1.1. General introduction

The fungi are ubiquitously found in environment and most of these are commensals, contaminants or non-pathogenic agents. Some of the fungi are beneficial to humans. A small proportion of these fungi are pathogenic to animals and plants. Only 5% of the total fungal species in the world have been identified, which constitute about 69,000 species out of an estimated 15,00,000 (Hawksworth, 2001). Of this, around 300 species are prominent primary pathogens of man and other mammals and 10 to 15 are frequently encountered in routine clinical practice. Most of the fungi exist as molds but there are number of pathogenic yeasts and many are dimorphic as well.

Cryptococcosis is a fungal disease found worldwide in human and animal populations. The causative agent is a heterobasidiomycetous encapsulated yeast, Cryptococcus spp. which is considered infectious only as a desiccated yeast cell or basidiospore as found in the environment (Benham, 1956). The major contributor to the emergence Cryptococcal infections is the increasing number of immunocompromised individuals. The significance of cryptococcosis has reached high levels after the emergence of Acquired Immunodeficiency Syndrome (AIDS) (Banerjee et al., 2001; Levitz & Boekhout, 2006). Following oral Candidiasis, Cryptococcosis is the second most common fungal infection in the immunocompromised host especially in HIV infected individuals with a risk of being the most severe infection. Cryptococcosis is not rare in HIV-negative patients. It is known to be associated in patients with chronic leukemia, solid tumors, those undergoing prolonged radiotherapy, extensive treatment with corticosteroids, cytostatics or antibiotics and organ transplant recipients (Mitchell & Perfect, 1995).
1.2. History

Isolation of cryptococcus from fermenting peach juice was carried out by Sanfelice during 1894 and it was in the same year the pathogenic potential in humans got first described from a tibial ulcer (Buschke, 1895). Due to lack of ascospores it was renamed as Cryptococcus hominis (Vuillemin, 1901). A case of cryptococcal meningitis was described in 1914 (Verse, 1914) and later in 1955 it could be recovered from pigeon droppings (Emmons, 1955). The sexual stage was discovered in 1976 (Kwon-Chung, 1976) and the whole genome sequencing of cryptococcus done in 2003.

1.3. Mycology

1.3.1. Taxonomy

Basidiomycetous yeasts are anamorphs of members of jelly fungi (Hymenomycetes; Tremellales) or of smuts (Ustilaginomycetes, Ustilaginales). Cryptococcus spp. are environmental fungi (Kwon-Chung, 1975) classified under;

- Phylum: Basidiomycota
- Class: Heterobasidiomycetes
- Order: Filobasidiales
- Family: Filobasidiaceae
- Genus: Filobasidiella

Cryptococcus is a large genus with species of diverse relationships. The pathogenic species *C. neoformans* is phylogenetically rather well delimited from the remaining species. Recently novel species, *Cryptococcus rahdhwai* was isolated from tree trunk hollow of *Ficus religiosa* in India (Khan, 2010).
1.3.2. Generic description of genus Cryptococcus

1.3.2.1. Morphological and physiological characters

Pseudo mycelium mostly absent; percurrent budding from spherical yeast cells; capsule may be present. Generative reproduction is mostly produced after mating of suitable partners. A clamped mycelium with thick-walled, brown teliospores is formed, which eventually germinate with a non-septate basidium (Holobasidium) or a septate basidium (Phragmobasidium), bearing sessile basidiospores. Ultrastructure reveals multilamellar cell walls, septa have dolipores or simple pores. They are recognized by presence enzyme of urease, extracellular DNAse and by the less widely used Diazonium Blue B (DBB) staining reaction, which is also positive. In addition, mostly extracellular starch-like compounds are produced, Inositol is mostly assimilated and sugars no fermentation. Cell wall contains xylose. (Barnett et al., 1990)

1.3.2.2. Life cycle

_Cryptococcus neoformans_ is a hetero-basidiomycetous yeast and can reproduce asexually through budding as well as sexually through mating type switching (Bergman, 1962). The anamorphic form or the asexual state of _Cryptococcus neoformans_ is characterized by the formation of budding yeast cells. These forms are haploid and unicellular yeasts obtained from body fluids of infected individuals. The teleomorphic form or the sexual state is characterized by the production of basidiospores belonging two tow mating types α and a. Co culture of yeast of each mating type can lead to conjugation which produces the teleomorph (Campbell et al., 2005). The teleomorph contains the hyphae which develop into specialized structures known as basidia.
Formation of uninucleate basidiospores occurs due to meiosis. Non encapsulated cells are released from the basidia, soon after which budding begins (Kwon-Chung, 1975; Campbell & Carter, 2006).

1.3.2.3. Classification

1.3.2.3.1. Conventional classification

Conventional nomenclature included three recognized varieties of Cryptococcus neoformans; C. neoformans var. grubii (serotype A), C. neoformans var. neoformans (serotype D) and C. neoformans var. gattii (serotypes B and C) as well as a hybrid of C. neoformans var. grubii and C. neoformans var. neoformans (serotype AD). Serotype AD is the least studied and has been reported in between serotype A and D (Kwon-Chung, 1975, Evans & Kessel, 1951).

1.3.2.3.2. Recent classification

Currently based on genetic variability and lack of evidence for genetic recombination between the two varieties proposed that C. neoformans should be divided into two distinct species as C. neoformans (serotypes A, D and AD) and C. gattii (serotypes B and C) (Kwon-Chung et al., 2002). The intergenic spacer regions and population structures of Cryptococcus neoformans species complex is found to differ (Diaz et al., 2005).

1.4. Cryptococcus neoformans species complex

The genus Cryptococcus includes over 37 species, however only C. neoformans and C. gattii are commonly considered to be most pathogenic causing Cryptococcosis in immunocompromised and immunocompetent individuals (Kwon-Chung & Varma, 2006). The characteristic features are shown in Table 1.1.
Dual infections with pigmented and albino strains of *C. neoformans* and unique hybrids of *Cryptococcus neoformans* and *Cryptococcus gattii* are reported (Bovers et al., 2006, 2008; Mandal et al., 2005). Extensive diversity is observed in the internal transcribed spacers and 5.8S rRNA gene of isolates of the species complex (Katsu et al., 2004).

<table>
<thead>
<tr>
<th>FEATURES</th>
<th><em>C. neoformans</em></th>
<th><em>C. gattii</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>var. grubii</td>
<td>var. neoformans</td>
</tr>
<tr>
<td>Serotypes</td>
<td>A</td>
<td>D</td>
</tr>
<tr>
<td>Mating Types</td>
<td>α</td>
<td>α and a</td>
</tr>
<tr>
<td>Telomorphs</td>
<td><em>Filobasidella neoformans var. neoformans</em></td>
<td><em>Filobasidella neoformans var. neoformans</em></td>
</tr>
<tr>
<td>Prevalence</td>
<td>Worldwide</td>
<td>Europe</td>
</tr>
<tr>
<td>Habitat</td>
<td>Avian Excreta</td>
<td>Avian Excreta</td>
</tr>
<tr>
<td>Immune status of Patient</td>
<td>HIV Positive</td>
<td>On Corticosteroids</td>
</tr>
<tr>
<td>Skin Involvement</td>
<td>Less Frequent</td>
<td>Frequent</td>
</tr>
<tr>
<td>Blastospores</td>
<td>Rounded</td>
<td>Rounded</td>
</tr>
<tr>
<td>Creatinine Dextrose Bromothymol blue Thymine media</td>
<td>No Growth</td>
<td>Growth with orange colour change</td>
</tr>
<tr>
<td>L-Canavanine Glycine Bromothymol Blue Media</td>
<td>No Change</td>
<td>No Change</td>
</tr>
<tr>
<td>Glycine Cycloheximide Phenol Red Agar</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>D-proline</td>
<td>Not Utilized</td>
<td>Not Utilized</td>
</tr>
<tr>
<td>D-tryptophan</td>
<td>Not Utilized</td>
<td>Not Utilized</td>
</tr>
<tr>
<td>Malate Assimilation</td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>

*Table 1.1. : Comparative differences among C. neoformans and C. gattii*
1.4.1. Serotypes

Based on immunological reactivity of cryptococcal capsule with immune sera owing to the antigenic variability of capsule in the proportions of xylose and glucoronic residues, degree of mannose substitution and percentage of O-acetyl attachments five serotypes A, B, C, D and AD are identified. (Evans, 1950; Evans & Kessel, 1951).

1.4.2. Varieties

Based on morphology, biochemistry and genetic characters Cryptococcus neoformans is further divided into varieties corresponding to the serotypes as; Cryptococcus neoformans var. grubii (serotype A) and Cryptococcus neoformans var. neoformans (serotype D). Cryptococcus neoformans var. gattii (serotype B and C) is now considered separate species Cryptococcus gattii (Kwon-Chung et al., 2002).

1.4.3. Mating types

In its telemorphic state the basidiospores of the yeast exist in two mating types 'alpha' (α) and 'α' which is determined by a single locus, two-allele system. The more virulent α type outnumber (>95) compared to the α type in both clinical and environmental isolates (Kwon-Chung & Bennett, 1978). Appropriate serotype A and D (A x A, A x D, D x D) or serotype B and C (B x B, B x C, C x C) can mate with each other (Kwon-Chung, 1975). The G α Protein Gpa1 and cAMP controls the mating and virulence of Cryptococcus neoformans (Alspaugh et al., 1997, 2002). It was found that the α strains preferentially disseminate to the central nervous system during co infection (Chang et al., 2001; Nielsen et al., 2005).
Hybrid strains are homozygous at mating-type locus (Cogliati et al., 2006). Same sex mating of α AD and α hybrids have been reported to occur in nature conferring hybrid fitness (Lin et al., 2008).

1.5. Non neoformans Cryptococcus with pathogenic potential

_Cryptococcus albidus_ and _Cryptococcus laurentii_ are found to be pathogenic to human beings and are implicated in more than 80% of the non neoformans cryptococcosis (Narayan et al., 2002; Cheng et al., 2001). Occasional reports of meningitis by _Cryptococcus adeliensis_ in leukemia patients have been reported in literature (Rimek et al., 2004). _Cryptococcus rajasthanensis_ sp. nov., an anamorphic yeast species related to _Cryptococcus laurentii_ has been recently isolated from Rajasthan, India. However its pathogenic role remain speculative (Saluja & Prasad, 2007). _Cryptococcus humicola, Cryptococcus curvatus_, and _Cryptococcus luteolus_ are rarely reported.

1.6. Ecology

1.6.1. _C. neoformans_ var _neoformans_ and var. _grubii_

Further seasonal variations in the climatic conditions were found to influence the prevalence of Cryptococcus from the decayed wood inside the tree hollows of diverse tree species (Granados & Castaneda, 2006; Randhawa et al., 2011). Cryptococcus neoformans have also been isolated from vegetables and fruits (Misra et al., 2000).

1.6.2. C. gattii

Cryptococcus gattii is mostly confined to the tropics and sub tropics of the world (Kwon-Chung & Bennett, 1984; Sorrell, 2001; Hagen & Boekhout, 2010). It was first isolated from eucalyptus trees (Eucalyptus camaldulensis and E. tereticornis) in Australia (Ellis & Pfeiffer, 1990b; Hermoso, 1999). Further C. gattii has been isolated from eucalypt species in many other parts of the world including California, India and Brazil (Montenegro & Paula, 2000; Nishikawa et al., 2003; Chakrabarti et al., 1997; Pfeiffer & Ellis, 1991) and in non-eucalypt trees from tropical and subtropical areas across the world (Nishikawa et al., 2003; Lazera et al., 2000, Callejas et al., 1998).

The common environmental isolates of C. gattii have been serotype B, but sometimes reports are indicative of serotype C isolated from almond trees (Terminalia catappa) in Columbia and vegetation in southern California (Pfeiffer & Ellis, 1991 & Callejas et al., 1998). It is been suggested that dispersal of infectious propagules could be linked to the flowering of the eucalypt trees (Ellis & Pfeiffer, 1990a).

Few studies speculated that there could be a alternative environmental sources of C. gattii that is yet to be identified, as molecular types isolated from clinical and environmental samples have been different in western and northern Australia (Chen et al., 1997; Sorrell, et al., 1996)
Cryptococcosis caused by *C. gattii* has been reported from regions where an the environmental source cannot be known, including parts of Australia, Africa and Papua New Guinea (Sorrell et al., 1996; Chen et al., 1997; Laurenson et al., 1996, 1997).

Both *C. neoformans* var. *grubii* and *C. gatti* have been isolated from the flowers and bark of eucalyptus tree in India (Gugnani et al., 2005).

### 1.7. Epidemiology of Cryptococcosis

The frequency and type of strains found in cases of cryptococcosis differ according to geographical areas as shown in Table 1.2. In large survey it was found that the overall cases around 80% were owing to *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii* (Kwon-Chung & Bennett, 1984). *C. gattii* is predominantly a primary pathogen infecting immunocompetent host even in endemic area and is rarely isolated as the etiological agent of cryptococcosis in AIDS patients (Walter & Atchinson, 1966; Chen et al., 2000; Speed & Dunt, 1995; Mitchell et al., 1995).

<table>
<thead>
<tr>
<th>Serotypes</th>
<th>Geographical Regions</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Worldwide including North USA</td>
</tr>
<tr>
<td>B</td>
<td>Australia, Southeast Asia (India), Central Africa, Brazil, Venezuela, California, Italy</td>
</tr>
<tr>
<td>C</td>
<td>Southern California, Colombia</td>
</tr>
<tr>
<td>D</td>
<td>Europe, Denmark, Italy, Switzerland, France</td>
</tr>
<tr>
<td>AD</td>
<td>Worldwide</td>
</tr>
</tbody>
</table>

*Table 1.2. : Geographical distribution of Cryptococcus serotypes*
The distribution of cryptococcosis in a region owing to *C. gattii* corresponds largely with their environmental distribution (Sorrell, 2001; Chen et al., 2000; Ellis & Pfeiffer, 1990b). In an survey of global human clinical cryptococcosis, isolates of *C. gattii* were not found in Austria, Belgium, Denmark, France, Germany, Holland, Italy, Switzerland, and Japan but were identified at an high prevalence in Australia, Brazil, Cambodia, Hawaii, southern California, Mexico, Paraguay, Thailand, Vietnam, Nepal and Central Africa (Kwon-Chung & Bennett, 1984; Tintelnot et al., 2004). Further studies have confirmed the high prevalence of *C. gattii* in tropical and sub-tropical regions including Brazil (Barreto-de-Oliveira et al., 2004; Nishikawa et al., 2003), Thailand (Poonwan et al., 1997), Papua New Guinea (Laurenson, 1996), Venezuela, South Africa and Mexico (Castanon-Olivares et al., 2000). A few number of human *C. gattii* cases have been reported from India, China, Taiwan, Peru, Argentina, Rwanda, Italy (Abraham et al., 1997; Sorrell, 2001; Li et al., 1993; Padhyee et al., 1993; Banerjee et al., 2001; Chen et al., 2000). In 2002 the first reported isolation of Cryptococcus *gatti* from a patient in Singapore was documented (Taylor et al., 2002).

The cases from Europe and non-endemic areas of North America are considered to be acquired elsewhere (Sorrell, 2001; Kwon-Chung & Bennett, 1984; Grosse et al., 2001). In North America *C. gattii* infection in North America is considered rare (Kwon-Chung & Bennett, 1984). Most of the isolates have been serotype B (Mirza et al., 2003; Brandt et al., 1996) with the exception of southern California where serotype C is more prevalent (Kwon-Chung & Bennett, 1984) and has been isolated from the environment (Pfeiffer & Ellis, 1991). Highly virulent *C. gattii* genotypes is reported to have emerged in northwest United States (Datta et al., 2009; Byrnes et al., 2010).
Cryptococcosis has been reported from most provinces in Canada commonly associated with immunosuppression and caused by *C. neoformans* (Sekhon et al., 1990). *Cryptococcus gattii* has been isolated once from an AIDS patient in Quebec who had a travel history to endemic region of *C. gattii* (St-Germain et al., 1988). Recently cryptococcosis outbreak of rare genotype of *C. gattii* was reported from the Vancouver Islands of British Columbia, Canada (Kidd et al., 2004; Fraser et al., 2006; Ma et al., 2009; Hagen et al., 2010) Figure 1.1.

**Figure 1.1**: News alerts on *C. gattii* outbreak in Vancouver  
*Courtesy: Ferry Hagen*

### 1.8. Predisposing factors

The occurrence of disseminated forms of Cryptococcosis is related to immune deficiencies of T cells (Kokturk et al., 2005). Before AIDS epidemic, most cases of cryptococcosis are related to cancer, especially malignancies (Friedman, 2005).
C. neoformans is found to occur in the respiratory tract of patients with bronchopulmonary disorders (Randhawa & Pal 1977). Treatment with corticosteroids agents was an important risk factor as well. AIDS now represents the most common risk factor for the development of Cryptococcal disease (Hajjeh et al., 1999). Another important T cell associated immunosuppression related to cryptococcosis is organ transplantation (Husain et al, 2001; Vilchez et al., 2003; Dromer et al., 2007). Serological evidence can be indicative of cryptococcosis reactivation in solid-organ transplant recipients (Saha et al., 2007).

1.9. Immunology

This particular fungus is of great interest as it traverses through all the areas of host immunity. Innate immune response involving alveolar macrophages is triggered as the infectious propagules reach the lung (Alvarez & Casadevall, 2006). Cell mediated immunity mediated by natural killer cells and both CD4+, CD8+ lymphocytes provide a protective immunity (Hildore et al., 1991; Miller & Mitchell, 1991; Hill & Harmsen, 1991; Huffnagle et al., 1991; Mody et al., 1993, Marr et al., 2006). The antibody mediated protection against Cryptococcus neoformans pulmonary infection is dependent on B cells (Rivera et al., 2005). It is found that interleukin-4 have a role in resistance to C. neoformans infections. Studies have demonstrated serum antibodies reactive with C. neoformans proteins in Cryptococcosis patients (Saha et al., 2008).

1.10. Virulence factors

Virulence factors of Cryptococcus neoformans can be regulated genetically or through environment conditions (Bahn et al., 2005; Casadevall, 2010).
The major virulence factors of Cryptococcus neoformans are considered to be the organisms ability to grow at 37°C, presence of capsular polysaccharide and production of melanin (Mc Clelland, 2005). In addition to various other virulence traits the mating type is also said to contribute in causing infection (Mitchell, 2004; Lin et al., 2008). The interactions of Cryptococcus neoformans with amoebae enabled the comprehension of intracellular pathogenic strategy of this yeast inside the macrophages (Steenbergen et al., 2001).

1.10.1. Growth at 37°C

This property is important for pathogenic potential of Cryptococcus (Perfect et.al., 2010). It was found that mutant C. neoformans that cannot grow at 37°C remained avirulent even in the presence of their virulence factors (Kwon-Chung, et al., 1982).

1.10.2. Capsular polysaccharide

A characteristic polysaccharide capsule of variable thickness generally surrounds the cryptococcal cell. In its natural environment the capsule is thinner and the yeast cell is smaller, while thicker capsule is found in infected tissues (Granger, et al., 1985). The capsule is demonstrated using India Ink preparation, as it exudes the India ink and forms a halo around the cell.

1.10.2.1. Components of the Capsule

It surrounds the cell wall and is composed of three kinds of antigenic polysaccharides. This exopolysaccharide is composed of a major component known as glucuronoxylomannan or GXM and a minor component called galactoxylomannan or GalX.
A third polysaccharide component is the Mannoprotein, which is a minor antigenic component (Levitz et al., 2001). The basic units of the capsule are xylose, mannose and glucuronic acid (Cherniak et al., 1991; Bose et al., 2003; McFadden et al., 2006). Radiological studies have revealed the radial differences in the capsular structure (Bryan et al., 2005) and equatorial ring like channels present in the polysaccharide capsule (Zaragoza et al., 2006). Variation in glucuronocylomannan structure in clinical isolates is found in recurrent cases of meningitis (Spitzer et al., 1993; Cherniak et al., 1995).

Glucuronoxylomannan or GXM consists of about 85% of the capsular mass. The backbone of this unit is made of mannose residues with α-1, 3 linkage and xylosyl and glucuronyl side groups. The GXM differs on the basis of addition of xylose and acetylation in different Cryptococcal strains. These difference characterized by specific antibody binding, divides Cryptococcus neoformans into the 5 serotypes. Galactoxylomannan or GalXM is responsible for around 8 to 9% of the capsular content. It consists of a α-1, 6 galactose polymer which had side chains of various lengths on alternate galactose residues. The constitution of the polysaccharide components varies based on the heterogeneity of the serotype. The third type of polysaccharide is the mannoprotein, which is a component of the cell wall. It is a minor component of the exopolysaccharide (Doering, 2000). Capsule associated gene like CAP60 has been identified (Chang et al., 1996, Chang & Kwon-Chung, 1998, 1999).
1.10.2.2. Properties and functions of the Capsule

The soluble polysaccharide of the capsule exerts various effects on the protective immune response like:

- They act as antigens that stimulate the cell mediated immunity (Cherniak & Sundstrom, 1994).
- preventing the leukocytes from reaching the site of inflammation thereby inhibiting production of proinflammatory cytokines.
- depleting the complement components by binding to them and serve as a protective barrier against phagocytosis (Kozel, 1977; Kozel et al., 1991).
- down regulating neutrophils and other lymphocytes (Monari et al., 2006).

Glucuronoxylomannan, Galactoxylomannan, and mannoprotein induce different levels of tumor necrosis factor alpha in human peripheral blood mononuclear cells (Chaka et al., 1997). In addition the capsule has anti phagocytotic property (Doering, 2009; Zargoza, et al., 2005). Phagocytosis is the engulfing and ingestion of foreign particles by phagocytic cells. During infection by *Cryptococcus neoformans*, the cells of the immune system like macrophages and neutrophils are unable to phagocytose the encapsulated yeast cells without opsonins. The lack of opsonins is due to the fact that the polysaccharide capsule has poor antigenicity and cannot produce an antibody response. Capsule free cells are phagocytosed to a higher extent as compared to encapsulated cells. Thus, the capsule provides a shield against action of immune effector cells. The capsule also interacts with antibodies (Feldmesser et al., 2000).
Chapter 1

Introduction and Literature Review

The capsular material is shed from the outer surface of the cell and is detected in body fluids like CSF and serum. This shed material can be considered as a factor of virulence and the increased production of it over time leads to the severity of the disease (Charlier et al., 2005).

1.10.2.3. Factors affecting capsule induction

Capsule size or thickness is determined by the genetics of the strain as well as by conditions of growth (Janbon, 2004; Maxson et al., 2007). Depending on the environmental conditions, the capsule of Cryptococcus neoformans shows a lot of variation (Zargoza, et al., 2005). Under in vitro conditions, the capsule size is much smaller than the size observed in in vivo conditions during mammalian infection (McFadden et al., 2006).

Another factor that induced capsule synthesis was a rich carbon dioxide atmosphere (Granger, et al., 1985; Zaragoza et al., 2003). It had great physiological importance as it determined the product of mammalian respiration as a factor. Diluted Sabouraud medium, either with water or PBS (phosphate buffered saline) showed much greater capsule formation than simple medium. Also the pH, if kept basic at 7 by addition of NaOH, enhanced the capsulation of cells.

The increase in capsule size differs based on the site of infection, as the cells in the lung have greater capsular volume than in the brain. At times, if the volume of the capsule increases, the cell size either reduces or increases, based on the type of environment conditions it is exposed to. The increase in capsular volume seems to be due to the reduction in growth rate of the yeast cells. This leads to reduced cell volume with an enhanced capsule (Doering, 2000).
Thus for a given strain, capsule production in vitro can be optimized by cultivation on solid or liquid medium in the presence of neutral pH, temperature of 37°C, elevated carbon dioxide, or decreased concentration of iron (Perfect, 2005).

Conversely, capsule production can be decreased in vitro by growth at lower temperatures, under conditions of high osmolarity (glucose or NaCl) or acid pH, or storage in soil. Under the same conditions of growth, some strains characteristically produce large, medium, or minimal capsules. But, capsules of cells under in vivo conditions show greater capsulation than cells grown in in vitro conditions. Some of the factors that reduce the volume of the capsule under in vitro conditions are the high glucose concentration and osmolarity of the medium (Doering, 2000).

**1.10.3. Melanin production**

The production and accumulation of melanin is a key to cryptococcal virulence mechanisms. Melanins are a negatively charged, insoluble compounds that contain free radicals. Basically, it is a pigment found in plants, animals, dimorphic fungi, molds and so on. It is a compound of high molecular weight formed by the oxidative polymerization of phenolic compounds (Liu et al., 1999; Suryanarayanan et al., 2004, Eisenman et al., 2005).

_Cryptococcus neoformans_ utilizes L Dopa or Caffeic Acid as substrates for the production of melanin. The melanin produced is stored in the cell wall of the fungus, hence giving it protection against environmental factors and immune cells.
This process is catalyzed by an enzyme known as phenol oxidase or laccase (Williamson, 1994; Garcia-Rivera, 2005). The presence of laccase gene CNLAC1 confer virulence to *C. neoformans* (Salas et al., 1996). It is a copper dependant enzyme and converts L Dopa to diaminobenzenes and finally to dopamine melanin. (Williamson, et al., 1998). The melanized cells are protected against free radical killing by immune cells through protection against oxygen and nitrogen based oxidants (Mednick et al., 2005). The pigment protects the fungal cells against high temperatures, UV radiation as well as phagocytosis by macrophages and neutrophils in case of an immune response (Wang & Casadevall, 1994). Melanin prevents the release of TNFα, which is required by macrophages for phagocytosis. Effects of antifungal agents on melanized *Cryptococcus neoformans* cells especially Amphotericin B and Voriconazole were shown to be at a lower degree than non melanized cells (Martinez, et al., 2007). This could be a factor contributing to the difficulty in treatment of Cryptococcal meningoencephalitis. High levels of catecholamines like dopamine and epinephrine are present in the central nervous system. Since phenol oxidase utilizes these catecholamines as substrates, it protects the cryptococcal cells from the destructive effects of catecholamines (Wang, 1996; Williamson, 1997).

The yeast *Cryptococcus neoformans* has been found to form DOPA melanin at the cell wall using laccase, and is a melanin producing fungus that does not synthesize the monomer to its own integrated cell wall melanin but forms the melanin at cell wall from exogenously supplied substrate (Wang, 1995). In this case, by growth in Caffeic Acid Ferric Citrate Test Agar, it is supplied with Caffeic Acid.
The Dopamine is converted dopaminoquinone by Laccase or phenol oxidase enzyme (Garcia-Rivera et al., 2005). After the formation of dopaminochromes and dihydroxyindole, finally Dopamine (Chaskes et al., 2008). Melanin is synthesized which is stored in the cell wall of the fungal cell.

1.10.4. Enzymes and Metabolites

Production of urease and superoxide dismutases are putative virulence factors. Polyl metabolic pathways leading to production of mannitol could contribute to virulence (Perfect et al., 1998). The major proteins secreted have been identified (Biondo et al, 2006). The role of Protease and Phospholipases as virulence determinants remain speculative (Hamilton & Goodley, 1996; Shea et al., 2006). Sialic acids in the cell surface of *Cryptococcus neoformans* is found to influence phagocytosis (Rodrigues et al., 1997).

*Cryptococcus neoformans* also show phenotypic switching and is considered to promote virulence (Jain et al., 2006; Guerrero et al., 2006).

1.11. Mechanism of Pathogenesis

Cryptococcosis is a disease contracted through the respiratory route by inhalation of a infectious particle, which could be a spore or a desiccated yeast cell (Neilson et al., 1977; Ruiz & Bulmer, 1980). A case of zoonotic transmission of *Cryptococcus neoformans* from a magpie to a immunocompetent host is reported in literature (Lagrou et al., 2005).
The invading microbe may either be cleared from the body in case of a healthy individual or it may remain dormant. The primary site of infection is the lungs. Natural killer cells, CD4+, CD8+ cells provide a protective immunity (Hill & Harmsen, 1991). As the immune system is compromised can disseminate. The ecological aspects and stages in pathogenesis is shown in Figure 1.2.

**Figure 1.2.** Salient ecological features and pathogenesis of Cryptococcosis
Their predilection to central nervous system leads to fatal meningoencephalitis if left untreated (Bicani & Harrison, 2004). The ability of cryptococcus to utilize catecholamines as substrates for melaninogenesis is speculated to be a reason for its neurotropism. They enter the central nervous system via transcellular penetration of blood brain barrier (Chang et al., 2004).

1.12. Spectrum of Clinical manifestations

By far the most common presentation of cryptococcosis remain meningoencephalitis with slight differences in non-AIDS patients (Kiertiburanakul et al., 2006; Akcaglar et al., 2007; Georgi, 2009; Zhu et al., 2010) and AIDS patients with respect to duration of symptoms, high antigen titer, extraneural involvement and opening CSF pressure etc. (Kovacs et al., 1985; Eng et al., 1986; Zuger et al., 1986; Chuck & Sande, 1989; Clark et al., 1990; Saag et al., 1992; Imwidthaya & Poungvarin, 2000, Mahoharan et al., 2001; Metta, 2002; Wadhwa et al., 2008). Recently multiple intracranial abscesses as an unusual feature in an immunocompetent host has been described and reviewed (Tore et al., 2010). In a review till 1965 it was found that primary pulmonary cryptococcosis remain asymptomatic (Campbell, 1966). It was also found that rarely patients may present with airway colonization, laryngitis and empyema (Duperval et al., 1977; Wasser & Talavera, 1987; Nadrous et al., 2004; Mulanovich et al., 2010). Endogenous cortisol production and diabetes mellitus was reported as a predisposing factor for pulmonary cryptococcosis (Thangakunam et al., 2008). Some studies have compared the clinical and radiographic characteristics of pulmonary cryptococcosis in immunocompetent and immunocompromised patients (Lindell et al., 2005; Chang et al., 2006).
There is a impressive array of cutaneous presentations of cryptococcosis with acneiform lesions, purpura, papules, vesicles, nodules, tumors, abscesses, ulcers, superficial granulomas, plaques resembling ecchymoses, sinus tracts, molluscum contagiosum-like lesions, cellulitis (Cawley et al., 1950; Borton & Wintroub, 1984; Hong et al., 2000; Vijaya et al., 2001; Kimura et al., 2001; Gauder, 1977; Gupta, 2004; Xiujia & Aie, 2005; Dharmashale et al., 2006; Sun et al., 2010; Elhence & Bansal, 2010). Infection due to trauma or clinical accidents has also been observed (Glaser & Garden, 1985; Casadevall & Mukherjee, 1994).

Osseous, ocular and involvement of prostrate has been documented. (Gugnani et al., 2002; Singh & Xess, 2010; Braman, 1981; Blackie et al., 1985). Peritonitis in HIV positive host and gastrointestinal cryptococcosis presenting as spontaneous jejunal perforation has been rarely reported in literature (Singh et al., 2011; Chaitowitz et al., 2003). Recently 15 cases of Cryptococcal lymphadenitis diagnosed using fine needle aspiration cytology has been reviewed (Srinivasan et al, 2010). Nephrotic and Cirrhotic patients also have been found to encounter cryptococcosis (Qadir et al., 2006; Franca et al., 2005).

1.13. Lab diagnosis

Through staining, culture and indication of presence of Cryptococcal antigen in CSF or serum, diagnosis of cryptococcosis is possible. Clinical specimens such as CSF, serum, corneal scrapings and other body fluids are most commonly tested for Cryptococcosis. Few rapid identification methods for diagnostic purposes are available (Muchmore et al., 1978; Cohen, 1984, Koshi et al., 1989; Saha et al., 2008). In a case Cryptococcal fungemia was diagnosed by blood (Sivasangeetha et al., 2007).
1.13.1. Direct examination

Direct microscopy with the use of India Ink shows round budding or adult yeast cells ranging from about 5 to 150 µm with a halo indicating the polysaccharide capsule (Mitchell & Perfect, 1995). Some modification of the india ink preparation is also found to be useful for diagnosis of capsulated cryptococcal cells from cerebrospinal fluid specimens (Zerpa et al., 1996). Interpretation of smears of purulent exudates using gramstain is not satisfactory (Bottone, 1980). Haematoxylin and Eosin, Grocott-Gomori’s methenamine silver staining, Periodic acid-Schiff staining and Mayer’s Mucicarmine staining are employed for tissue sections to look for cryptococcus. Certain unusual morphologies of cryptococcus spp. in tissue specimens was observed in few cases (Gazzani, 2010).

1.13.2. Culture and serology

For the primary isolation of Cryptococcus neoformans, Blood or Chocolate Agar, Sabouraud Dextrose Agar, Brain Heart Infusion Agar, Birdseed Agar or Staib’s Medium are used. These cultures are viable for around 3 weeks and can be visualized after 48 hours to 72 hours incubation at 37°C (Mitchell & Perfect, 1995).

The growth of Cryptococcus neoformans is mucoid, white, cream or buff coloured on Sabouraud dextrose agar (Mitchell & Perfect, 1995). It produces a brown colour effect due to formation of melanin when grown on a medium containing extract of Niger seeds or Caffeic acid test agar as shown in Figure 1.3.
Brown coloured colonies are formed after a period of time due to conversion of substrate (dopamine) to melanin by enzyme phenoloxidase which confirms the presence of *Cryptococcus neoformans* (Shaw & Kapica, 1972). *C. neoformans* and *C. gattii* can be differentiated on the basis of their growth on different media such as L-canaeamine glycine bromothymol blue (CGB) agar where *C. gattii* turns cobalt-blue color and *C. neoformans* remains the same color (Klien et al., 2009). Latex agglutination test for cryptococcal capsular polysaccharide antigen which is both sensitive and specific is used for serological diagnosis (Bloomfield, 1963; Kralovic, 1998; Khyriem, 2003, Kontoyiannis, 2003).

### 1.13.3. Typing methods

Species and serotype identification has been attempted using multiplex PCR (Ito-kuwa et al., 2007; Enache-Angoulvant et al., 2007; Leal et al., 2008). The chemotyping of *C. neoformans* by quantitative analysis of 1H nuclear magnetic resonance spectra of glucuronoxylomannans with a computer-simulated artificial neural network has been successful (Cherniak, 1998). In few studies the authors performed electrophoretic karyotyping (Ngamwongsatit et al., 2005; Esposto et al., 2009).
Hybridization probes for conventional DNA fingerprinting used as single primers in the polymerase chain reaction helped to distinguish strains of *Cryptococcus neoformans* (Meyer et al., 1993; McEwen et al., 2000). Molecular typing methods like; PCR fingerprinting, Random amplification of polymorphic DNA analysis, dispersed repetitive DNA were successful employed for typing and distinguishing isolates of *C. neoformans* and *C. gatti* (Dromer et al., 1994; Sorrell et al., 1996; Boekhout et al., 1997; Meyer et al., 1999, 2003; Casali et al., 2003; Almeida et al., 2007; Olivares et al., 2009). Suspension array is employed for rapid identification of varieties and genotypes of *C. neoformans* species complex (Diaz & Fell, 2005). Representational difference analysis (RDA) is helpful in identifying genomic differences between *C. neoformans* and *C. gatti*. In one study the authors have highlighted the utility of PCR method for the diagnosis of neurocryptococcosis (Paschoal, 2004). In PCR fingerprinting single primers specific to microsatellite (GTG)$_5$ and (GACA)$_4$ or minisatellite specific core sequence of wild page M13 (5'-GAGGGTGGCGGTTCT-3') are used to produce species and strain specific multilocus profiles (Meyer, 1993).

### 1.13.4. Antifungal susceptibility

Though acquired drug resistance is less encountered among clinical isolates of cryptococcus, in vitro susceptibility testing for antifungal agents could provide clinicians with a valuable adjunct to designing appropriate treatment strategies (Yildiran et al., 2002; Assing et al., 2003; Lee & Fothergill, 2003; Datta et al., 2003; Abdel-Salam, 2005; Khyriem et al., 2006).
Various methods and media has been assessed for the drug resistance among clinical *Cryptococcus neoformans* isolates (Jessup et al., 1998; Lozano-Chiu et al., 1998; Ochiuzzi et al., 2010). In antiretroviral-naive and antiretroviral-experienced patients treated with amphotericin B or fluconazole the fungal burden, fungicidal activity and outcome of cryptococcal meningitis were documented (Bicanic et al., 2007).

One study evaluated the minimum inhibitory concentration for Amphotericin and Fluconazole of 265 isolates from HIV positive patients before and after 2 or more months of antifungal therapy (Arechavala et al., 2009). It is found that the *C. neoformans* serotype A and *C. gattii* serotype B isolates differ in their susceptibilities to fluconazole and voriconazole (Khan et al., 2009).

### 1.13.5. Animal models

Animal models have been an important tool in the development of drugs for cryptococcosis. In vitro susceptibility testing provides an initial screening of agents but cannot always predict efficacy in treating severely immunocompromised hosts with unique sites of infection. Although animal model cannot give 100% efficacy in predicting the effect of drugs on the human system they are useful to approximate the clinical relevance. Earlier studies on immunology of cryptococcosis utilized the guinea pig and rat, but later the swiss albino mice was found to be a better model for pathogenicity testing (Chander, 2002; Sukroongreung et al., 1998). Mice are inexpensive, well characterized immunologically, and genetically stable, and similar to immunocompromised patients. The immunosuppressed rabbit model of cryptococcal meningitis and nematode, *Caenorhabditis elegans* has also been used (Mylonakis et al., 2002, 2004; Tang et al., 2005).
1.13.5.1. Caenorhabditis as a model organism

*Caenorhabditis elegans* is a eukaryotic, freeliving, transparent, nematode of about 1 mm in length Figure 1.4. It is a multicellular organism that can be used as a model organism for scientific research (Stiernagle, 2006).

![Image of C. elegans in Adult stage](image)

*Figure 1.4.* : *Caenorhabditis elegans in Adult stage.*

Its habitat is the soil and feeds on bacteria such as E. coli OP50 or fungi, but for laboratory purposes it feeds on a lawn of bacteria spread on NGM (Nematode Growth Medium). The worm exists as a hermaphrodite and contains both sperm and eggs. Hence, it can self fertilize and produce around 300 progeny, leading to propagation of homozygosity of alleles, ensuring genetically identical generations.

*C. elegans* hatch after approximately 14 hours and grow rapidly. The first larval stage or L1 proceeds through four molt cycles before becoming adults. Under crowded situations or lack of food, the worms choose an alternate form called the dauer larva. In this stage, it can survive up to months. When conditions improve, the life cycle resumes and the worm enters the fourth larval stage or L4 before becoming an adult with an overall life span of 2 to 3 weeks (Mylonakis et al., 2002).
The body plan of *Caenorhabditis elegans* is simple and consists of few tissues. The head contains the brain and the organ necessary for feeding, the pharynx. The body of an adult hermaphrodite worm consists of the intestine, uterus and spermatheca. It is cylindrical in shape and contains layers of epithelial cells surrounded by a cuticle made of many proteins like collagens. The muscles of the body are arranged in four rows, two along the ventral side and two along the dorsal side. The interior is a pseudocoelomic space or fluid filled space surrounding the intestine and gonad. It has no circulatory system and allows oxygen from the environment to diffuse through it. The presence of a hydrostatic skeleton is determined as the body is under internal hydrostatic pressure. The coordinated contractions of the muscles of the body wall allows the movement of the worm in sinusoidal waves (Mylonakis et al., 2002).

The whole genome of Caenorhabditis has been sequenced. The genome of the worm is relatively small (97 megabases) while the human genome is around 3000 megabases. Hence, manipulation of sequences and genes can be done through easy and routine procedures that are not easily available in other eukaryotes. *Caenorhabditis elegans* is easy to grow and maintain in petri dishes with a life span of about 2 to 3 weeks. This transparent nematode or roundworm serves a model organism, especially in pathogenicity studies as it can feed on both bacterial and fungal lawns (Stiernagle, 2006).

*Caenorhabditis elegans* is a remarkable nematode that can be used as a model organism for a number of studies based on apoptosis, drug discovery, signaling, metabolism and the mechanism of aging in mammals (Kaletta & Hengartner, 2006). Due to its small size, assays can be carried out in microtitre plates either in solid or liquid medium and can be propagated in large numbers.
Killing assays consisting of transferring the nematodes from a lawn of *E. coli* OP50 to a lawn of pathogenic bacteria or fungus in solid or liquid media are carried out to test the virulence factors and drug studies. It has a short generation time and produces about 300 progenies per self fertilizing hermaphrodite (London et al., 2006).

*Caenorhabditis elegans* is the fastest and most sensitive to cost effective and high throughput methods. Since the worm is transparent, in vivo fluorescence markers can be used to study many processes. The advantage of using *C. elegans* is that instead of studying isolated process, the entire organism and its behavioral response can be observed (Berger et al., 2007).

Drugs enter the nematode in three different routes; by a slow ingestion process, by uptake through skin and uptake through exposed sensory neuronal endings. The different routes are illustrated in Figure 1.5. First, the chemosensory neurons choose appropriate food sources. Next, the drug is taken up by aspiration through the pharynx, whose activity depends on food availability and is controlled by several pathways. Microvilli present on the intestinal lumen absorb the drug into the intestinal cells. The drug is then distributed rapidly from the body cavity and it finally reaches its target.

*Figure 1.5.*: An illustration of various drug entry routes into *C. elegans*
The increasing incidence of fungal infections, the discovery of new antifungal agents, and the emergence of drug resistance in fungi all contributed to a pressing need for new model systems to study the mechanisms of fungal virulence.

Studies showed that Caenorhabditis feeds on cryptococci (Mylonakis et al., 2002). Different types of yeasts exert different effects on Caenorhabditis elegans. However, Cryptococcus neoformans kills C. elegans. Paramecium species ingest and kill the cells of C. neoformans (Frager, 2010). Cryptococcal cells are cleared from the system, when exposed to liquid medium. Yeasts like Candida albicans persist in the gut and break the nematode cuticle through filamentation. After ingestion of the yeast, the accumulation in the intestine may or may not lead to killing of the nematode, as other factors such as secretion of toxins may be responsible.

1.14. Management of Cryptococcosis

Cryptococcosis has emerged as one of the leading cause of central nervous system mycoses with 100% mortality rate if left untreated (Saag, 2000). Current antifungal therapy for cryptococcosis consists of long term antifungal drug treatments, high costs and severe side effects.

1.14.1. Antifungal therapy

For an effective management of the Cryptococcosis, Amphotericin (AmB), Flucytosine (5FC) and Fluconazole (FLU) are administered for an induction and maintenance therapy (Hay, 1995; Powderly, 2006; Perfect et al., 2010).
Until recently, it has not been possible to predict the clinical response to Amphotericin B treatment. The use of combination chemotherapy for cryptococcal meningitis is evolving as the treatment of choice (Milefchik et al., 2008). Of late the combination of Amphotericin B with Fluconazole has demonstrated promising clinical outcome (Brouwer, 2004; Sathishchandra et al., 2007). In a recent randomised controlled trial conducted in India liposomal amphotericin B for treatment of cryptococcal meningitis in HIV/AIDS patients was evaluated (Jadhav et al., 2010).

1.14.1.1. Antifungal agents and toxicity

Fungal diseases were not recognized as important pathogens until recently and the study of resistance to antifungal agents has lagged behind that of antibacterial resistance for several reasons. These developments and the increase in fungal infections required a desperate need for new, safer, and more efficient agents to combat serious fungal infections. The use of potent antifungal agents come with a cost and risk. Many agents such as Amphotericin B has toxic effects on human.

However, newer agents such as azoles like, Fluconazole has reduced toxicity and increased efficacy (Ghannoum & Rice, 1999). Several antifungal studies have been carried out against Cryptococcus neoformans using standard drugs and the MIC of each has been determined (Souza et al., 2005; Duin et al., 2004).

Amphotericin B was believed to be the best in treatment of fungal infections for around 30 years, but due to its side effects, a need for a new class of drugs was realized. It belongs to the class of antifungals called Polyenes (Duin et al., 2002).
Polyenes are amphiphilic molecules with multiple conjugated double bonds. Some examples of polyenes are Nystatin and Natamycin. Susceptibility to polyenes was found to be linked to the sterol content in the outer membrane of the cell, which resulted in production of aqueous pores consisting of 8 Amphotericin B molecules linked hydrophobically to the membrane sterols. Finally, the hydroxyl groups turn inwards, leading to altered permeability and leakage of cytoplasmic components and eventually the death of the fungal cell. Amphotericin B has a narrow therapeutic index and this imposes a limit on its clinical use. To reduce side effects, liposomal formulations of Amphotericin B has been created which allows transfer of higher doses of the drug with reduced toxic effects to mammalian cells. Some examples of liposomal preparations are ABELCET, Amphoteck and AmBisome (Chander, 2002).

The average dose of conventional Amphotericin B to treat fungal infections in human beings is 0.50-1.5 mg/kg IV once a day. After side effects such as cardiac arrest, renal damage and loss of vision and hearing, the need for new lipid formulations of Amphotericin B were brought forward. The lipid formulation, although administered at a higher dose of 3-1.5 mg/kg once a day, reduced the side effects (Duin et al., 2002).

Another class of antifungals is the Azole based antimycotic drugs or Azoles. Original compounds such as Miconazole were replaced by triazoles like Fluconazole and Ketoconazole which had better clinical efficacy and safety. But overall, azoles had lesser side effects and greater efficiency. Azole drugs inhibit enzyme 14 a demethylase, a cytochrome P450 CYP51 enzyme necessary for the formation of ergosterol (Duin et al., 2004).
Thus lack of ergosterol in the fungal membrane leads to disruption of cell membrane and impairment in function and eventual inhibition of fungal growth. Triazoles and Echinocandins have lesser toxicity to mammals and are more potent. These drugs became a vital part of antifungal therapy. Administered at a dose of 400 mg/kg once a day, azoles cause side effects such as gastrointestinal disorders, dizziness and seizures.

1.14.1.2. Combination of antifungal agents with analogues

The use of compounds derived from the body, that are a part of the innate immune system, to enhance the action of the antifungal agents would help reduce the dosage and eventually the side effects of these agents (Nierenberg et al., 2010). The innate immune system through compounds such as Lactoferrin and Histatins seem to have antifungal effects against yeasts such as *Candida albicans* (Kuipers et al., 1999; Venkatesh & Rong, 2008). Thus, the use of compounds derived from the immune system would reduce the use of antifungal drugs through reduction in dosage and bring about lesser side effects and more efficiency. These compounds have the ability to act against fungus that have developed a resistance to certain azoles or polyenes.

Gomesin, a peptide produced by the spider *Acanthoscurria gomesiana* was found to be a potent anticryptococcal agent that acts in synergism with fluconazole (Barbosa et al., 2007). Recently an analogue Astemizole was identified to have promoted fungicidal activity of fluconazole against *C. neoformans var. grubii* and *C. gattii* (Vu & Gelli, 2010). More research needs to be done on such immune substances to outpace the drug resistant and opportunistic fungi causing life threatening diseases, by enhancing fungicidal activity and stability.
1.14.1.2.1. Xanthine and Hypoxanthine

Hypoxanthine and Xanthine are not incorporated into the nucleic acids as they are being synthesized but are important intermediates in the synthesis and degradation of the purine nucleotides. They are the precursors of uric acid, the end product of purine metabolism (Brychkova, 2008). Hypoxanthine and Xanthine are found in body fluids, body tissues and other organisms. Inborn errors of metabolism are characterized by elevated excretion of Xanthine and hypoxanthine; Isolated Xanthine dehydrogenase (XDH or Xanthine oxidase) deficiency. Elevations of Xanthine and hypoxanthine and abnormally low levels of uric acid are found in both disorders. Allopurinol, a Xanthine oxidase inhibitor that prevents conversion of Xanthine to uric acid, is used to treat hyperuricemia (Pacher et al., 2006). Xanthine is a product on the pathway of purine degradation. It is created from guanine by guanine deaminase and from hypoxanthine by Xanthine Oxidase and is a spontaneous deamination product of adenine.

1.14.3. Prevention, Prophylaxis and Vaccine strategies

Prevention of cryptococcosis in mass population is challenging. In high risk population prophylactic fluconazole administration had been carried out (Nightingale et al., 1992; Powderly et al., 1995). The influence of monoclonal antibodies on complement deposition on the capsule of pathogenic fungi by both classical pathway activation and steric hindrance has been tried experimentally (Zaragoza & Casadevall, 2006). Highly effective glucuronoxylomannan-tetanus toxoid conjugate vaccine of Cryptococcus neoformans in a murine model had been in preclinical developmental stages (Devi et al., 1991; Devi, 1996; Marshall, 1995; Datta & Priofski, 2006).