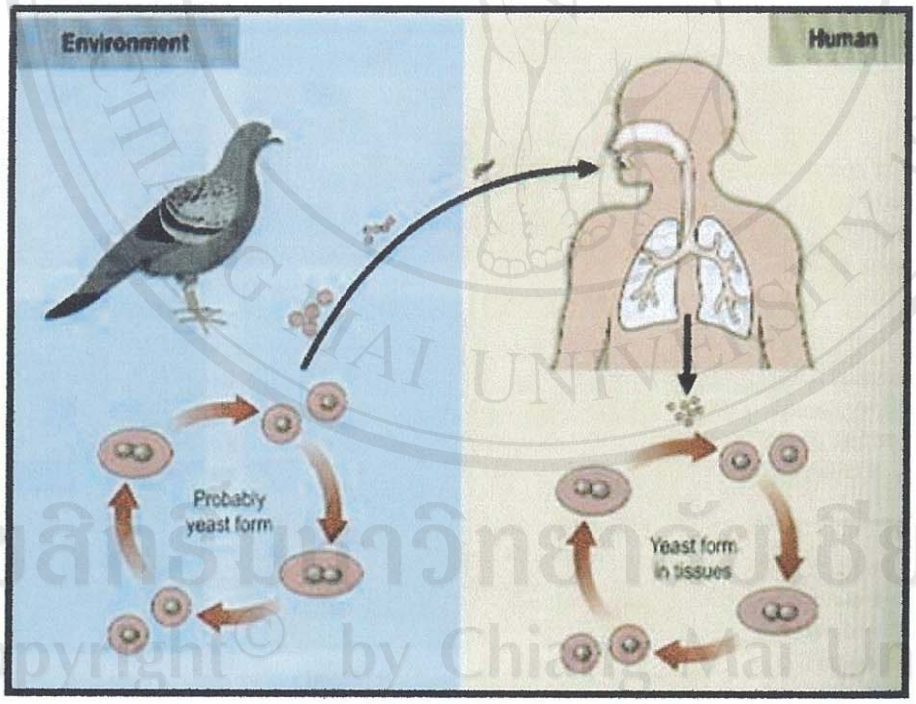

Figure 3. *C. neoformans* infection.

Thus, the lung is the primary site of infection; however, the organism has a high predilection for systemic spread to the brain and meninges that is organ of most dramatic involvement. It appears that *C. neoformans* has an affinity for central nervous system tissue, but the reasons are not known. Polacheck et al. (1990) have suggested that this is due to the ability of *C. neoformans* to utilize catecholamines that are present in nervous tissue for melanogenesis, a precess in which phenoloxidase present in strains of *C. neoformans* may be important. Thus *C. neoformans* can survive in central nervous tissue, cause infected foci, and ultimately break into the meninges to cause active meningitis. Additional factors most probably exist that determine this affinity for central nervous tissue, but they are not recognized at the moment (Segal and Baum, 1994).

This statement is intuitively supported by experience with other fungal organisms but not other yeasts. Thus, part of this statement is conjectural for man. It is known that *C. neoformans* can be found in aerosolized infected soil in particles ranging from 0.5 to 2.0 μm in diameter, ideal for aspiration and sedimentation at the alveolar level (Neilson et al., 1977). This observation supports the concept of a *pulmonary route of initial infection. C. neoformans* is the leading cause of fungal meningitis and is an important cause of morbidity and mortality in patients with AIDS and in transplant recipients. *C. neoformans* also produces systemic disease in patients who have no apparent underlying immunologic disorder.

Primary pulmonary infections are frequently asymptomatic and may be detected as incidental findings on a routine chest radiographic examination. A solitary pulmonary nodule that can mimic a carcinoma is most commonly observed; the correct diagnosis is usually made when the mass is resected. *C. neoformans* can also produce a symptomatic pneumonia characterized by diffuse pulmonary infiltrates.

Cryptococcal meningitis, which is caused by hematogenous spread of yeast from the lungs to the meninges surrounding the brains, is the most frequently diagnosed form of cryptococcosis. Symptoms usually include the combinations of headache, mental status changes, and fever lasting several weeks. Cryptococcal disease of the central nervous system may occasionally take the form of an expanding intracerebral mass that causes focal neurologic deficits. Other common

manifestations of disseminated cryptococcosis include skin lesions and osteolytic bone lesions (Murray et al., 2002).

F. Laboratory diagnosis

1. Direct Microscopic Examination

Clinical specimens—spinal fluid, tissue, sputum and respiratory samples, scrapings, punch biopsies, aspirates from cutaneous lesions, and other appropriate specimens should be examined directly, a particularly useful and rapid diagnostic is an India ink preparation. *C. neoformans* appears as a single cell or budding yeast surrounded by a clear halo because of the exclusion of the ink particles by the polysaccharide capsule (Fig. 4A). Although *C. neoformans* can become fragile, collapse, or be crescentic when dried or fixed, the encapsulated cells are rapidly distinguished in a colored colloidal medium such as India ink when mixed with fluids such as cerebrospinal fluid (CSF). A modified India ink preparation for *C. neoformans* in CSF employs 2% chromium mercury and India ink, which allows for identification of both external and internal features in the yeast (Zerpa et al., 1996). This simple test has been found to be positive in more than 80% of patients with AIDS and cryptococcal meningitis and in 30 to 50% of patients with non-AIDS cryptococcal meningitis. The examination may even be improved by centrifuging the CSF specimen (i.e., 500 rpm for 10 min) and using the pellet for staining.

Histopathologically, the 5- to 20- μm encapsulated yeasts in tissues have been relatively east to identify because of their prominent capsules. Yeasts have been identified from various body sites and tissues with histological stains ranging from the nonspecific Papanicolaou, hematoxylin and eosin, and acridine orange preparations to more specific fungal stains such as Calcofluor, which stains fungal chitin, or a Gomori methenamine silver stain. Even a Gram stain may show *C. neoformans* as a poorly stained gram-positive yeast. However, because of the polysaccharide capsule possessed by *C. neoformans* and in most cases the enlargement of this capsule during infection, several specific stains have been developed, such as the mucicarmine, periodic acid-Schiff, and alcian blue stains, that identify the capsular material surrounding the yeasts (Fig. 4B). On the other hand, the size and narrow budding characteristics of this yeast may still be helpful in predicting *C. neoformans* within a

lesion, even without a positive capsular stain. The Fontana-Masson stain, which appears to identify melanin, has been used occasionally to specifically identify *C. neoformans* in tissue when the capsule is not apparent (Kwon-Chung et al., 1981 ; Ro et al., 1987).

In clinical specimens, yeast cells of *C. neoformans* are mostly globose in shape, although some may be oval to lemon-shaped, and var. *gattii* cells may actually be elliptical. At times when specimens are dried, heat-fixed, and stained, *C. neoformans* cells will collapse or become crescent-shaped, making them more difficult to identify. *C. neoformans* generally does not produce hyphae in tissue, but occasionally—under certain conditions, such as severe temperature elevations (38 to 40 °C). Chains of cells will then form with unusual shapes that can mimic pseudohyphae or actually appear to possess true hypha formation on direct examination (Erke and Schneidau, 1973).

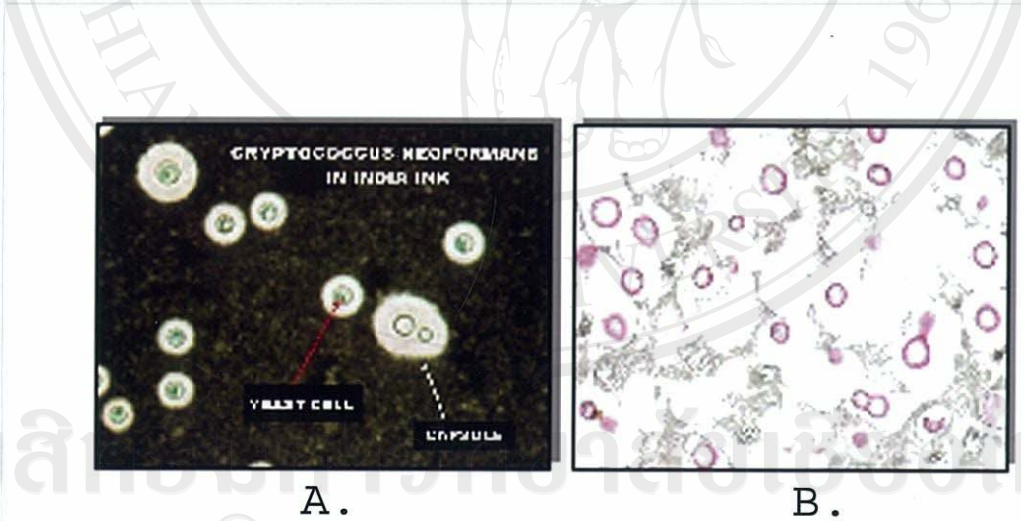

Figure 4. A. India ink preparation showing capsules of *C. neoformans.*
B. Mucicarmine stain of lung tissue.

Furthermore, with sputum or pus, it may be reasonable to digest the specimens with 10% potassium hydroxide to remove tissue artifacts, making yeast cells easier to identify. A brain tissue smear may display the yeast cells readily without sodium hydroxide digestion (Kwon-Chung and Bennett, 1992).

2. Growth characteristics and culture methods

C. neoformans is a mammalian pathogen in large part because, of all the cryptococcal species, it is the only species that can routinely grow at 37 °C. Some strains of *Cryptococcus albidus* and *C. laurentii* can grow at 37 °C, but this characteristic is not common for these species. On the other hand, most strains of *C. neoformans* optimally grow at 30 to 37 °C, and their growth is tightly regulated at these temperatures. *C. neoformans* var. *gattii* appears to be even more sensitive to these high temperatures than *C. neoformans* var. *neoformans*, and strains will die rapidly when environmentally exposed to 40 °C. The yeast grows well in acidic media (pH 5-7) but does not tolerate alkaline pH conditions above 7.6 and will die. The yeast also grows well aerobically, but growth stops in anaerobic conditions. Furthermore, as the pCO_2 tension rises in the environment to host level (i.e., from 0.04 to 5%), capsular synthesis is induced in most strains (Granger et al., 1985). The growth of *C. neoformans* will be detected on most standard fungal and bacterial media; it is not a particularly fastidious yeast, and colonies on agar plates can usually be observed by visual inspection in 48 to 72 h. Some strains of *C. neoformans* var. *gattii* may require 5 to 7 days to grow and may not be easily identified at higher temperatures (35 to 37 °C).

Standard fungal media such as Sabouraud glucose agar work well for the isolation of *C. neoformans*, but when antibiotics are used in the medium to reduce bacterial contamination, cholramphenical is a better choice than cycloheximide. The yeast will grow on artificial media as opaque, creamy and mucoid colonies that may turn orange-tan or brown after prolonged incubation (Fig. 5A) (Casadevall and Perfect, 1998).

In addition to Sabouraud's agar, it is also recommended for direct culturing from clinical material to use the niger seed (*Guizottia abyssinica*, birdseed) agar, first

described by Staib in 1962 (Staib, 1962) as a medium for identification of *C. neoformans*, since the fungus grows on this medium in the form of distinct brown colonies. This unique characteristic, not shared by other *Cryptococcus* species or *Candida*, *Torulopsis*, *Trichosporon*, or *Rhodotorula* species, results from oxidation of caffeic acid in the medium to melanin, a reaction mediated by the enzyme phenoloxidase produced by *C. neoformans* (Fig. 5B). The same principle was used to construct synthetic media, which contain L-dopa or dopamine on which *C. neoformans* will produce brown-black colonies. The selective medium is of particular importance when attempting to isolate the fungus from clinical material, such as sputum, which may be contaminated with other yeast species (Segat and Baum, 1994). It is also advisable to concentrate the specimen by centrifugation (e.g., CSF) or membrane filtration (urine) (Staib and Seibold, 1989). Recovery of *C. neoformans* from blood, similar to the situation with *Candida*, has increased by use of the centrifugation-lysis method (Tarrand et al., 1991).

Canavanine-glycine-bromothymol blue (CGB) agar can be used to separate *C. neoformans* var. *neoformans* from var. *gattii* (Kwon-Chung et al., 1982b). This medium exploits the biochemical differences between the two varieties. All isolates of var. *gattii* can utilize glycine and are resistant to canavanine. The medium turns blue as a result of alkalinization by the ammonia released during the degradation of glycine (Fig. 5C). *C. neoformans* var. *neoformans* cannot utilize glycine as either a carbon or a nitrogen source and be simultaneously resistant to canavanine in the same medium (Casadevall and Perfect, 1998).

Identification of the organism as *C. neoformans* is supported by the fact that it is an encapsulated yeast that grows well at 37 °C. The most important biochemical characteristics of *C. neoformans* are that it contains urease, does not assimilate nitrate, does not possess fermentative capability and utilizes galactose, maltose, galactitol, and sucrose. However, it will not assimilate lactose or melibiose, and its growth is strain variable with erythritol (Casadevall and Perfect, 1998).

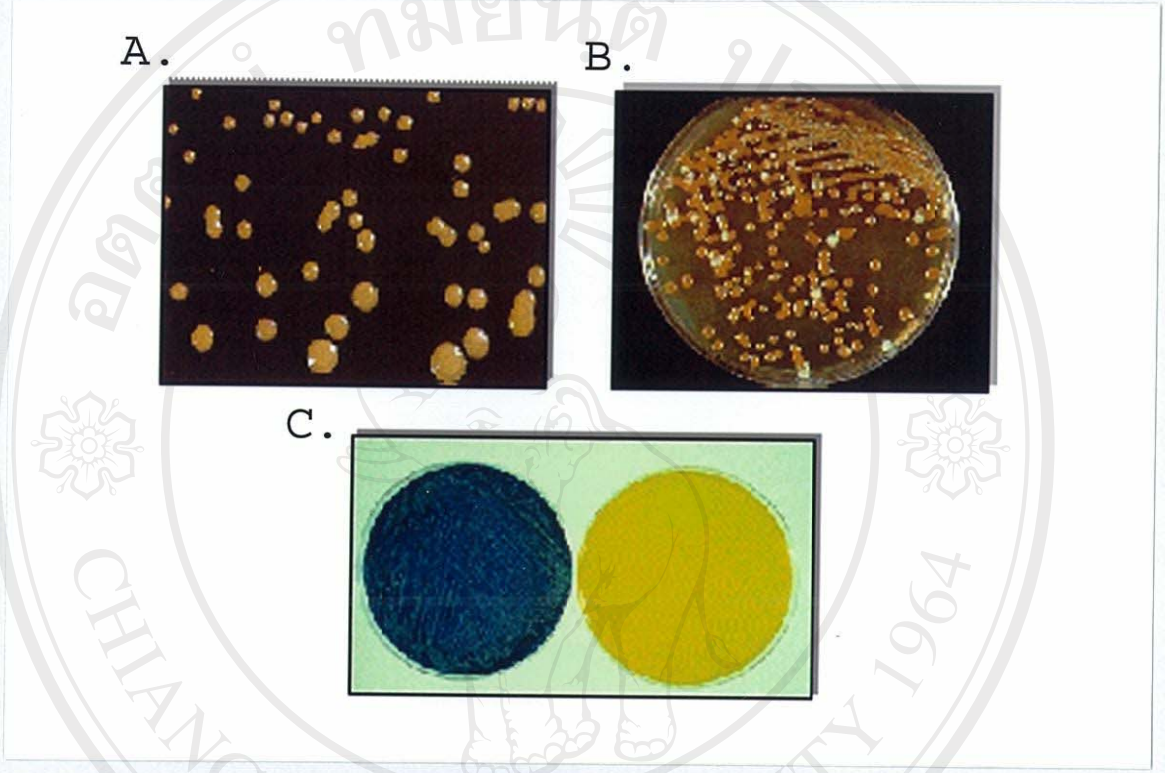

Figure 5. A. On Sabouraud's dextrose agar colonies are cream colored, smooth, mucoid yeast-like in appearance. B. Bird seed agar plate showing brown colonies of *C. neoformans* and white colonies of *Candida albicans.* C. CGB agar turns blue for *C. neoformans* var. *gattii.*

3. Serology

The serologic test for the diagnosis of cryptococcosis is both specific and sensitive. During infection, the capsular polysaccharide becomes solubilized in the body fluids, and this antigen can be detected and quantified with specific rabbit anti-*C. neoformans* antiserum. The most commonly employed method for either screening or titration is a latex agglutination test. The reagents for the test are commercially available in kit form. Latex particles are coated with the specific hyperimmune rabbit immunoglobulin and mixed with dilutions of patient CSF, serum, or urine (Bloomfield et al., 1963). A positive agglutination at a dilution of 1:4 strongly suggests cryptococcal infection. Titers of ≥ 8 usually indicate active disease, and most patients with AIDS have higher antigen titers. Controls include latex particles coated with normal rabbit globulin to detect nonspecific agglutination, which occurs in serum with rheumatoid factor (Bennett and Bailey, 1971).

Several enzyme immunoassays (EIAs) have also been developed to detect either antigen or antibody. In comparison with the latex agglutination test, reading EIAs is less subjective, the EIA is unaffected by prozone reactions, and the EIA may detect antigen earlier and in smaller amounts. The EIA dose not react with rheumatoid factor, and hence the specimen does not require pronase treatment. However, EIAs require more time to perform than the latex agglutination test, which can be completed in several minutes. Finally, the EIAs may detect antigen earlier in infection and at lower antigen concentrations. There may also be fewer false-positive tests with the EIAs. Finally, there is no correlation between high titers by EIA or by latex agglutination. However, both tests will identify all *C. neoformans* serotype infections. A comparison between a commercial EIA (Premier; Meridian Diagnostics, Cincinnati, Ohio), which employs a monoclonal antibody that recognizes all serotypes, and standard latex agglutination tests showed 92 to 97.8% agreement between them (Frank et al., 1993 ; Gade et al., 1991 ; Sekhon et al., 1993 ; Tanner et al., 1994).

4. Molecular Identification

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*. Strains can be identified by using pulsed-field gel electrophoresis or contour-clamped homogeneous-field gel electrophoresis to separate chromosomes and distinguish electrophoretic karyotypes of *C. neoformans* (Dromer et al., 1994 ; Perfect et al., 1989, 1993 ; Polacheck and Lebens, 1989 ; Wickes et al., 1994).

Several probes have been studied to distinguish the variety, serotype, or strain of *C. neoformans*. Genomic DNA can be digested with an appropriate restriction endonuclease, separated by electrophoresis, blotted, and hybridized with the species- or variety-specific probes CND 1.7 and CND 1.4, respectively (Polacheck et al., 1992). Other probes that distinguish individual strains include the plasmid probe UT-4p (Dromer et al., 1994 ; Varma and Kwon-Chung, 1992), the *URA5* gene (Currie et al., 1994), a repetitive element, CNRE-1 (Currie et al., 1994 ; Spitzer and Spitzer, 1992, 1994), and simple DNA repeats (Haynes et al., 1995).

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). Species-specific probes and primers have been developed (Mitchell et al., 1994b). Strains can also be identified by the RFLP patterns of genomic and mitochondrial DNA (Casadevall et al., 1992 ; Currie et al., 1994). Strains are much more quickly identified by banding patterns generated with the techniques of randomly amplified polymorphic DNA (Crampin et al., 1993 ; Haynes et al., 1995 ; Yamamoto et al., 1995) or by PCR fingerprinting (Meyer et al., 1993 ; Mitchell et al., 1994), in which a simple repeat is used as a single primer for amplification. In addition to DNA-based methods of identification, strains can be distinguished by multilocus enzyme analysis, in which cytoplasmic extracts are subjected to starch gel electrophoresis and stained for 10 different enzymes (Brandt et al., 1993). From the electrophoretic mobilities of these enzymes, the variety and serotype of *C. neoformans* can be identified.

Variations in the structure of capsular polysaccharide among serotypes have also been documented.

The use of excellent histological staining methods for the capsule, its relatively facile growth and identification features, and an excellent serological test make this yeast less likely to be an initial focus for molecular diagnostics. Molecular techniques will simply be incorporated into diagnostic strategies for *C. neoformans* when nonculture methods are routine practice.

G. Molecular Biology

The early development of molecular biological techniques for studying a pathogen generally involves questions of epidemiology in relationship to pathogenicity. Investigators in the late 1980s and the 1990s have used the newly acquired ability to type individual strains of *C. neoformans* by molecular techniques. Several molecular typing systems have been developed and used for *C. neoformans* infections. These typing systems can identify unique strains from both clinical and environmental sources and in some instances are used to determine relapse from true reinfection isolates. These molecular techniques include multilocus enzyme typing (Brandt et al., 1995, 1996); electrophoretic karyotyping with pulsed-field gel electrophoresis (Kwon-Chung et al., 1992 ; Perfect et al., 1989, 1993 ; Polacheck and Lebens, 1989); restriction fragment length polymorphisms (RFLPs); DNA fingerprinting by hybridization of restricted DNA with genomic repetitive DNA fragments (Currie et al., 1994 ; Polacheck et al., 1992 ; Spitzer and Spitzer, 1992 ; Varma and Kwon-Chung, 1992 ; Varma et al., 1995), mitochondrial DNA probes (Varma and Kwon-Chung, 1989), and oligonucleotide probes to microsatellite sequences; and random amplified polymorphic DNA (RAPD) analysis and PCR fingerprinting (Crampin et al., 1993 ; Meyer et al., 1993a, 1993b ; Meyer and Mitchell, 1994 ; Sorrell et al., 1996).

Several recent reports have established that genotypic variation in *C. neoformans* can be identified by molecular techniques.

Pulsed-field gel electrophoresis using a CHEF (contour-clamped homogeneous electric field) apparatus has been used to separate chromosomes of *C. neoformans*. A consistent finding in these studies was the significant polymorphic

variation in chromosome size between strains (Perfect et al., 1989, 1993). A variety of karyotype studies have found between 8 and 13 chromosomes in *C. neoformans* strains, with its predicted genome size of approximately 23 Mb. The genome size appears to be larger than that of *Saccharomyces cerevisiae* or *Candida albicans*. In *C. neoformans* var. *neoformans*, there are generally between 8 and 12 chromosomes; the largest chromosome is ≥ 2.2 Mb and the smallest is about 770 Kb, but smaller chromosomes have been noted (Perfect et al., 1989, 1993 ; Polacheck and Lebens, 1989). Similarly, *C. neoformans* var. *gattii* strains most commonly have between 11 and 13 chromosomes, with several small chromosomes in the range of 400 to 700 Kb (Kwon-Chung et al., 1992 ; Perfect et al., 1989). Pulsed-field gel electrophoresis has been used to demonstrate polymorphisms in the chromosome-like band pattern of 13 *C. neoformans* var. *neoformans* strains (Wickes et al., 1994). Perfect et al. used pulsed-field gel electrophoresis as an epidemiological tool for *C. neoformans* infections. In his study of over 40 isolates from both clinical and environmental sources, 90% had a unique chromosome banding by this technique. There was no conserved pattern associated with body site of infection, geographical location of the isolate, or human immunodeficiency virus status. Karyotypes of individual isolates remained stable during both in vitro passage and in vivo infections. Karyotype was used to exclude the possibility of nosocomial spread of *C. neoformans* in one clinical situation and supported relapse in two other cases. Because of its variable sizes between isolates, karyotyping of *C. neoformans* is a convenient method for molecular identification of different strains (Perfect et al., 1993).

The well-described molecular epidemiological strategy of using repetitive elements to probe for RFLPs in many pathogenic yeast species has also been developed for epidemiological studies with *C. neoformans*. First, the use of repetitive, multiple-copy ribosomal DNA (rDNA) genes of *C. neoformans* has been explored. The cryptococcal rDNA gene complex has been cloned (Restrepo and Barbour, 1989) and sequence (Fan et al., 1994). The *C. neoformans* rDNA repetitive fragment (approximately 8 Kb) contains genes in a 16S, 5.8S, and 23S arrangement, and these genes are transcribed in the same direction.

RFLPs with other repetitive elements can distinguish between strains of *C. neoformans*. For example, Polacheck et al. (1992) identified middle-repetitive DNA

sequences from *C. neoformans* (CND 1.4 and CND1.7) that can be used to distinguish certain hybridization patterns were found to be more common in AIDS versus non-AIDS isolates with the use of these repetitive probes, none of the molecular typing methods have conclusively proved that there is a unique genotype(s) from isolates that is AIDS-specific. The CNRE-1 probe contains a member of dispersed family of repetitive element (Currie et al., 1994 ; Spitzer and Spitzer, 1992, 1994 ; Spitzer et al., 1993). Spitzer and Spitzer identified this repetitive element and showed that *C. neoformans* possesses multiple copies of CNRE-1 that are located on at least seven chromosomes in some strains. There are at least 10 to 20 members of this family with a minimum length of 5 to 10 Kb. The function of CNRE-1 is not known, but it does not possess features typical of a transposable element (Spitzer and Spitzer, 1994). Since more than 70% of isolates within a geographically constrained population had an RFLP pattern different from that of CNRE-1, this probe has some discriminating power to identify different clinical strains. Fortunately, the probe also does not appear to change hybridization patterns during infection or with antifungal treatment (Currie et al., 1994 ; Spitzer and Spitzer, 1993). It has been used in concert with specific gene sequences from the *URA5* gene to help identify clonal populations of *C. neoformans* within a geographically restricted environment (Currie et al., 1994) and to compare the genetic diversity of isolates from Brazil with those from New York (Franzot et al., 1997). In the first study (Currie et al., 1994), isolates within the same CNRE-1 group showed fewer differences in nucleotide sequence of the *URA5* gene than did those from different CNRE-1 groups; RFLP analysis of environmental (pigeon excreta) and clinical *C. neoformans* var. *neoformans* isolates in a limited geographic area distinguished 6 strains among 8 environmental isolates and 12 strains among 17 clinical isolates. Clusters of patients with three strain types accounted for 47% of clinical isolates. Despite this diversity, two strains were shared by environmental and clinical isolates. The isolates from environmental sites heavily contaminated with pigeon excreta and from patients had identical RFLPs implies that some patients infected by strains of *C. neoformans* var. *neoformans* which are genetically related to those found in pigeon excreta. This observation suggests that pigeon excreta could potentially be reservoir for pathogenic *C. neoformans* var. *neoformans* for immunocompromised individuals. In the second study (Franzot et al.,

1997), there were differences in discriminating power between CNRE-1 and *URA5* gene sequence and karyotypes, but these molecular studies showed that there were differences in local diversity between the two sites but that some isolates were closely related to each other, suggesting global dispersal of some pathogenic strains.

Varma and Kwon-Chung (1992) identified another DNA probe (UT-4p) that has also been used to examine the diversity of *C. neoformans* strains (Varma and Kwon-Chung, 1992 ; Varma et al., 1995). This 7-Kb linear plasmid contains the cryptococcal *URA5* gene, and telomere-like sequences (TTAGGGGG) have been added to the ends of the plasmid. The acquisition of repetitive DNA sequences that are present on each chromosome and polymorphisms within the *URA5* gene made this probe useful for strain identification. Initial work with the probe found that 21 of 26 strains had unique DNA fingerprints and that the fingerprints of isolates within a serotype group were more similar to each other than to those from another serotype (Varma and Kwon-Chung, 1992). Although there were no unique patterns in AIDS or non-AIDS patients in 156 isolates, the patterns could be grouped into 9 to 12 distinct fingerprint patterns depending on the *C. neoformans* variety (Varma et al., 1995). In a study of 40 serotype D strains from France by Dromer et al. (1994), at least 10 groups were identified with the UT-4p probe and RFLPs. There was some suggestion that certain risk groups, such as drug addicts and homosexuals, had isolates from the same fingerprint group; also, five of seven isolates from cryptococcal pneumonia patients had the same fingerprint group. These findings suggest that this probe might identify characteristics of the genotype that predict body localization or even risk factors for infection, but further studies are needed to confirm this finding. The DNA RFLPs for UT-4p in serotype D strains were also found to be stable during human infection and its treatment.

Synthetic oligonucleotide probes homologous to microsatellite sequences have been used to identify genotypes of isolates. Although a series of oligonucleotide probes, including 5'-$(GT)_8$-3', 5'-$(GTG)_5$-3', 5'-$(GATA)_4$-3', and 5'-$(GACA)_4$-3', have been effectively used (Haynes et al., 1995 ; Meyer et al., 1993 ; Meyer and Mitchell, 1995) found that the oligonucleotide 5'-$(GGAT)_4$-3' yielded the most consistent and reproducible hybridization signals. With this highly discriminatory probe, all patients were found to have different isolates by RFLP analysis.

Garcia-Hermoso and coworker (1999) reported that possibility of geographical clustering when they used RAPD or PCR-fingerprinting with $(GACA)_4$ primer, and RFLPs with CNRE-1 probe for regrouping strains from control samples of *C. neoformans* var. *grubii* (serotype A) environmental isolates according to their geographical origins. The two typing techniques were then used to analyze 103 isolates from 29 patients diagnosed with cryptococcosis in France. Nine of the 29 patients originated from Europe. Results showed a statistically significant of clustering of isolate subtypes from patients originating from Africa compared to those from Europe. They concluded that the patients had acquired the *C. neoformans* infectious strain long before their clinical diagnoses were made. Viviani and collaborators (1997) in Italy studied seventy-three *C. neoformans* isolates and eight other yeast strains by PCR-fingerprinting for distinguishing among *C. neoformans* serotypes and other yeasts. Fingerprinting produced by priming with $(GACA)_4$ differentiated *C. neoformans* from all other yeasts tested and identified the five *C. neoformans* serotypes. Four major bands of molecular size 800, 540, 475, 410 base pairs were recognized for serotypes A, AD, and D. Two of them were identified by five different genotypic patterns in which at least one of the two-bands specific for serotype A and D were presents in different combinations. On repeated and simultaneously performed genotype and serotype testing of nine strains, the genotypic pattern did not change, whereas serotyping was unstable in three cases. PCR-fingerprinting using $(GACA)_4$ as a primer proved more stable than serology in discriminating *C. neoformans* serotypes A, D, and AD and was able to distinguish among serotype AD strains.

The use of PCR technology combined with repetitive primers (arbitrary primed PCR) has allowed investigators to identify the DNA fingerprints of different fungal strains rapidly, specifically, and with a minimal number of DNA purifications. These procedures have been adapted and refined for use in *C. neoformans*. The first PCR fingerprint studies used a variety of RAPD fragments within a strain. This work reflects the use of arbitrary single primers to identify the known polymorphic sequence structure of DNA from individual yeast strains. This PCR fingerprinting technique has been used to amplify hypervariable repetitive DNA sequences in *C. neoformans*. PCR fingerprinting with the oligonucleotide primers $(GTG)_5$, $(GACA)_4$,

and the phage M13 core sequence (GAGGGTGGXGGXTCT), but not with $(CA)_8$-generated polymorphic bands, has been successful in all *C. neoformans* strains tested. Strains of serotype A, B/C, or D could also be classified to serotype by their PCR fingerprint pattern. Thus, this method has the sensitivity to detect both inter- and intravarietal differences.

By these PCR methods, some amplified bands have been sequenced, and it appears that they generally represent inter-repeat sequences that are likely to be located in the noncoding regions of the *C. neoformans* genome. The high degree of homology among some of these minisatellite sequences and the primers produces such strong binding for PCR fingerprinting that these sequences may be more reliable and discriminating than standard RAPD fragments, which may use shorter primers (6 to 10 nucleotides) and arbitrary sequences. Passo et al. (1997) revealed that the genetic relatedness of clinical and environmental *C. neoformans* strains in the Maltese Islands, Italy was investigated by random amplified polymorphic DNA fingerprinting with four primers. The clinical strains isolated over the period of 1 year from AIDS patients showed identical fingerprints. The electrophoretic patterns of the two clinical strains were also the most common patterns among the environmental strains, but the patterns among the environmental strains showed a wide variability and no correlation with the site of isolation. RAPD analysis using five primers separated 344 *C. neoformans* isolates into 19 subtypes but could not distinguish isolates of *C. neoformans* var. *gattii* from *C. neoformans* var. *neoformans* (Crampin et al., 1993). On the other hand, RAPD analysis with 12- to 22- mer pairs and PCR fingerprinting with a single primer derived from the microsatellite core sequence of the wild-type phage M13 found only three major genetic profiles in isolates of *C. neoformans* var. *gattii* (Sorrell et al., 1996). Yamamoto et al. (1995) used RAPD profile analysis with three primers that revealed six patterns among 21 clinical isolates and three patterns among 8 environmental (pigeon excreta) isolates in the southern Japanese prefecture of Nagasaki. Two environmental isolates from two locations associated strongly with two patients revealed identical RAPD patterns for isolates from each patient. These results suggested that clinical and environmental isolates belong to the same pool of *C. neoformans* isolates and that these isolates had certain geographic locations, although the number of isolated strains was limited. PCR fingerprinting has evolved

as the most popular method for DNA fingerprinting of the infectious fungi. Using random primers of approximately 10 bases, amplicons throughout the genome were targeted and amplified. Amplified products were separated on an agarose gel and stained with ethidium bromide (Soll, 2000). Only one study by RAPD analysis had suggested that *C. neoformans* isolates from AIDS patients and other immunocompromised hosts might have a common genetic profile, and this result awaited confirmation (Chen et al., 1996).

However, all of these typing systems have been used singly and the isolate discrimination data obtained have not been substantiated by a second confirmatory technique.

H. Treatment

Cryptococcosis is a disease of variable clinical expression and variable course which may occur in patients whose immune response is normal or suppressed; thus, results of therapy have been difficult to determine. The ideal has always been to find a highly effective drug with little toxicity that can be given orally and crosses the blood-brain barrier.

In the late 1950s, amphotericin B (AMB) was found to be effective against a variety of pathogenic fungi including *C. neoformans*. Thus, the stage was set for the use of this drug in treating cryptococcal meningitis. In the 1960s, a second drug appeared that was effective against *C. neoformans*, 5-fluorocytosine (5-FC). With two such drugs available, it was clear that combination therapy would be tried, since results with either drug alone were less effective (Segal and Baum, 1994).

A new era in the treatment of fungal infections appeared in the late 1960s with the appearance of the imidazole drugs. The first of these that had clinical anti-cryptococcal effectiveness was miconazole, as typified by the case of Weinstein and Jacoby (1980). This drug was not universally effective, however, and had significant toxicity. A much larger experience developed with ketoconazole. Although murine cryptococcosis was improved by ketoconazole treatment the persistence of positive culture from CSF raised questions regarding its clinical utility in man. This skepticism was never countered by clinical experience, and the summary by Sugar

et al. (1990) clearly stated the greater usefulness of AMB treatment and the variable results with ketoconazole.

In the late 1980s, the development of the triazoles, a new family of similar drugs, provided early clinical experience that gave promise of results that equal those achieved with AMB (Iwata et al., 1990 ; Larsen et al., 1990). Use of these drugs achieved not only clinical success but sterilization of CSF in some cases of meningitis.

Cyclosporin A is an important drug that suppresses foreign tissue rejection and is of critical importance to organ survival in human recipients of transplanted organs. In fact, it is arguably the most important drug in this arena of medicine. Cyclosporin A has been found to have an impact on experimental cryptococcal infection, a matter with important clinical implications due to the frequency of *C. neoformans* infections in organ transplant recipients. Mody et al. (1988) found that cells involved in the delayed-hypersensitivity response to *C. neoformans* were not modified by cyclosporin A but a T cell, which enhanced the effector cell response against *C. neoformans*, was inhibited by cyclosporin A, thus causing an overall decrease in the immune response in mice to *C. neoformans*.

In summary, cryptococcosis is a disease with broad clinical importance, the immunologic and therapeutic implications of which are only superficially understood. The widespread residence of the organism in nature and the growing susceptible human population emphasize the urgency of dealing with this problem in the most energetic manner possible.