Chapter I: Introduction
1.1 GENERAL

The fungi are widely found in environment. An estimated 1.5 million species of fungi are known, out of which only 70,000 species are identified till date. About 400 species have been reported which are known to cause diseases in humans. Fungal infections came to be known during the mid of 19th century and since then have risen over the last century mainly due to a progressive increase in the number of impaired immunity patients. The AIDS has altered the incidence and prevalence of fungal infections. During this period, there has been a paradigm shift in epidemiology scenario about fungal diseases. Impaired immunity against fungi may result from one or more factors including immune defects due to AIDS, cancer and immunosuppressive therapy. Opportunistic fungi, such as *Aspergillus*, *Fusarium* and *Penicillium* spp are becoming more prevalent in organ transplant carriers (Chander, 2009).

Cryptococcosis, caused by *Cryptococcus* is one of the most common opportunistic fungal infections in the AIDS patients and was earlier considered as "sleeping giant". These pathogens are responsible for an estimated one million cases of Cryptococcal meningitis globally per year in AIDS patients, resulting in approximately 6, 25,000 deaths (Park *et al*., 2009). It is now considered as "awakening giant" and after the outbreak in Canada; it is predicted as the "Mycosis of the future".

*Cryptococcus* belongs to class basidiomycetes and has a distinctive feature that is formation of polysaccharide capsule which sets it apart from the other medically important yeasts. The cells of *Cryptococcus* are round or oval shaped and covered by a layer of thick polysaccharide material. Members of the genus *Cryptococcus* generally are considered to be yeasts that are nonfermentative, assimilate inositol and produce urease. The life cycle of *Cryptococcus* involves asexual and sexual forms. The asexual form exists as yeast and reproduces by budding.

*Cryptococcus* consists of two mating types, MATα and MATa (Wickes *et al*., 1996). *Filobasidiella neoformans* is the sexual stage of *C. grubii* and *C. neoformans*. Sexual stage of *C. gattii* is known as *Filobasidiella bacillisporus*. It is ubiquitous in nature and causes infection, mainly in immunocompromised patients (Casadevall *et al*., 1998).
Sanfelice (1894) first reported *Cryptococcus neoformans* in peach juice. Soil is the natural habitat for *Cryptococcus*. It is found in the environment in association with avian and vegetation populations (Emmons, 1951; Vikins *et al.*, 2002; Randhawa *et al.*, 2003). Decaying woods and eucalyptus trees are the environmental niche for *Cryptococcus*. Emmons (1955) reported the association of *C. neoformans* with pigeon droppings. Contamination of soil with bird excreta especially with pigeon droppings provides a favourable environment for its growth. Pigeons (414°C) provide creatinine for growth of *Cryptococcus* but the body temperature of pigeons is higher than that needed for growth of *Cryptococcus* and therefore they do not suffer from the disease (Chander, 2009).

*Cryptococcus* has been divided into five serotypes, viz., A, B, C, D and AD, on the basis of capsular reactions. Earlier, the genus was divided into three varieties; *C. neoformans* var. *grubii*, *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii*. Presently, *C. gattii* is considered as a different species. *Cryptococcus* consists of two mating types; MATα and MATa. Majority of the *Cryptococcus* isolates belong to the mating type MATa (Wickes *et al.*, 1996).

Environmental and clinical isolates of *Cryptococcus* species complex have been divided into eight molecular types; VNI and VNII (serotype A), VNIII (serotype AD), VNIV (serotype D), VGI, VGII, VGIII and VGIV (serotype B and C) with the help of Restriction Fragment Length Polymorphism (RFLP) analysis (Meyer *et al.*, 1999). Soil contaminated with pigeon droppings is the ideal source for isolation of strains of serotypes A and D (Halliday *et al.*, 2003). Strains of serotype A have also been isolated from decaying woods (Nishikawa *et al.*, 2003).

*Cryptococcus* infections are believed to be acquired by inhalation of airborne yeast or basidiospores from environmental sources and getting deposited in the alveoli of the host lungs (Hull *et al.*, 2002). These spores can cause severe disease in immunocompromised persons. The lungs, skin and brain are most commonly infected sites. Unlike *C. neoformans*, *C. gattii* spores can cause infection in healthy people as well as in organ transplant patients. Both can cause severe neurological and pulmonary infections (Hull *et al.*, 2002).

Out of 70 recognised species of *Cryptococcus*, *C. neoformans*, *C. grubii* and *C. gattii* are best known for their clinical importance. *C. neoformans* is well known for causing cryptococcosis.
*C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii* are pathogenic to human beings and commonly found in clinical specimens (Morgan *et al.*, 2006). Meningoencephalitis and lung infections are caused by *C. neoformans* var. *neoformans* or *C. neoformans* var. *grubii* and *C. gattii* in immunocompetent hosts (Sharp, 2009). Immunocompromised patients are mainly infected by *C. neoformans* var. *grubii* serotype A. About 90% of Cryptococcal infections and 99% cryptococcosis cases in AIDS patients have been reported by *C. neoformans* var. *grubii* alone (Xu *et al.*, 2002).

*Cryptococcus* infections are caused by the formation of biofilms and occasionally by free-living cells. Microbes form colonies attached in a community in close relationship. Fungal infection is more complex than bacterial infection and therefore difficult to treat. Due to the different properties of biofilms, it is more resistant towards antifungal therapy (Donlan, 2002).

Virulence of *C. neoformans* is due to several factors like capsular polysaccharide, urease production, phospholipase, melanin synthesis and mating types (Chander, 2009). *Cryptococcus* is pathogenic because of the presence of polysaccharide capsule and ability to produce phenol oxidase and to grow at 37°C (Cherniak *et al.*, 1994). Capsule production helps in protecting the organism against phagocytosis (Breen *et al.*, 1982). Phospholipase is involved in the establishment of Cryptococcal infection by causing damage to the cell membrane (Chen *et al.*, 1997).

Few infection cases of other *Cryptococcus* species are also reported. Cases of *C. albidus* cutaneous infection (Narayan *et al.*, 2000), infection in the eye and blood of lymphoma patients (Garelick *et al.*, 2004; Ramchandren *et al.*, 2004) are reported. Cases of *C. laurentii* disseminated disease of central nervous system (CNS) (Vlchkova-Lashkoska *et al.*, 2004) and fungemia in a neonate and cancer patients (Cheng *et al.*, 2001; Averbuch *et al.*, 2002) are also reported.

Cases of *C. flavescens* in an AIDS patient (Ikeda *et al.*, 2004) and *C. diffluens* from patient with atopic dermatitis (Sugita *et al.*, 2003) are also reported. *C. diffluens* case of subcutaneous infection was reported by Kantarcioglu (2007). A case of systemic cryptococcosis caused by *C. humicolus* was reported in a child (Shinde *et al.*, 2004). *C. curvatus* was recovered from the cerebrospinal fluid of a 30-year-old HIV-infected male patient (Dromer *et al.*, 1995).
Chander (2009) has described several aspects of Cryptococcus and pointed out that amphotericin B has been successfully used against Cryptococcus because of its ability of rapidly decreasing load of yeast cells in the central nervous system. Flucytosine has also been found as a useful antifungal agent. Flucytosine is used in combination with amphotericin because combined action of both the drugs reduces the toxicity effect. Drug resistant isolates of C. neoformans has also been reported. The isolates show drug resistance against amphotericin and fluconazole in vitro (Chander, 2009).

Polymerase chain reaction (PCR) fingerprinting, Restriction Fragment Length Polymorphism (RFLP) and Random Amplified Polymorphic DNA (RAPD) are reliable methods for genetic characterization of clinical and environmental isolates (Feng et al., 2008). Fingerprinting methods were used for comparison of environmental and clinical isolates of C. neoformans (Meyer et al., 2003; Trilles et al., 2003). A close relationship among environmental and clinical isolates has been proved within the given geographical area in Australia (Sorrell et al., 1996), where a genetic concordance between Eucalyptus (100%) and clinical isolates (92%) of C. gattii has been explained with the help of RAPD analysis. It provides strong evidence that there is a direct link between reservoir (environment) and infection (Sorrell et al., 1996).

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

A case of meningitis caused by C. adeliensis was reported in Germany (Rimek, 2004). Tintelnot (2005) reported the successful isolation of C. adeliensis from pigeon droppings in Germany. Both report of C. adeliensis from the clinical and environment represents close relationship between the clinical and environmental strains.

Until recently, C. gattii was considered to be a tropical or semitropical organism occurring only in Australia. It was not known to be present in temperate climates like western Canada. C. gattii caused an outbreak (1999) affecting humans and animals in British Colombia (BC) and
Vancouver leading to death of humans. Infections were found as well in domestic animals, *i.e.* dogs, cats, and horses (Fyfe *et al*., 2008).

In British Columbia (Canada), about 218 cases of *Cryptococcus* infections in humans were recorded (MacDougall *et al*., 2011). The average incidence of cryptococcosis has been reported higher in Vancouver (Canada) than in the rest of the world. The reason behind the recent outbreak in Vancouver Island could be due to the favourable climatic conditions. Presently changing climate has a large impact on the distribution of *Cryptococcus*; as the environmental niche of this pathogen varies from tropical to temperate regions (Galanis *et al*., 2010).

Indian states; Punjab, Haryana, Uttar Pradesh, Tamil Nadu and Union Territory (Chandigarh and Delhi) have a wide prevalence of *C. neoformans* and *C. gattii*. A variety of tree species have been studied for distribution and isolation of *Cryptococcus* species. 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; 2003; 2008).

A report on the status of cryptococcosis in India reveals more cases from Northern states while HIV prevalence is low here as compared to Southern or Western states (Jaiswal *et al*., 2002). In India, clinical cases of HIV associated cryptococcosis increased from 20% (1996) to 50% during 2000-2004 (Banerjee, 2005). India has an estimated population of 3-9 million people living with HIV/AIDS (UNAIDS, 2006). Himachal Pradesh (H.P.) is also affected with HIV-AIDS epidemic. There were 540 full blown cases of AIDS in Himachal Pradesh, as per survey of Health Official Project (2009). Most of the cases were from Shimla, Kangra, Hamirpur, Bilaspur, Mandi, Kullu, Chamba, Una, Sirmaur and Solan districts. Cases of HIV/AIDS reported were 4998 as in December, 2010. In December, 2012, 6,481 positive cases of HIV were detected of whom 2065 have AIDS (www.hpsacs.org/aids.asp).

In India, clinical cases and samples have been studied frequently due to public health concern. However, the environmental sampling is quite limited. Thus, there is a need to focus on environmental isolation of *Cryptococcus*. 
The present study has been carried out in Solan. This region for sampling was selected because of its temperate climate. In the past, most of the studies in India have been carried out in warm or tropical areas such as Punjab and Delhi (Chakrabarti et al., 1997; Randhawa et al., 2001; 2003; 2008). Presently changing climate has a large impact on the distribution of Cryptococcus and the environmental niche of this pathogen is varying from tropical to temperate regions. After the Cryptococcus outbreak in the very cold city of Vancouver, Canada (Kidd et al., 2004), it has become important to study this pathogenic or rare yeast from cold climate areas also. Solan has heavy vegetation with a large number of tree species. Therefore, this study was undertaken so as to isolate and characterize the environmental and clinical samples to know the prevalence of this pathogen in Solan city of Himachal Pradesh with the following objectives:

1.2 OBJECTIVES OF THE RESEARCH

1. Collection and screening of environmental samples for the isolation of Cryptococcus species.

2. Identification and characterization of environmental and clinical isolates of Cryptococcus species complex.

3. Molecular characterization of the environmental isolates of Cryptococcus species complex.