Chapter II: Review of Literature
REVIEW OF LITERATURE

Cryptococcus belongs to class basidiomycetes. It causes infections mainly in immunocompromised patients thus gaining prevalence as one of the most life-threatening pathogen in immunocompromised patients. C. neoformans and C. gattii are closely related pathogenic fungi that can cause respiratory and neurological diseases in humans. These pathogenic species are clustered in the Filobasidiella genus within the order Tremellales (Casadevall et al., 1998).

2.1 THE DISCOVERY

It was in 1894, that Sanfelice first reported C. neoformans in peach juice in Italy. In the same year, it was also reported from the tibial lesion of a female by Otto Busse and Abraham Buschke in Germany. In the following year, it was demonstrated that it was pathogenic in laboratory animals (c.f. Knoke et al., 1994).

In the early 1900s, many clinical case descriptions of C. neoformans created an increased awareness of this fungus as a human pathogen. Stoddard and Cutler (1916) published the first monograph of 10 reviewed cases of C. neoformans infections and described some animal studies also. Freeman (1931) pointed out that the first description of cryptococcosis was made by Zenker in 1861. He argued for Zenker that the first clinical description of the case which was provided by Zenker was same as that of Cryptococcus meningitis. However, credit for the discovery of Cryptococcus was given to Busse and Buschke because they provided the first detailed clinical, pathological and morphological description along with the saved culture of this organism for further studies. Cox and Tohurst (1946) published the second monograph on Cryptococcus. It describes 12 new clinical cases from Australia (Cox et al., 1946).

Cryptococcus has received a series of names in the past. Many names were proposed for Cryptococcus during the period 1894-1975. Saccharomyces hominis was the first name given by Busse and Buschke (c.f. Knoke et al., 1994). In early 1900s, term „European blastomycosis“ was proposed in place of cryptococcosis. In 1901, Vuillemin used the term Cryptococcus to refer to the genus of the organism (c.f. Benham, 1950). The epithet „neoformans“ was assigned for species because of its tendency to form tumour like lesions in experimental animals.
Benham (1930) made efforts to differentiate it from other pathogenic fungi and gave satisfactory explanations for clarifying the classification of *Cryptococcus*. She studied many of the yeast isolates from humans and grouped them as Cryptococci (Benham, 1935). Name *C. neoformans* was supported by Benham (1950) on the basis of the name „Cryptococcus” which was given by Vuillemin (1901) and the „neoformans” by Sanfelice (1895). Benham and Lodder (1950) were credited to clarify the taxonomic relationship of *Cryptococcus* with other yeasts (c.f. Drouhet, 1997).

By 1950, the term *Cryptococcus* had come into use and was quickly adopted in medical field as well as in literature. In 1950, Emmons for the first time isolated it from pigeon droppings and soil contaminated with avian excreta (Emmons, 1960). This observation provided the clue to many to study the relationship between *Cryptococcus*, cryptococcosis and birds. It laid the foundation of the present concept that the infection can be acquired from environmental sources.

In mid of 1970s, Kwon-Chung (1975) provided the detailed taxonomical status of the organism and also proved the sexual reproduction in lab. Staib (1971) reported isolation of *Cryptococcus* from ripe peach fruit (Polacheck, 1991). By the late 1970s, it became clear that the incidence of cryptococcosis was rising rapidly. In recognition of this rising incidence of infection, Kaufman termed cryptococcosis ‘the awakening giant’ among mycological infections (Kaufman et al., 1977).

### 2.2 VIRULENCE FACTORS

Virulence is the infectiousness of a microorganism and its ability to overcome the natural defence of a host. In case of fungi, identification of certain virulence factors and the corresponding genes is more complex and even less defined than bacteria.

Virulence of *C. neoformans* is due to several factors like capsular polysaccharide, urease production, phospholipase, protease, melanin synthesis and mating types. *Cryptococcus* is pathogenic because of the presence of polysaccharide capsule and ability of producing phenol oxidase and growing at 37°C. The capsule is a major virulence factor. Capsule production helps in protecting the organism against phagocytosis (Breen et al., 1982; Fromptling et al., 1982; Kwon-Chung et al., 1986; Cherniak et al., 1994; Hull et al., 2002).
To cause infection in humans, a *C. neoformans* isolate must grow at 37°C in an atmosphere of approximately 5% CO₂ and at a pH of 7.3 to 7.4. Of all the *Cryptococcus* species, *C. neoformans* and *C. gattii* can grow well at mammalian host temperature of 37°C. This virulence phenotype of growth at host body temperature in *C. neoformans* appears to be requisite for survival in the mammalian host. To survive at 37°C, the organism must have an intact gene that encodes the *C. neoformans* calcineurin catalytic subunit. *C. gattii* and *C. albidus* are also able to grow at human body temperature of 37°C (Cherniak et al., 1994). *C. albidus* is also reported for some cases of infection (Melo et al., 1980; Gluck et al., 1987). Ability of *C. neoformans* and *C. gattii* to grow at 37°C is a characteristic feature in distinguishing these two species from other species (Kabasawa et al., 1991; Kidd et al., 2004).

Calcineurin is a serine-threonine specific phosphatase that is activated by Ca²⁺-calmodulin and is involved in stress responses in yeast. Although calcineurin mutant strains of *C. neoformans* can grow at 24°C, they cannot survive *in vitro* at 37°C, in 5% CO₂, or at alkaline pH. Since these are similar to conditions in the host, one would predict that the calcineurin mutant would not survive in the human host. 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 (Odom et al., 1997). Ability to grow at physiological temperatures is essential for the virulence of *Cryptococcus*.

The production of brown pigmentation due to phenol oxidase activity has been studied on medium Staib’s Niger seed agar, Pal’s sunflower seed agar or on medium containing chemicals such as caffeic acid or L-3, 4-dihydroxyphenylalanine (DOPA) for the identification of *C. neoformans* (Chaskes et al., 1978a; Staib et al., 1987; Khan et al., 2004). Melanin production is a unique characteristic property of *C. neoformans*. The ability to produce melanin pigment is one of the widely used criteria for the identification of *C. neoformans* and for the evaluation of virulence (Kwon-Chung et al., 1986; Polak, 1990). Caffeic acid was able to cause brown pigmentation of *C. neoformans* colonies, particularly in the presence of ferric citrate (Pulverer et al., 1971). These compounds caffeic acid and ferric citrate were incorporated in paper discs as substrates for the phenoloxidase enzyme activity in the rapid identification test of *C. neoformans* by Hopfer (1975b).
Melanin protects the cells from the unfavorable environmental and host conditions (Wang et al., 1994). *C. neoformans* must have the enzyme phenoloxidase. Wild *C. neoformans* mutants lacking melanin were found less virulent in mice as compared to the melanized strains (Polacheck et al., 1990). It was found that the gene CNLAC1 encodes this enzyme and the disruption of CNLAC1 may result into the loss of virulence (Williamson, 1994). *C. neoformans* could produce sufficient levels of melanin to effectively protect the organism from oxidative compounds produced by macrophages (Jacobson et al., 1993). All the above mentioned evidences indicate that melanin production is an effective protective component in the survival of *C. neoformans*.

*Cryptococcus* is able to produce an extracellular DNase which protects them in the environment from the biotic factors and may also act as a virulence factor to cause disease in the mammalian host (Perfect et al., 2006). Extracellular DNase is also considered as a virulence factor in *Staphylococcus* and *Streptococcus* pathogenesis. DNase is required by *Streptococcus* in the establishment and enhancing the invasion of innate immune system of the host, specifically to avoiding the killing caused by neutrophils ((McCarty, 1948; Sumby et al., 2005). In cryptococcosis, neutrophils have been observed at the inflammatory site in response to the infection and found in close association with the infected tissue (Fazekas et al., 1958).

It was suggested that extracellular phospholipase activity produced by *C. neoformans* may disrupt mammalian cell membranes and allow the yeast cells to penetrate into host tissues which are the essential steps to establish the infection (Chen et al., 1997; 2000). Extracellular phospholipase produced by many bacteria and fungi are implicated in their pathogenicity to the host by causing damage to the cell membrane (Barrett-Bee et al., 1985; Meyers et al., 1992). *Candida albicans* also produce phospholipase which is associated with the mucosal invasion (Ibrahim et al., 1995). *C. neoformans* isolates from AIDS patients showed higher phospholipase activity than in pigeon excreta isolates (Casali et al. 2003). *C. neoformans* PLB can readily cleave lung surfactant components and facilitate tissue invasion by the organism (Goerke, 1974).

Ability of *C. neoformans* to produce a protease that could easily digest human proteins and also capable of digesting casein was reported by Muller (1972) and Brueske (1986). Microscopic examination of the lesions produced in mice suggested that the Cryptococcal cells degrade collagen bundles in the dermis. It may contribute to degradation of proteins involved in tissue
integrity and host immunity (Salkowski et al., 1991). Collagen fibril degradation has been observed in experimental murine models (Goldman et al., 1994). Ability of Cryptococcus to produce protease and phospholipase may be considered as potential virulence components.

Urease plays an important role in virulence of Cryptococcus. Infection with C. neoformans in mice model suggests that urease may help in dissemination of the organism to the central nervous system (Olszewski et al., 2004). With the help of a knockout strain of C. neoformans, it was concluded that migration into the mouse brain is a urease dependent process (Shi et al., 2010). C. neoformans / gattii produce various enzymes that allow the survival of the yeast not only in the environment, but in the animal or human hosts.

Phenotypic switching was first described in Candida albicans (Slutsky et al., 1985; 1987; Radford et al., 1994). In more recent years, phenotypic switching has also been demonstrated in other fungi including C. neoformans (Eissenberg et al., 1996; Sinha et al., 2000). It was proven that C. neoformans has the ability to undergo rapid changes in vitro (Franzot et al., 1998; Guerrero et al., 2006). Importance of phenotypic switching was associated with the setting of human disease by several findings.

2.3 INFECTION

AIDS patients and those undergoing immunosuppressive therapy are at increased risk of cryptococcosis. It is known for causing a form of meningitis and meningoencephalitis in people suffering from AIDS or HIV (Casadevall et al., 1998; Litvintseva et al., 2005).

Fungal meningitis is a secondary infection in AIDS patients. Meningoencephalitis is normally caused by C. neoformans in immunocompromised individuals. In some cases only fungemia is reported. Less commonly, organ transplant, cancer and long term corticosteroid treatment may lead to cryptococcosis. Long term use and abuse of drugs and diabetic patients can easily get infected with this pathogen (Chayakulkeeree et al., 2006).

2.4 C. neoformans /grubii and C. gattii

Cryptococcus neoformans is divided into two varieties C. neoformans var. grubii (serotype A), C. neoformans var. neoformans (D) and hybrid (AD). Filobasidiella neoformans is the sexual stage of C. grubii and C. neoformans. Both are opportunistic pathogens in immunocompromised patients. Immunocompetent subjects are less prone to these pathogens (Casadevall et al., 1998).
C. grubii is distributed world-wide. Emmons reported the association of C. neoformans with pigeon droppings in 1955. It is found mainly in association with pigeon droppings and soil contaminated with pigeon excreta.

Strains of serotype A have also been isolated from decaying woods ((Nishikawa et al., 2003). C. neoformans is well distributed in Europe and South America. Immunocompromised patients are mainly infected by C. neoformans var. grubii serotype A. About 90% Cryptococcal infections and 99% cryptococcosis cases in AIDS patients have been caused by serotype A. Serotype D is also responsible for causing infections among immunocompromised patients, Hybrid serotype AD strains have been isolated from the environment as well as from patients in North America (Xu et al., 2002).

Cryptococcus gattii infection was first reported in tropical regions; Africa, Australia and United states. The sexual stage is known as Filobasidiella bacillisporus. It is well distributed in tropical/subtropical and temperate regions and endemic in Africa and Australia. C. gattii is a primary pathogen for immunocompetent individuals (Casadevall et al., 1998)

2.5 ECOLOGY AND EPIDEMIOLOGY OF CRYPTOCOCCUS SPECIES COMPLEX

Ellis and Pfeifer (1990) reported the first isolation of Cryptococcus gattii in Australia from Eucalyptus tree. Cryptococcus gattii has been reported from different countries; Argentina, Austria, Brazil, Cambodia, Canada, China, Colombia, Congo, Egypt, France, Germany, Greece, Hawaii, India, Italy, Japan, Malaysia, Mexico, Nagasaki, Nepal, Netherland, Papua New Guinea, Paraguay, Peru, Singapore, South Africa, Spain, Taiwan, Thailand, United Kingdom, United States, Uruguay, Venezuela and Vietnam. C. gattii, C. grubii and C. neoformans had been successfully isolated from all these countries (Ellis et al., 1990; Sorrell, 2001; Colom et al., 2005; Viviani et al., 2006).

In the very beginning, both the environmental isolation and clinical incidences linked with Cryptococcus were studied only in the tropical and sub-tropical countries. But, it has been also isolated from the cold environment after the outbreak of infection on Vancouver Island (Canada) and Northwest Pacific of USA during 1999 (Kidd et al., 2004).
A large number of tree species were sampled for environmental isolation of *Cryptococcus* spp complex. These trees include angiosperms and gymnosperms. *C. gattii* has been isolated all around the world from different tree species: *Pinus* spp, *Terminalia catappa*, *Cedrus* spp, *Eucalyptus* spp, *Douglas fir*, *Arbutus*, *Ficus*, *Mimusops elengi* and *Manilkara hexandra*. *Cryptococcus* has been isolated from both the angiosperms (77%) and gymnosperms (23%). Near about 54 tree species has been found positive for the isolation of *Cryptococcus* species (Springer et al., 2010).

*Cryptococcus* is mainly isolated from sources like bark, fissures, trunk hollows, leaves, flowers and debris of all these trees. Air and water sampling was also positive to some extent. In Japan and also in India, *Cryptococcus* has been isolated from some fruits and vegetables (Misra et al., 2000; Perfect et al., 2002; Kidd et al., 2007b; Boddy et al., 2008; Loperena et al., 2010).

Changing climate like global warming affects the distribution of *Cryptococcus*. Now, the environmental niche for this pathogen varies from tropical to temperate regions. A few species of *Cryptococcus* named *C. victoriae* and *C. statezillae* have been isolated from the very cold environment of Antarctica (Watson, 2002).

*C. gattii* caused an outbreak (1999) affecting humans and animals in British Colombia (BC) and Vancouver leading to death of humans (Fyfe et al., 2008). Genotypic characterization has shown that all clinical and environmental isolates belonged to VGII of *C. gattii*. Total 218 cases were recorded (MacDougall et al., 2011). An annual incidence of infection is 6.5 and 27.9 cases per million in British Colombia (BC) and Vancouver Island respectively. It is the “highest endemic incidence” of this disease reported ever worldwide. Spread of the disease from Vancouver to lower regions of BC may be associated with travelling, traffic, car wheels and some abiotic factors like wind and water (Kidd et al., 2004; Kidd et al., 2007a, b; British Columbia-CDC, 2008).

*C. gattii* VGII has also been recovered from Pacific Northwest of United States. It is believed that distribution of *C. gattii* VGII from Vancouver Island to North Pacific occurred through tourism and movement of soil and woody products through wind and water. But no one knows how *C. gattii* reached Washington from Vancouver and got adapted to temperate environment causing infection (Kidd et al., 2007b).
A rare genotype is responsible for causing an outbreak on Vancouver Island. PCR-fingerprinting pattern of VGI clinical isolates has shown high resemblance of this rare genotype strain with some Australian isolates. However, it has not been recovered from the Vancouver environment. It may therefore be possible that infection is acquired from other or different environments (Kidd et al., 2004).

2.6 SEASONAL OR CLIMATIC EFFECT

*Cryptococcus* species complex is distributed throughout the environment and is greatly affected by different seasons. All the species can be isolated in every season, although has a large impact on population size which changes with season. The species are largely affected by changing seasonal patterns as hot summer is less favourable for the growth of *C. gattii* compared to *C. neoformans* (Granados et al., 2005).

Humidity, precipitation, rainfall, soil moisture, temperature, salt concentration, wind and nutrient availability affect the distribution of different serotypes. *C. grubii* decreases during rainy season as compared to other seasons like summer and winter. Its recovery from bird faeces decreases during rainy season. It means, rainy season or humidity greatly affects the prevalence of *C. grubii*. Isolation of *C. grubii* and *C. gattii* is favoured by autumn and summer (Montenegro et al., 2000).

Population density of *C. gattii* decreases during winter. Different serotypes are adapted to different environments like serotype A has shown a wide prevalence in Medellin (Colombia) but the serotype B was found as predominant serotype in city Bogota (Colombia). These variations in the prevalence of serotypes are related with the climatic pattern. Bogota has a temperate climate as compared to Medellin which having a mild-warm climate. These different types of climatic conditions provide optimum incubation and ecological niche for the well adaptation and growth of serotypes. All these finding suggest that different *Cryptococcus* species are adapted to survive in different climatic conditions. These adaptations and climatic requirements are largely responsible for the changing niche and geographical distribution (Granados et al., 2005).

Environmental distribution pattern is different for all the different serotypes A, B and C. Serotype B is favoured by high humidity and low solar radiations as compared to serotypes A and C which require high temperature and low humidity. It may be assumed that serotypes A and C require dry seasons for growth but the serotype B has been successfully isolated in Bogota
(Colombia) from the temperate environment with low temperature and high humidity. It provides support to the fact that ecological niche is changing from tropical to temperate zone and can easily explain the reason of cryptococcosis outbreak in Vancouver Island (Granados et al., 2005).

Dry droppings provide the favourable niche for the yeast growth. C. grubii is more frequently isolated from the dry pigeon droppings rather than fresh droppings (Ruiz et al., 1981). Serotype B is isolated in Bogota (Colombia) from the Eucalyptus trees, so its isolation may be possible from this reason because of close association of serotype B with Eucalyptus but in Medellin (Colombia), serotype A is more prevalent and also isolated from Eucalyptus. Cucuta (Colombia) has a hot environment, but not a single positive sample of serotype B has been obtained from 388 Eucalyptus samples (Granados et al., 2005).

All these findings are informative and explain the prevalence of different serotypes in different climatic conditions and could easily explain the prevalence of serotype B with temperate regions. Changing climate and global warming have changed the natural habitats or niche for the pathogen over a wide geographical area from very hot environment to mild warm and temperate regions of the world.

2.7 INFECTION AMONG ANIMALS

In Australia, New Zealand and Brazil, infections are more common among animals like dogs, cats, horses and goats. Animals get more infected with Cryptococcal infections as compared to human beings. A large number of domestic and zoo animals have been infected with this pathogen including dolphins, koalas, ferrets, cheetah, kiwi, squirrels, cockatoo and parrots. It also requires more attention towards the proper analysis of veterinary samples. Animals may be largely responsible for spreading of infection among humans (Malik et al., 2003; Duncan et al., 2006a, b; Bowles et al., 2009).

A zoonotic transmission of Cryptococcus is also reported in Boston, Massachusetts (USA). The isolates from cockatoo and a patient (also immunocompromised) having same biochemical profile has also been reported. Both the isolates from the patient and cockatoo had the same pattern when genotyped with RAPD. Therefore, it is suggestive that immunocompromised patients should avoid exposure to the pet birds and animals (Nosanchuk, 2000).
2.8 SPREADING OF CRYPTOCOCCUS

It may be assumed that the environmental spreading of infection by yeast among human beings occurs through contaminated plant particles (Figure 2.1). Honeybee colonies may harbour \textit{C. neoformans} var. \textit{grubii} on eucalyptus trees (Ergin \textit{et al.}, 2004).

There are many factors for spreading of \textit{C. gattii} infection from environment to humans and animals. It may be associated with human activities like hunting, disturbance of soil, tree limbing and wood chipping in the forests. Due to these activities, environmental spreading of the pathogen becomes easier and it easily gets distributed throughout the environment by wind (Duncan \textit{et al.}, 2006b).

Water and air sampling is found less positive for \textit{C. gattii}, but the air sampling and water sources near the positive trees give positive results as the pathogens get easily dispersed in the environment by wind and water sources also get contaminated. Sea water and lakes harbour these pathogens easily and get contaminated by infected water inhabitants and soil contaminants. Birds play an important role in spreading by contaminating trees with droppings (Kidd \textit{et al.}, 2007a). \textit{C. gattii} VGII has also been recovered from Pacific Northwest of United States (Kidd \textit{et al.}, 2007b).

2.9 ANTIFUNGAL RESISTANCE

The occurrence of antifungal resistance among clinical and environmental isolates of \textit{C. neoformans} and \textit{C. gattii} has been evidenced during the last decades. It leads to the necessity to perform the susceptibility testing to improve the clinical diagnosis in severely ill patients (Aller \textit{et al.}, 2000; Rex \textit{et al.}, 2001). Different levels of resistance to fluconazole, itraconazole, and amphotericin B has been reported in the \textit{C. neoformans} and \textit{C. gattii} (Costa \textit{et al.}, 2010; Verma \textit{et al.}, 2010). Only few studies of \textit{C. laurentii} strains have been published (Pedroso \textit{et al.}, 2006). Since the Amphotericin B is considered as the gold standard drug to cure Cryptococcal infections of CNS. An amphotericin B resistant \textit{C. laurentii} strain was also reported from a HIV patient with meningoencephalitis from Italy (Manfredi \textit{et al.}, 2006). A decreased profile of susceptibility to the azoles has been shown by the clinical and environmental isolates of \textit{C. laurentii} (Bernal \textit{et al.}, 2010).
Figure 2.1 Diagrammatic view of spreading of Cryptococcus from environment to host
2.10 EPIDEMIOLOGICAL RELATEDNESS

Fingerprinting methods are used for comparison of environmental and clinical isolates of \textit{C. neoformans}. A close relationship among environmental and clinical isolates has been proved. It provides strong evidence that there is a direct link between reservoir (environment) and infection. Analysis of environmental isolates from animals and \textit{Eucalyptus} spp (Australia) has been carried out by using random amplification of polymorphic DNA (RAPD) method. Animal and \textit{Eucalyptus} isolates of \textit{C. gattii} exhibited one type of profile. Genetic divergence has been confirmed by using Fluorescent Amplified Fragment Length Polymorphism (FAFLP) among serotypes A, D and the clinical isolates. FAFLP typing confirms the divergence between the varieties (Sorrell \textit{et al.}, 1996; Meyer \textit{et al.}, 2003; Trilles \textit{et al.}, 2003).

In Nagasaki (Japan), isolates from patients and environmental sources within a given area were studied by the RAPD. Both the clinical and environmental isolates had the same RAPD patterns and were identical with each other, which suggest a relationship between clinical and environmental isolates (Yamamoto \textit{et al.}, 1995).

In North Carolina (USA), genotypes isolated from the patients were the same as the genotypes from the environment within the same area. This may suggest a relationship between human and environmental sources of infections. After a comparative analysis of clinical and environmental isolates of \textit{C. neoformans} in a given area with the help of fingerprinting, it has been shown that both types of isolates are closely related in the studied areas (Litvintseva \textit{et al.}, 2005).

Molecular type VNI has shown its presence in all clinical and environmental samples which were isolated from five regions of Colombia. It presents a close relationship among clinical (patients) and environmental isolates (Franzot \textit{et al.}, 1997).

In New York (USA), RFLP analysis revealed that the isolates of \textit{C. neoformans} from patients and as well as from environmental source (pigeon excreta) shared genetic characters to a greater extent. Both the clinical and environmental isolates of \textit{C. neoformans} have shown a close genetic relationship and therefore it may be assumed that pigeon excreta can act as a reservoir for \textit{C. neoformans} (Currie \textit{et al.}, 1994).
Several methods have been used to identify yeast and to characterize them further. Fungichrom method is easy to use for the faster identification of yeast and phenotypic characterization. Genotypic characterization has been carried out by using URA5 DNA, RAPD, RFLP and Luminex technology for the typing of clinical and environmental isolates. Multilocus sequence typing is helpful and reliable for the identification of genes. PCR finger typing is very useful method for clinical and environmental *C. neoformans* species genotyping. These methods are useful in the epidemiological studies and the characterization of the strains (Jain *et al.*, 2005; Escandon *et al.*, 2006; Landlinger *et al.*, 2009; Meyer *et al.*, 2009).

### 2.11 IDENTIFICATION AND CHARACTERIZATION

Positive urease activity is helpful in the preliminary identification of *Cryptococcus* although many other types of yeasts like *Rhodotorula*, *Trichosporon* and *Tremella* also show positive urease activity (Seeliger, 1956; Cazin *et al.*, 1969). Indian ink is helpful in the observation of the capsule (Murray, 1999). Bacterial species are able to reduce nitrate to nitrite or nitrogenous gases (Edwards *et al.*, 1972). Nitrate reduction test is also helpful in the identification of *Cryptococcus* as different species of *Cryptococcus* named *albidus*, *ater*, *diffluens*, *kuetzingii* and *terreus* have the ability of reducing nitrate (Rhodes *et al.*, 1975; Kabasawa *et al.*, 1991).

One of the traits better known is the versatility of the yeast using different carbon sources as energy. *C. neoformans* and *C. gattii* are not able to assimilate lactose, mellibiose and erythritol. In contrast, *C. gattii* can use fumaric acid and succinic acid as sole carbon sources, and malic acid and D-proline as sole nitrogen sources (Dufait *et al.*, 1987). Genus *Cryptococcus* is also known as non-fermenter (Huppert *et al.*, 1975; Rippon, 1988). Staib’s agar, L-DOPA agar, Caffeic agar can be used for the identification of *C. neoformans* as dark brownish colony is the characteristic feature of *C. neoformans* (Chaskes *et al.*, 1978a). CGB (canavanine glycine bromothymol blue) test is very helpful in distinguishing *C. gattii* from the *C. neoformans* because later was negative for this test (Kwon-Chung *et al.*, 1982). *C. gattii* can assimilate glycine as nitrogen sources and it can resist canavanine, while *C. neoformans* can not. These biochemical differences are the base for the creation and preparation of the canavanine glycine bromothymol blue medium (Kwon-Chung *et al.*, 1982).
CDBT (creatinine dextrose bromothymol blue thymine) medium was developed by Kwon-Chung et al (1978). It differentiates *C. neoformans* var. *neoformans* from *C. neoformans* var. *grubii*. It may also be used in separating *C. gattii* from *C. neoformans* as the former produced the blue coloration of the medium but later produced yellow colonies. Therefore, it can also be used for species differentiation. Littman (1947) developed Littman oxgall agar for the primary isolation of pathogenic fungi. *C. neoformans* is able to produce purple-gray colonies on this medium (Littman, 1947).

*C. gattii* can assimilate D-tryptophan thereby producing a brown diffusible pigment while *C. neoformans* was unable to assimilate tryptophan (Baro et al., 1998). Pigmentation intensity gradually increases with time (Chaskes et al., 2008). Tryptophan-derived pigments were not the melanins and growth on M-FDTG (fructose D-tryptophan glycine) medium can be used to rapidly differentiate *C. gattii* from *C. neoformans*. Glycine serves as a sole source of carbon and nitrogen which is utilized by *C. gattii*, not by *C. neoformans*. These components help in the pigment induction by D-tryptophan (Mukamurangwa et al., 1995). Various studies revealed that *C. gattii* could assimilate D-proline and D-tryptophan whereas *C. neoformans* is not able to assimilate these amino acids (Dufait et al., 1987; Mukamurangwa et al., 1995). Additionally, other *Cryptococcus* species are able to produce light brown or pink pigments when cultured on L- or DL-tryptophan (Chaskes et al., 1978b).

Kwon-Chung suggested that urease inhibition may be used as varietal differentiation test for *C. neoformans*. The effect of EDTA (Ethylene Diamine Tetraacetic Acid) on the two varieties of *C. neoformans* appeared due to the greater inhibition of urease synthesis in *C. neoformans* var. *gattii* (Kwon-Chung et al., 1987).

### 2.12 MOLECULAR TYPING AND CHARACTERIZATION

In the 1990’s, most of the molecular studies and DNA typing techniques have been used to understand the epidemiology, pathogenicity and distribution of the yeast. These techniques included karyotyping, Random Amplification of Polymorphic DNA (RAPD) (Meyer et al., 1993; Sorrel et al., 1996), and Restriction Fragment Length Polymorphism (RFLP) of the genomic DNA (Currie et al., 1994). In order to better understand the yeast relationship with its ecological niche, researchers have used the 5.8S rDNA with the internal transcribed spacer (ITS) to
characterize the yeast, because the 5.8S rDNA has proven to be more specific to identify the *Cryptococcus* species than the other sequence of the rDNA (Katzu *et al*., 2003).

The nuclear ribosomal RNA (rRNA) genes (small subunit, large subunit and 5.8S) are organized in a cluster but separated by two internal transcribed spacer regions (ITS1 and ITS2): Typically, in eukaryotic genomes the rRNA genes are repeated in tandem arrays in the order of several hundred, or possibly thousands of copies (Hillis *et al*., 1991).

DNA sequence analysis of the D2 region of the large subunit and the internal transcribed spacer 2 (ITS2) region of the ribosomal DNA have both been widely used to genotypically identify and classify molds and yeasts. The identification of fungi is frequently based on microscopic and phenotypic criteria. These methods are very subjective and often can only taxonomically classify an organism to the genus level or higher. With genotypic methods of identifying microorganisms becoming more commonplace, DNA sequencing is the clear choice for rapid and accurate identifications for yeasts and molds. There are two sequencing targets in the ribosomal RNA operon which are D1/D2 region of the large ribosomal subunit (LSU) and the internal transcribed spacer regions (ITS1/ITS2) that have been commonly used for identification of fungal unknowns. The D1-D2 expansion region of the LSU has traditionally been the target of choice for yeast identification, while the ITS region is more commonly used for filamentous fungi taxonomy. The ITS region has recently been recommended as a marker for fungal bar coding. Differences in a specific region of ITS2, has also been shown to correlate with species delineation (Muller *et al*., 2007).

The ITS region is now perhaps the most widely sequenced DNA region in fungi. It has typically been most useful for molecular systematic at the species level, and even within species (e.g., to identify geographic races). Combined sequence analysis system of D1/D2 and ITS region is recommended for species identification, while species definition requires classical biological information such as life cycles and phenotypic characterization (Scorzetti *et al*., 2002).

In case of fungi, especially the yeasts, both the ITS and LSU gene are being used. Two gene ITS and LSU amplification systems also increased the sequencing costs. During barcode database development, both markers would have to be sequenced. Although it is clear that LSU is superior to the ITS for recognizing species in some groups of yeasts. But in combined system ITS/LSU both gave the similar results in the identification process (Schoch *et al*., 2011).
These days, ITS gene is used as barcode in the fungi and yeast identification at the species level. As compared to other ribosomal genes, the internal transcribed spacer (ITS) region has the highest probability of successful identification for the broadest range of fungi and yeast. It is very helpful in easily distinguishing the interspecific and intraspecific variation among species. The nuclear ribosomal large subunit also has good resolution for species identification especially among the ascomycete group of fungi, but slightly inferior to the ITS. The nuclear ribosomal small subunit has poor resolution in the identification of fungi. ITS will be formally proposed for adoption as the primary fungal barcode marker to the consortium for the Barcode of Life (Schoch et al., 2011).

2.13 PIGMENTATION

A hallmark feature of several pathogenic microbes is the distinctive color of their colonies when propagated in the clinical laboratory. Such pigmentation may contribute to the virulence of the microbes by allowing them to evade host immune by causing damage to the cells and tissues.

Several microorganisms' pigments that promote microbial virulence include the golden staphyloxanthin of *Staphylococcus aureus*, blue-green pyocyanin of *Pseudomonas* species and the dark brown pigments of *C. neoformans* and *Aspergillus* species. These pigments help in the identification of the pathogens therefore proven as important features in the diagnosis of infections. Pigments may also provide a clue to the nomenclature of the microorganisms. Such pigmentation of the colony phenotypes proved useful in the preliminary clinical diagnosis (Liu et al., 2009).

*Rhodotorula* and *Cryptococcus* species have many similar physiological and morphological properties. Both the species produced urease enzyme and failed to ferment carbohydrates. *Rhodotorula* species differ from *Cryptococci* by their inability to assimilate inositol and their obvious carotenoid pigment (Huppert et al., 1975; Larone, 1987; Rippon, 1988).
2.14 PREVALENCE IN INDIA

Indian states; Punjab, Haryana, Uttar Pradesh, Tamil Nadu and Union Territory (Chandigarh and Delhi) have a wide prevalence of *C. neoformans* and *C. gattii*. Tree species *i.e.* *Eucalyptus camaldulensis*, *Syzygium cumini*, *Ficus religiosa*, *Butea monosperma*, *Tamarindus indica*, *Polyalthia longifolia*, *Mimusops elengi* and *Manilkara hexandra*, etc have been studied for distribution and isolation of *Cryptococcus*. Both the species (*C. neoformans* and *C. gattii*) have been successfully isolated from decaying wood in trunk hollows, bark and soil near the base of all these trees (Chakrabarti *et al.*, 1997; Randhawa *et al.*, 2001; Grover *et al.*, 2007; Randhawa *et al.*, 2008).

Clinical cases and samples have been studied seriously due to public health concern, although, environmental sampling is much limited. So there is a large need of focus on environmental study. Because all the clinical cases which were studied in Australia, Boston, British Columbia, California, Colombia, Nagasaki, New York, North Carolina, Oregon, Vancouver and Washington have shown that all the clinical isolates share the same biochemical profile and genetic pattern with the environmental isolates (Currie *et al.*, 1994; Yamamoto *et al.*, 1995; Sorrell *et al.*, 1996; Franzot *et al.*, 1997; Kidd *et al.*, 2004; Litvintseva *et al.*, 2005). Environmental distribution and clinical incidences of *Cryptococcus* are inter-related with each other.

It is therefore imperative that studies are conducted to define the environmental sources of *Cryptococcus* for all regions where disease is either detected or likely to be detected. With more number of AIDS being reported, environmental sources of *Cryptococcus* now acquire increased importance so that more pragmatic preventive and efficient clinical methods can be suggested and adopted for its avoidance and control.