6. DISCUSSION

Opportunistic infections are major life-threatening complications in immunocompromised patients, especially with AIDS cases. Cryptococcosis is one of the most commonly recognised opportunistic infection in such patients. Formerly, cryptococcosis was a rare disease and it used to occur mostly in patients with defects in cell-mediated immune response. In recent years, a sharp rise in the incidence of cryptococcosis has been observed and this increase in incidence has been found parallel with the sharp increase in the incidence of AIDS all over the world (Levitz, 1991). Zuger et al. (1986) reported that, about 50% of the patients with cryptococcosis were the AIDS-defining opportunistic infection in HIV positive patients. The duration of survival and quality of life of these patients depends onto a large extent on effective diagnosis, treatment and prevention of such infections.

THE PREVALENCE OF CRYPTOCOCCOSIS IN HOSPITALISED AIDS PATIENTS AT CHENNAI

The prevalence of cryptococcosis in patients with AIDS varies according to geographical region. The prevalence of cryptococcosis in HIV infected patients is 3 to 6% in Europe, 6 to 10% in the USA and 10 to 30% in some tropical countries, particularly in Central Black Africa, predominantly with meningeal infection (Zuger et al., 1986; Holmberg and Meyer, 1986; Dismukes, 1988; Murphy and Denning, 1994).
A number of isolated reports on human cryptococcosis has been reported from all over India (Balakrishna Rao et al., 1952; Sinha and Barua, 1960; Padhye and Thirumalachar, 1961; Aikal et al., 1967; Basu Mallik et al., 1967; Randhawa and Paliwal, 1977; Randhawa and Pal, 1977; Talwar and Meera, 1986; Mani and Rajendran, 1995; Banerjee et al., 1995; Chakrabarti et al., 1995; Aher et al., 1996). However reports from South India were very few (Subramanian et al., 1965; Sharma et al., 1985; Rajkumar et al., 1992; Asha et al., 1995) and rarely from AIDS patients (Kumarasamy et al., 1995). Hence, an attempt was made to fulfil this lacuna on the epidemiology of cryptococcosis in India.

PULMONARY CRYPTOCOCCOSIS

Since C. neoformans is not a saprophyte of the respiratory tract like Candida albicans or other species of budding fungi, it is important to rule out C. neoformans in the differential diagnosis, whenever yeast-like fungi are isolated in clinical specimens especially from, HIV infected individuals (Staib, 1987).

Pulmonary cryptococcosis is largely ignored because of its vague symptoms. It is well documented that pulmonary cryptococcosis in a normal host can resolve spontaneously or tends to be localised to the lung and most of the AIDS patients with pulmonary cryptococcosis have disseminated disease and 60 to 70% of such patients have concomitant meningeal involvement (Kerkering et al., 1981; Clark et al., 1990). In the present study out of the 117 AIDS patients, C. neoformans was identified in 2 cases with the prevalence
rate of 1.7% (95% CI 0.3-5.5) (Table 5). However follow-up studies could not be done with the culture proven pulmonary cases, as one patient died of severe diarrhoea a week after the diagnosis and another patient left the hospital without prior intimation. The antigens could not be detected in these cases and the failure in detection of cryptococcal antigen in the serum and isolation of fungus from other sites (extrapulmonary) was probably related to the stage of the infection during which the specimen was collected. This is in agreement with the observations made by Jensen et al. (1985). One patient had the past history of pulmonary tuberculosis and treatment.

According to Khanna et al. (1996) pulmonary tuberculosis may be one of the co-factors for cryptococcal infection, in addition to immunocompromised state, as tuberculosis is the commonest secondary infection in AIDS patients in India.

Moreover, the involvement of lungs in the AIDS as well as in non-AIDS patients due to C. neoformans has been almost clinically inconspicuous and the primary stage of this infection was never diagnosed before the detection of the fungus in specimens from the respiratory tract (Staib et al., 1987). Therefore the clinician should initiate the search for C. neoformans in the specimens from the respiratory tract of HIV infected persons and persons with related immunodeficiencies.
ROLE OF MEDIA ON THE RECOVERY OF C. NEOFORMANS FROM PULMONARY SPECIMENS OF AIDS PATIENTS

Early diagnosis and treatment of less life-threatening forms of the disease such as pulmonary disease, could lessen the impact of the disease for any given individual. Pulmonary cryptococcosis has been reported for only a small number of patients with AIDS, despite the fact that lungs are probably the major route of entry of the organisms (Waffer and Talavera, 1987). This low frequency may be artifactual in that conventional fungal sputum cultures from these patients frequently yield C. albicans, because of oral contamination of the specimens, since both oral C. albicans colonisation and disease (thrush) are extremely common in AIDS patients (Piot and Colebunders, 1987).

Moreover, the colony morphologies of C. albicans and C. neoformans may be similar on conventional primary isolation media in the first day or two of growth, and C. albicans may occur in greater numbers on the plate, thereby obscuring the presence of C. neoformans colonies. The present study also showed that, the direct plating onto selective media may increase the sensitivity and this finding supports the earlier study by Denning et al. (1990). This present study also demonstrated the superiority of Staib's agar over another selective medium, Sunflower seed agar (Table 13).
MENINGEAL CRYPTOCOCCOSIS

Cryptococcal meningitis may present in an indolent manner or as acute illness in AIDS patients. In the present study with the hospitalised AIDS patients from southern region of India, the prevalence of cryptococcal meningitis was found to be 11.4% (95% CI 7.1-17.1). Another study from Vellore (South India) by Koshi et al. (1989) showed the prevalence of 9.6% with non-AIDS patients, predominantly with liver cirrhosis. Kumarasamy et al. (1995) studied the spectrum of opportunistic infections in 100 AIDS patients and one patient (1%) was found to be having cryptococcal meningitis. To best of our knowledge, no other information is available regarding the prevalence.

Fever and headache, the most common symptoms, are present in about 80 to 90% of patients. Other non-specific symptoms including nausea, vomiting and malaise was observed in less than 50% of patients. Alteration of mental status in 20% and photophobia in 19 to 28% are common (Dismukes, 1988). Sometimes, only headache (Kovacs et al., 1985; Mitchell et al., 1995) or fever (Rozenbaum and Goncalves, 1994) was the only clinical symptom observed in AIDS patients. Neck stiffness more frequently occurs in the non-immunocompromised patients (75%) than patients with AIDS (32.8%) or other predisposing factors (47.4%) as reported by Rozenbaum and Goncalves (1994). In the present study, the major symptoms observed were headache (94.4%), fever (72.2%) and stiff neck (33.3%). Thus the findings in the present study
agrees with the observations made in AIDS patients with cryptococcosis by
earlier investigators.

According to Johnson et al. (1992), the clinical course of cryptococcal
meningitis in AIDS shows some important differences from the features of
illness in non-AIDS patients. Complications such as raised intracranial
pressure and visual impairment, that are recognised in non-AIDS patients may
be less frequent than in those with AIDS. Accordingly, in the present study,
22.2% of the patients were presented with visual impairment (Table 6). Thus
monitoring of these patients by regular fundoscopy and visual acuity
assessment is recommended in order to detect early signs of raised intracranial
pressure, before the development of significant visual disturbance.

OUTCOME OF TREATMENT

Therapy for acute or first episode cryptococcal meningitis in AIDS
patients has been associated with high failure and relapse rates, as therapy for
other opportunistic infectious diseases in this group of severely
immunocompromised patients (Dismukes, 1988). In the present study, despite
the treatment with amphotericin B all the patients with cryptococcal
meningitis did not survive.

Moreover, amphotericin B regimen must be individually tailored for each
patient with AIDS, because of the complexities in this population such as
concurrent opportunistic infections, HIV related renal diseases and multi-drug
therapy regimens (Dismukes, 1988).
Kovacs et al. (1985) reported that, the treatment results in 24 patients with AIDS and first-episode cryptococcosis, including 18 with meningitis, only 42% of the patients responded to treatment. In these 24 patients, two different therapeutic regimens were used, amphotericin B alone and amphotericin B with 5-flucytosine. There was no difference in the efficacy of these two regimens, although the number of patients who received each regimen was small. Another retrospective study by Chuck and Sande (1989) revealed that, the addition of 5-flucytosine offered no therapeutic benefit and was associated with a greater number of toxic side-effects. In contrast, other investigators (Eng et al., 1986; Chakrabarti et al., 1995) reported the successful use of combination therapy with 5-flucytosine. In addition, the azoles were used successfully in patients whose amphotericin B treatment failed or who developed dose-limiting toxic effects of amphotericin B and was found favourable because they are orally active and had minimal toxicity (Dismukes, 1993; Powderly, 1993).

ANALYSIS OF CLINICAL DATA FROM VARIOUS CENTRES

The retrospective analysis of available clinical data obtained along with the isolates received from various centres revealed the important information regarding age and sex-wise distribution, and predisposing factors. Age and sex-wise distribution revealed male preponderance, which corroborates with other workers (Talwar and Meera, 1986; Lo, 1976; Richardson, 1976; Chakrabarti et al., 1995) and the maximum number of cases in the age group of 26-35 years as reported earlier by Zugar et al. (1986) and Rozenbaum and Goncalves
Although this may be partly explained by greater outdoor exposure for men, there is an evidence that estrogens might inhibit the growth of *C. neoformans* (Mohr *et al.*, 1972).

The present retrospective analysis of clinical data revealed that, the HIV infection (77.3%) is the major form of predisposing condition for cryptococcosis (Table 8). Before the AIDS era, cryptococcosis taking a disseminated course with CNS involvement was very rare. It occurred chiefly against a background of primary conditions such as Hodgkin's disease, sarcoidosis, tuberculosis, systemic corticosteroid therapy and organ transplants. Since 1980, AIDS has become the most frequent predisposing condition for cryptococcosis (Kovacs *et al.*, 1985; Zuger *et al.*, 1986; Staib, 1987; Dismukes, 1988; Chuck and Sande, 1989).

On the contrary, the report by Chakrabarti *et al.* (1995) reveals, renal transplantation and steroid therapy were the major predisposing condition other than HIV infection.

**LABORATORY FINDINGS**

Cryptococcosis in AIDS patients is usually associated with profound immunodeficiency with the CD4 count almost invariably less than 100 cells/mm³ (Crowe *et al.*, 1991). The analysis of immune function markers such as CD4 cell counts in the present study revealed that the mean and standard deviation of the cell counts for 16 patients was 109 ± 84.3. Review of
A retrospective study by Brandt et al. (1996) indicates that, the range 3-155 with the median 25 of CD4 cell counts were associated with cryptococcal meningitis.

However, the CSF indices observed in the present study were slightly abnormal (Table 12). Similar observations were also made by some workers earlier. For example in the series of 18 patients with AIDS and cryptococcal meningitis reported by Kovacs et al. (1985), the leucocyte count was ≤ 5 cells/mm³ in 11 of 17 patients tested, the protein level was <45 mg/dL in 5 of 16, the glucose level was >40 mg/dL in 11 of 16, and in three patients, these CSF indices were absolutely normal. In an another series, of 22 patients reported by Zuger et al. (1986) the leucocyte count was <20/mm³ in 15 of 22, protein <40 mg/dL in 14 of 22, and glucose >50 mg/dL in 14 of 22 patients. Thus, the clinician should not be misled by a normal or only slightly abnormal CSF indices in AIDS patients with cryptococcosis and greater diagnostic reliance should be placed on the more specific tests such as culture, detection of capsular antigen of C. neoformans etc.

**DIAGNOSIS OF CRYPTOCOCCOSIS**

In patients with and without AIDS, specific studies such as direct microscopic examination with negative stain, culture and cryptococcal antigen are usually positive with various sensitivity (Dismukes, 1988). In the present study, the sensitivity of negative staining, culture and detection of antigen were found to be 77.8%, 100% and 100% respectively (Table 15).
Though the negative staining is an established method of detecting the encapsulated \textit{C. neoformans} cells in the CSF specimen, the sensitivity of this method is less than the culture and antigen detection. Similar findings were also reported by Stockstill and Kauffman (1983) with the sensitivity of 56.3\%, Kovacs \textit{et al.} (1985) with the sensitivity of 82.4\%, Zuger \textit{et al.} (1986) with the sensitivity of 68.2\%, Mitchell \textit{et al.} (1995) with the sensitivity of 62\% and Khanna \textit{et al.} (1996) with the sensitivity 75.6\%. There is no doubt that, culture of CSF is the most sensitive method of diagnosing cryptococcal meningitis. On the contrary, some investigators reported the false negativity with the culture method (Zuger \textit{et al.}, 1986; Rozenbaum and Goncalves, 1994).

**ISOLATION OF \textit{C. NEOFORMANS} FROM EXTRANEURAL SITES**

Isolation of \textit{C. neoformans} from any site should be regarded as significant and an indication for further evaluation and initiation of therapy (Powderly, 1993). The high degree of susceptibility of HIV infected patients to \textit{C. neoformans} infection, spreading from a primary focus in the lung with few symptoms, means that colonies of the organism can be expected in a multiplicity of body regions and organs. Hence at the first signs of extrapulmonary and extracerebral manifestations difficulties of diagnosis can arise. In addition to involvement of the lung and CNS, manifestations have been observed in AIDS patients in the heart, lymph nodes, spleen, liver, bone, joints, kidneys, prostate gland, pancreas, thyroid, adrenal glands, eyes, intestine, skin and blood (Staib, 1987; Rozenbaum and Goncalves, 1994). Gupta and Deshmukh (1996) reported that cryptococcal infection involves skin in
about 10% of patients as a non-specific lesion. Diamond and Bennett (1974) reported, the isolation of *C. neoformans* from extraneural sites before therapy which provided useful prognostic information and it was very well correlated with the treatment failure.

Similarly in the present study, the isolation was possible from extraneural sites such as blood (50%), urine (33.3%) and sputum (5.6%). On the contrary, Staib and Seibold (1988) reported the 100% isolation of *C. neoformans* from urine. However the sample size was very less (3 patients) to rely on their percentage positivity in urine specimens.

Kovacs *et al.* (1985) showed that among 18 AIDS patients with cryptococcal meningitis, positive extraneural cultures were seen in 9 (50%). Similarly Zuger *et al.* (1986) noted positive extraneural cultures in 36.4% of the patients at initial presentation. Another study from India by Chakrabarti *et al.* (1995) reported that the isolation from blood (37.2%) and urine (14%), of the patients with cryptococcal meningitis. Thus blood culture seems to be an important means of extraneural isolation in AIDS patients with cryptococcal meningitis. However growth and identification of the organisms require several days. Thus the use of rapid diagnostic tests such as detection of antigen, has a great impact on the management of cryptococcosis in HIV infected individuals.

In the present study, the cryptococcal antigen was detected in CSF specimens of all the patients with cryptococcal meningitis and 16 cases (88.9%) were positive for antigen in serum specimens (Table 15). Hence, testing for
antigen in serum may be useful as a initial screening test for febrile patients. The present study is substantiated by the earlier study of Eng et al. (1986), in which 98% of the AIDS patients with cryptococcal meningitis were positive for antigen in serum specimens. Because of the high sensitivity of antigen detection, serum testing for antigen has been used increasingly as a method of screening for invasive disease, especially in AIDS patients.

However, the reports from Denmark (Hoffmann et al., 1991) and Zaire (Desmet et al., 1989) provide contrasting results. In these population, among whom there is a high incidence of cryptococcal meningitis, the serum antigen was detected only in 12% of HIV positive patients, 66% of whom had culture proven meningitis. Hence, the routine screening of serum is not useful for these patients.

**DIAGNOSTIC VALUE OF CO-A TEST**

Rapid diagnosis of cryptococcal meningitis is necessary for timely therapy and improved prognosis. The LA test, which is sensitive and widely used as a rapid test, but may yield false positive reactions due to the presence of rheumatoid factors. However application of Co-A test for detection of antigen proved to be a simple, specific, sensitive and cost effective. Furthermore, it was proved to have early diagnostic as well as prognostic value, as revealed by response to therapy and decrease in Co-A titre (Koshi et al., 1989). In the present study, the CSF cryptococcal antigen was detected in 94.4% of culture proven cryptococcal meningitis cases by Co-A test and the CSF antigen was
negative by Co-A test in one patient, which was positive by LA test. It was also found to be effective in detecting the antigen in serum specimens (88.9%). However, this is in agreement with the earlier study by Koshi et al. (1989) showing high sensitivity.

SKIN TEST

The present study reveals that, the application of skin test in AIDS patients (Table 15) with cryptococcosis may be of value for assessing immunological status of the AIDS patients, rather than diagnostic importance. The observation of negative delayed type skin reactivity is not unique and it might be due to immunodeficiency as indicated by CD4 cell counts in the present study (Table 11).

On the other hand, the use of skin test for the epidemiological studies were proved to be effective (Walter and Atchinson, 1966; Newberry et al., 1967) and to some extent for the diagnostic purpose in earlier days, elicited positive reactions in upto 91% of patients with cryptococcosis (Atkinson and Bennett, 1968). Graybill and Alford (1974) reported that one-third of the patients with cryptococcosis had positive delayed skin test reactions to cryptococcocin, of which 4.6% of the patients had underlying disease such as renal transplant, sarcoidosis, Hodgkin's disease, etc.
THE OCCURRENCE OF C. NEOFORMANS IN ENVIRONMENTAL SPECIMENS

Emmons (1951 and 1955) found fecal matter of the pigeon colonised by C. neoformans. This observation was followed by a worldwide research into the problem of this habitat. In many parts of the world this habitat could be confirmed and brought into relation with the occurrence of cryptococcosis. The association of C. neoformans with bird's excreta was explained that, the bird's urine substances such as purines, urea and creatinine were utilised in highly concentrated form by C. neoformans due to its osmophilic capacity.

It was furthermore stated that, creatinine was exclusively utilised (with a few exceptions) by C. neoformans in contrast to the other species of the genus Cryptococcus (Staib, 1963b). Many studies have shown that excreta of pigeons and less frequently, that of other birds serve as an important natural reservoir of C. neoformans infections. The recovery of C. neoformans from pigeon excreta from other parts of India is also well documented (Gugnani, et al., 1972; Pal, et al., 1979; Pal, 1989a; Dhindsa, et al., 1994).

The results of the present study also revealed the association of C. neoformans with the environmental specimens (Table 18). 9.6% of the specimens of pigeon droppings collected from 5 different locations in this part of the country (South India), yielded the growth of C. neoformans, which is very less when compared with another study from Vellore. The Vellore (South India) study revealed higher occurrence rate (54.5%) of C. neoformans in pigeon droppings (Asha et al., 1995). In the present study, only 16.7%
of the specimens of pigeon droppings from Vellore yielded the growth of C. neoformans. This difference may probably be due to the differences in the seasonal variation or specimen collection sites. Apart from pigeon droppings, the other suggested environmental specimen is the Eucalyptus tree. However the negative finding from the specimens of Eucalyptus tree examined might be, attributed either to actual scarcity of the fungus in South India or to an inadequacy of the number of specimens examined. In the previous study from North India (Chakrabarti et al., 1997), of 696 Eucalyptus tree specimens tested, 5 (0.7%) yielded the growth of C. neoformans var gattii.

**EFFECT OF MEDIUM AND ADDITIVES ON THE RECOVERY OF C. NEOFORMANS FROM ENVIRONMENTAL SPECIMENS**

The present study confirms that, the Staib's agar with 0.01% of biphenyl is suitable for the isolation of C. neoformans from highly contaminated specimens such as pigeon droppings (Table 19). But, the higher concentration of biphenyl (0.1%) inhibits the growth of C. neoformans and the development of BCE on this medium. Similarly, the addition of methyl violet to the Staib's agar also performed well, showing higher CFU/gm of the specimen. This observation is in agreement with the Rubio et al. (1984), who designed the medium for the recovery of C. neoformans from environmental specimens. Another selective medium, Sunflower seed agar yielded the growth only 75% of the positive specimens. On the other hand, the investigator who designed Sunflower seed agar, has successfully isolated C. neoformans from the excreta of different avian species, fruits and vegetables (Pal and Mehrotra, 1984; Pal,
1989a and 1989b). However for isolation on Sunflower seed agar, the specimen should contain *C. neoformans* in large numbers. These observations suggest the superiority of Staib’s agar with biphenyl 0.01% and Staib’s agar with methyl violet, to conventional and other selective media for the effective isolation of *C. neoformans*.

**CHARACTERISATION OF C. NEOFORMANS**

**Antifungal Susceptibility Testing**

The lack of eradication of cryptococcal infection in AIDS patients might have important consequences for therapy. Prolonged treatment can convert the isolates progressively less susceptible to antifungal therapy. Along with increased immunodeficiency, this can cause therapeutic failure. Consequently methods for the routine susceptibility testing of *C. neoformans* isolates are needed either to facilitate choices among antifungal drugs or to monitor current therapy.

Despite antifungal therapy, the mortality occurs in about 20 to 50% (Kovacs et al., 1985; Maher and Mwandumba, 1994). Moreover, the acquired resistance are increasingly recognised with amphotericin B (Powderly et al., 1993), 5-flucytosine (Staib and Seibold, 1988) and fluconazole (Bandt et al., 1996) during the treatment.

Wingard et al. (1991) reported that prolonged use of fluconazole for treatment or prophylaxis of mucocutaneous candidiasis and this has led to replacement of fluconazole sensitive *Candida albicans* strains with non-
C. albicans yeast resistant to this drug. Moreover, the low dose oral fluconazole prophylaxis for oral candidiasis in this patient does not prevent the development of cryptococcal meningitis. Several studies suggest fluconazole is effective using doses higher than required in the treatment of oral candidiasis, in the long-term treatment and management of AIDS patients with cryptococcal meningitis (Coker et al., 1992).

In the present study, a wider range of MIC was observed with fluconazole. This is probably due to the increasing resistance, during the treatment for oral candidiasis in AIDS patients as fluconazole is widely used. However, 90% of the isolates were inhibited by fluconazole at the concentration of 16 μg/ml. Other drugs have shown narrow range of MICs to these isolates. However the study of Chaturvedi et al. (1988) with 12 isolates of C. neoformans from North India are comparable with the present study.

BIOTYPING OF THE ISOLATES

The characteristics of C. neoformans that differentiate it from other yeast species are its polysaccharide capsule and urease activity. However, isolates lacking a capsule or urease activity are not uncommon (Ruane et al., 1988). Similarly, although melanin formation (BCE) is a stable character of C. neoformans, phenoloxidase-negative C. neoformans has also been reported (Kabasawa et al., 1991). However there was no BCE-negative strains observed in the present study, although varying intensity of the melanin production was observed with different isolates.
THE PREVALENCE OF VARIETIES AND SEROTYPES OF C. NEOFORMANS FROM CLINICAL SOURCES

Kwon-chung and Bennett (1984a) reported the varieties and serotypes of 725 clinical isolates of C. neoformans from various parts of the world. Isolates from regions with temperate climate such as the USA (excluding Southern California and Hawaii), Europe and Japan belonged nearly exclusively to var neoformans.

In another study by Shadomy et al. (1987) showed that 86% of the clinical isolates from America and 57% of isolates from China were var neoformans.

Other investigators with smaller numbers of clinical isolates have reported similar trends (Bennett et al., 1977; Muchmore et al., 1980; Mishra et al., 1981; Swinne, 1984; Swinne et al., 1986; Ellis, 1987).

In contrast, there was a high frequency (35 to 100%) of var gattii from tropical and subtropical regions such as Southern California, Australia, Southeast Asia, Brazil and Central Africa (Kwon-chung and Bennett, 1984b). It was suggested that the presence of var gattii predominantly in tropical and subtropical regions may be a consequence of the association of this variety with Eucalyptus trees, as these trees are not found in temperate climates (Ellis and Pfeiffer, 1990b; Mc Clatchie, 1902).
According to a recent study by Dromer et al. (1994), the serotype A of var *neoformans* is most common throughout the world and serotype D is uncommon in USA, but is more frequent in Europe, particularly in Italy (49%) and in France (>20%) and nearly all the isolates from Southern California belong to serotype C (Shadamy et al., 1987).

In India from northern region, the study (Padhye et al., 1993) with 18 clinical isolates revealed that, 15 isolates (83.3%) were var *neoformans* and remaining 3 isolates (16.7%) were var *gattii*. The results of serotyping revealed, of the 18 isolates, 13 (72.2%), 2 (11.1%) and 3 (16.7%) were serotype A, AD and B respectively. From southern region, Asha et al. (1995) reported that, out of 11 clinical isolates, 10 (91%) were var *neoformans* (serotype A) and one (9%) was var *gattii* (serotype B).

In the present study, the serotyping results revealed that 87.2%, 4.3% 3%, 4.9% and 0.6% of the isolates were serotype A,D,AD,B and C respectively. The state-wise analysis of the prevalence of serotypes revealed that, 85 to 100% of the clinical isolates from all the states studied were of serotype A (Table 25). Thus, the epidemiological situation of *C. neoformans* in India is similar to that of the USA and Japan (Bennett et al., 1977, Hironaga et al., 1983). The serotype D was observed only in Karnataka (5.3%), Maharashtra (4.3%) and Tamilnadu (3.8%).
There is no other published data available on the prevalence of varieties and serotypes of clinical isolates of *C. neoformans* from India. However in the present study, 94.5% of the Indian clinical isolates were var *neoformans* and remaining 5.5% of the isolates were of var *gattii*. Therefore this is probably the third documented report of the var *gattii* after Padhye *et al.* (1993) from North India and Asha *et al.* (1995) from South India and, the first report of the occurrence of serotypes C and D with the Indian clinical isolates of *C. neoformans*. Thus, the present study confirms the earlier observations that the predominant variety and serotype among the Indian clinical isolates are var *neoformans* and serotype A.

The incidence of cryptococcosis in AIDS patients varies according to geographic regions. The clinical isolates from such patients have been identified predominantly as var *neoformans*, even in areas where the var *gattii* is endemic (Swinne *et al.*, 1986; Shimizu *et al.*, 1986; Rinaldi *et al.*, 1986; Bottone *et al.*, 1987; Staib *et al.*, 1987; St-Germain *et al.*, 1988), whereas var *gattii* has a propensity for causing disease in immunocompetent hosts and is gradually increasing (Speed, 1993; Fisher *et al.*, 1993; Mitchell *et al.*, 1995). The reason for this disparity between isolates from patients with and without AIDS is speculative but likely relate to differences in pathogenicity.

However in the present study, var *neoformans* was predominantly associated with HIV positive patients than HIV negative patients (*P* = 0.007 Fisher's exact test) (Table 23) as in the previous studies. Isolation of var *gattii* from AIDS patients is rarely reported (Kapend’a *et al.*, 1987; St-Germain *et al.*, ...)
1988; Clancy et al., 1990; Rozenbaum et al., 1990). This study also suggests that although var gattii has a relative predilection for healthy hosts, var gattii does cause the infection in HIV positive patients and it must be probably due to incidental exposure to this variety.

It is interesting to note that, the higher prevalence of var gattii (7.9%) and the occurrence of serotype C (1.3%) was observed only from the state of Karnataka.

THE PREVALENCE OF VARIETIES AND SEROTYPES OF C. NEOFORMANS FROM ENVIRONMENTAL SOURCES

Isolates of C. neoformans var neoformans are most readily isolated from droppings of pigeons and other avian species. But, the ecological niche of var gattii was an enigma until 1990, when Ellis and Pfeiffer isolated the fungus from E. camaldulensis in Australia and var gattii was never isolated from bird’s droppings (Summerbell et al., 1992). In India, although several investigators isolated C. neoformans from environmental sources, the isolates have not been characterised except a few. However these investigators (Pal, 1989a and 1989b; Dhindsa et al., 1994; Asha et al., 1995; Pal, 1995) characterised the isolates only upto varietal typing, all of which were found to be var neoformans. In the present study also all the isolates from pigeon droppings were identified as var neoformans. Bennett et al. (1977) reported the prevalence of serotypes among the isolates from environmental sources from USA and the majority of the isolates (84.6%) were serotype A, 14.5%
serotype D and 0.8% were of untypable strains. The present study on the environmental isolates from India revealed that out of the 12 isolates, 66.7%, 8.3% and 25% were serotype A, D and AD respectively.

EFFICACY OF THE METHOD DETERMINING VARIETAL STATUS

The differentiation of varietal status of *C. neoformans* was established with colour change on CGB agar and assimilation of D-proline, although negligible colour change on CGB agar was observed with some var *neoformans* isolates. In previous report (Bennett *et al.*, 1978), 62% of serotype B and 45% of serotype C isolates were identified by GCE on modified Staib’s agar and in the present study, 50% of serotype B isolates and none of serotype C produced GCE.

Kwon-chung *et al.* (1982b) and Shadomy *et al.* (1987) reported the superiority of CGB agar over GCP agar, although the developers of GCP agar claimed a 100% accuracy in differentiating the varieties (Salkin and Hurd, 1982). This present study also agrees with their findings, although less number of isolates were used in the present study.

Therefore these observations indicate that, the use of CGB agar and D-proline assimilation test are effective for the determination of varietal status of the isolates of *C. neoformans*. 
VIRULENCE STUDIES

The different serotypes A, D, AD, B and C of *C. neoformans* from clinical sources from Karnataka state, were studied for their virulence pattern. The results show that, var *neofomans* was more virulent than var *gattii* (Table 26). Among the serotypes of var *neofomans*, serotype AD seems to be more virulent than serotypes A and D, although the prevalence of serotype AD was less in Karnataka state than other serotypes.

The present study revealed that, the virulence of the isolates of *C. neoformans* differed according to their origin from different states of India (Table 27). For example, the higher virulence was observed with the isolates of Maharashtra and the lower virulence was observed with the isolates of West Bengal. However, there is no appreciable correlation between the prevalence pattern of serotype and virulence of the isolates in each state. Thus, the higher prevalence of serotype A, must be due to some other factors rather than virulence.

THE PREVALENCE OF MATING TYPES AMONG CLINICAL AND ENVIRONMENTAL ISOLATES

The results of mating typing revealed that, the majority (86.7%) of clinical isolates and all (100%) environmental isolates belonged to ‘α’ mating type of *F. neoformans* var *neofomans* (Table 28). This observation substantiates the earlier reports in India that, ‘α’ type of *F. neoformans* var *neofomans* occurs at high frequency both in clinical and environmental
isolates of *C. neoformans* (Pal, 1989b and 1995; Pal et al., 1991). Similar results have been observed in other countries such as USA and Japan (Kwon-chung and Bennett, 1978; Hasegawa, 1982; Hironaga et al., 1983).

The experimental studies by Kwon-chung *et al*. (1992c) revealed that, the higher frequency of ‘α’-type with the clinical isolates are probably due to the high virulence of ‘α’ strains than the ‘a’ strains. They also emphasised that, the higher frequency of ‘α’ type among environmental isolates is due to better survival of ‘α’ strains in the digestive tract of the birds than the ‘a’ strains.

Although, self-fertile isolates were reported earlier by a few investigators (Erke, 1976; Hironaga *et al*., 1983), in the present study self-fertility behaviour was not observed with any of the isolates.

**RAPD PROFILES OF THE ISOLATES OF *C. NEOFORMANS* FROM CLINICAL AND ENVIRONMENTAL SOURCES**

In the present study, an attempt was also made to observe the molecular similarities between clinical and environmental isolates from the same geographical region. The results of the present study revealed that, 50% of clinical isolates and 33.3% of environmental isolates had profile II, despite the diversity (Table 29). However, the association between both clinical and environmental isolates were not statistically significant (*P* > 0.05, χ² test). Thus, further investigation is necessary with larger number of isolates.
However, several investigators reported the similarities between clinical and environmental isolates by creatinine auxanogram (Staib and Heissen, 1989), karyotyping by pulsed field electrophoresis, RFLP analysis (Currie et al., 1994) and DNA fingerprint analysis (Varma et al., 1995; Hermoso et al., 1997). Therefore, further RAPD analysis and other above said molecular studies with many cryptococcal isolates is essential before arriving at a final conclusion regarding the similarities and dissimilarities among the clinical and environmental isolates of C. neoformans.