Chapter 11
Opportunistic Pathogenic Yeasts

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Contents

Abstract Advances in medical research, made during the last few decades, have improved the prophylactic, diagnostic and therapeutic capabilities for variety of infections/diseases. However, many of the prophylactic and therapeutic procedures have been seen in many instances to exact a price of host-vulnerability to an expanding group of opportunistic pathogens and yeasts are one of the important members in it. Fortunately amongst the vast majority of yeasts present in nature only few are considered to have the capability to cause infections when certain opportunities predisposes and these are termed as 'opportunistic pathogenic yeasts.' However, the term 'pathogenic' is quite tricky, as it depends of various factors of the host, the 'bug' and the environment to manifest the clinical infection. The borderline is expanding. In the present century with unprecedented increase in number of immune-compromised host in various disciplines of health care settings, where any yeast, which has the capability to grow at 37°C (normal body temperature of human), can be pathogenic and cause infection in particular situation.

Spectrum of infective yeasts varies in different geographical region and mainly depends upon nature of immune suppression of the patients and prevailing yeast in the environment. Opportunistic yeast pathogen mostly reported are *Candida* spp. (*albicans*, *tropicalis*, *krusei, parapsilosis, kefyr, glabrata, dubliensis, rugosa* and others), *Cryptococcus neoformans* (var *grubii*,var *neoformans* and var *gattii*), *Trichosporon* spp. and occasionally others like *Geotrichum* spp, *Pichia* spp. etc.

Among these, *C. albicans* has been regarded as the most common agent of invasive yeast infection.

The population of patients at risk has expanded to include those with a broad list of medical conditions, such as solid-organ and hematopoietic stem cell transplantation (HSCT), cancer, receipt of immunosuppressive therapy, HIV/AIDS, premature

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birth, advanced age, and major surgery. Furthermore, the etiology of these infections has changed. In the 1980s, yeasts (particularly *Candida albicans*) were the most common causative agents of invasive mycoses. However, presently non *albicans* species of *Candida* (NAC) account for >50% of infections. In addition, infections caused by other yeasts, such as *Trichosporon* species, have been reported. This chapter intends to high light important predisposing factors responsible for increase incidence of opportunistic yeast infection, its clinical significance, diagnostic approach for early detection of pathogenic yeast, guide line of therapy and epidemiology of important opportunistic yeast pathogen specially that of *Candida* and *Cryptococcus*.

Keywords Opportunistic yeast pathogen, candidosis, cryptococcosis, *Candida*, *Cryptococcus*, immune suppression

11.1 Introduction

Advances in medical research, made during the last few decades, have improved the prophylactic, diagnostic and therapeutic capabilities for a variety of infections/diseases. However, many of the prophylactic and therapeutic procedures have been seen in many instances to exact a price of host-vulnerability to an expanding group of opportunistic pathogens and yeasts are important members in it. Yeasts being ubiquitous in origin can be easily driven by environmental change linked to demography and the speed at which development is impacting human activities like road building, agricultural changes, population movements etc. Fortunately amongst the vast majority of yeasts present in nature, only few are known pathogens which can cause infection in human and animal. However the scenario has changed during last two decades. Many types of yeasts which were not known to cause infection earlier (termed as non pathogenic yeast), now shown to have the capability to cause infections when certain opportunities predisposes and these are termed as ‘opportunistic pathogenic yeasts’ (OPY). In last two to three decades OPY are increasingly reported from clinical manifestation of various disease conditions especially in immune-compromised patients (ICP).

The yeasts responsible for opportunistic infection (OI) differ in characteristics from that of conventional communicable pathogen. These are mainly low or non virulent, though the borderline is expanding. However the term ‘pathogenic’ is quite tricky as it depends on various factors of the host, the ‘bug’ and the environment to manifest the clinical infection. In recent years the definition of ‘pathogen’ is changing and the concept is evolving purely on the basis of ‘host-parasite’ interaction in particular situation. (Casadevall and Pirofski, 2002). Hence, these could be, non pathogenic in an individuals with intact immune system (*Candida albicans*) or known pathogen presenting in a different way than usual in immune-competent individuals (*Cryptococcus neoformans*) (Banerjee, 2005). Presently, with an unprecedented increase in number of immune-compromised patients (ICP) in

various disciplines of the health care system, particularly the current pandemic of HIV/AIDS, OPY have assumed great significance, where any yeast, which has the capability to grow at 37°C (normal body temperature of human), can be pathogenic and cause infection in a particular situation.

The severity of infection caused by these OPY depends mainly on the type and state of immune-suppression of the host, nature and bolus of infective yeast, the route of its entry and final site of lodgment. Many of these OPY have particular tissue tropism (e.g. *C. neoformans*) which is reflected in the characteristic diseases they produce. The clinical manifestation ranges from benign and localized lesion (either transient or chronic) to disseminated and sometimes fatal infection.

Spectrum of infective yeasts varies in different geographical regions and mainly depends upon the nature of immune suppression of the host and prevailing yeast in the environment (Advani et al., 1996; Musial et al., 1988; Banerjee et al., 1992, 1997; Handa et al., 1996; Rastogi, et al., 1999; Goswami et al., 2000; Jagarlamudi et al., 2000; Lattiff et al., 2004; Marques et al., 2000).

Opportunistic pathogenic yeast mostly reported are Candida spp (*albicans, tropicalis, krusei, parapsilosis, kefyr, glabrata, dubliensis, rugosa* and others) (Odds, 1988), *Cryptococcus neoformans* (var *grubii,* var *neoformans* and var gattii) (Casadevall and Perfect, 1998) Trichosporon spp. (Rippon, 1988; Ramos et al., 2004) and occasionally others like Geotrichum spp, Pichia spp. etc. While in present HIV/AIDS era, azole resistance *C. albicans* and non *albicans* Candida (NAC) has been highlighted. (Musial et al., 1988; Lattiff et al., 2004).

This chapter intends to highlight important predisposing factors responsible for increased incidence of OPY, its clinical significance, diagnostic approach for early detection, guidelines for therapy and epidemiology of important opportunistic yeasts like *Candida* and *Cryptococcus*. Emphasis will be provided in the background of Indian scenario.

Important opportunistic infections and pathogenic yeast associated with lesion:

11.2 Candidosis

Candidosis is one of the common endogenous opportunistic yeast infections. Candidosis arise in subjects who are predisposed like extremes of age, illness, debility or local reduction of host resistance to an overgrowth of their own yeast flora. Incidences of all types of candidosis, and especially deep-seated infections, have risen throughout the eras of antibiotic and immunosuppressive chemotherapy. Nosocomial candidosis is a distinct entity (Burnie et al., 1985; Pfaller, 1996). Of the causative agents, most common and virulent species is *C albicans*, (Odds, 1988) though other non-albicans Candida (NAC) species are increasingly being reported from all groups of patients. Incidence and prevalence of it varies in different geographical area. (Burnie et al., 1985; Wingard, 1995; Nguyen et al., 1996; Colombo et al., 1999 Krcmery and Barnes, 2002; Gutierrez, 2002; Almirante, 2006). In the HIV/AIDS era, reports of

fluconazole resistance *C. albicans* have come out and this creates lot of therapeutic problem. (Maenza et al., 1997; Yang et al., 2003) *C. albicans* causes mostly superficial mucocutaneous infection (Odds, 1988) though systemic infection is not uncommon (Sood et al., 1998; Pfaller et al., 2000; Leleu et al., 2002; Tortorano et al., 2004). Candidosis is significant in immunodeficiency in that it is commonest fungal infection found in neutropenia, (Kralovicova et al., 1997; Jagarlamudi, et al., 2000), cancer (Viscoli et al., 1999), transplant (Fotedar and Banerjee, 1996) and HIV/AIDS (Kumarasamy et al., 1995; Aggarwal et al., 1997; Lattiff et al., 2004). Extensive oesophageal candidosis is an AIDS defining condition.

Oropharyngeal candidosis (OPC) is at the top of the list of opportunistic infections in HIV disease reported worldwide before the era of highly active anti retroviral therapy (HEART), same is also true for Indian HIV positive patients (Lattiff et al., 2004; Banerjee, 2005). Our three studies on OPC in HIV/AIDS since 1992, with increase number of patients in each series, show still prevalent isolate as *C. albicans*, though emergence of non *albicans* Candida species, in concordance with experience of other investigators, has also been reported (Mirdha et al., 1993; Rastogi et al., 1999; Solomon and Ganesh, 2002; Lattiff et al., 2004; Banerjee, 2005).

Though oral candidosis, unless is very extensive, is not diagnostic of AIDS, it is of prognostic value as its presence indicates progression of the immunodeficiency. Even in immune-competent individuals certain other conditions predisposes for occurrence of mucocutaneous candidosis. As for instance, diabetes mellitus where patients have increased risk of developing vulvovaginal candidosis (VVC) (Reed, 1992; Peer et al., 1993; Goswami et al., 2000; Goswami et al., 2006). In persons with systemic infections, Candida species are now the fourth most commonly isolated pathogens from blood cultures (Pfaller et al., 2000).Yeast fungemia, especially that with Candida, occurs frequently in patients with indwelling catheters (Strinden et al., 1985; Sood et al., 1998; Fatkenheuer et al., 2003) and can result in endocarditis (Nguyen et al., 1996; Beynon et al., 2006) or pyelonephritis (Hall, 1980; Seidenfeld et al., 1982.), artificial heart valves, or other prosthetic devices (Kojic and Darouiche, 2004). Urinary tract infection (UTI) by different Candida species is a common clinical entity (Mirdha et al., 1998(b)). In advanced countries with the introduction and wide spread use of fluconazole overall incidence of candidemia caused by *C. albicans* has been decreased but it has increased the occurrence of NAC like *C. glabrata*. (Fidel et al., 1999). A retrospective study between 2001 to 2003 at AIIMS revealed that incidence of cultured proved candidemia increased from 7% (2001) to 10% (2002) to 10.3% (2003) with increase rate of isolation of NAC like *C. tropicalis*, *C. parapsilosis* and *C. glabrata* (Fig. 11.1).

Candida infection of the eye results from injury to cornea (keratitis) or can involve the retina (endopthalmitis) as one of the manifestation of candidosis spread by hematogenous dissemination (Chignell, 1992).

Candida species is readily isolated on most laboratory media, of which the most commonly used and economical media is Sabouraud dextrose agar (SDA). Since common Candida species isolated from clinical specimen are not inhibited by antibiotics or cycloheximide (except *C. glabrata*, some strain of *C. krusei* and

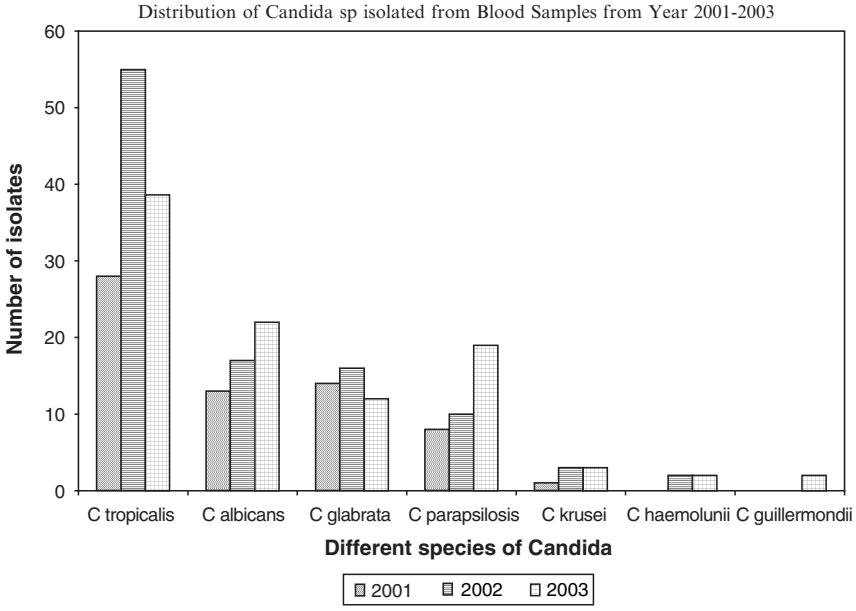


Fig. 11.1 Depicting rate of isolation of NAC like *C. tropicalis*, *C. parapsilosis* and *C. glabrata* from blood in the year 2001, 2002 and 2003

C. parapsilosis, which are cycloheximide sensitive), the use of media containing these antimicrobials are very helpful in isolation of Candida, specially from specimens which are not sterile (e.g. skin, sputum and urine), though there is difference in opinion of its use as yeast isolation media (Barnett et al., 1983). Most pathogenic strains grow well in 37°C as also at room temp (22°C–25°C).

Triphenyl terazolium chloride (TTZ) medium is a useful indicator screening medium which can identify Candida up to species level. *C. albicans* is unable to reduce tetrazolium dyes, so its colony appear creamish white, while other Candida species give colonies with various degrees of pink or red coloration. *C. tropicalis* consistently produce maroon color colony on it (Fig. 11.2).

Young colonies are white with soft consistency; the surface and margins of the colonies are smooth, although rough surfaced strains have been described. Old colonies frequently show a fringe of submerged mycelium which appears as feathery outgrowth deep in the agar.

In a wet mount, masses of budding cells and fragments of mycelium, often with budding cells attached indicative of presence of yeast like organism. Gram stain of smears shows Gram-positive budding yeasts with both pseudo-mycelium and true mycelium (Fig. 11.3).

The traditional specific test for rapid identification of *C. albicans* is the ‘germ tube’ test, in which *C. albicans* alone produces hyphal outgrowth from blastospore when incubated at 37°C in serum for 2–3 h. Germ tube test is a rapid screening test

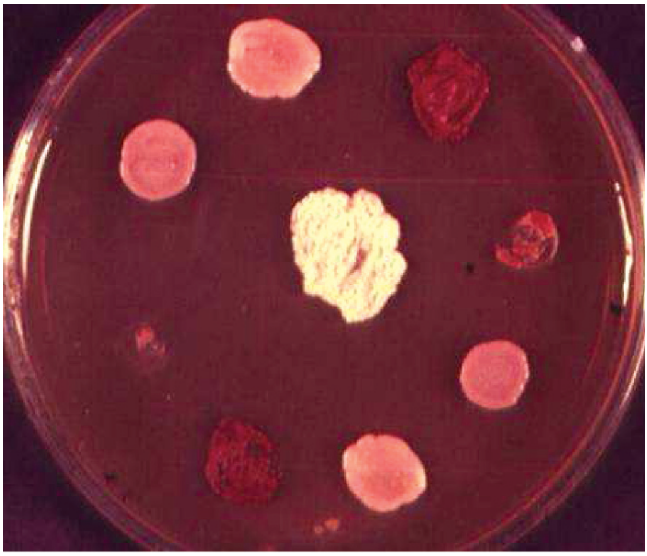
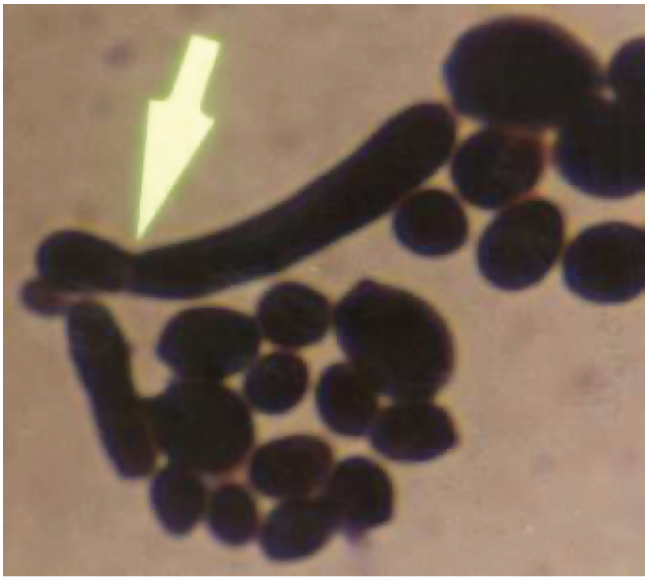


Fig. 11.2 TTZ medium with growth of different Candida species

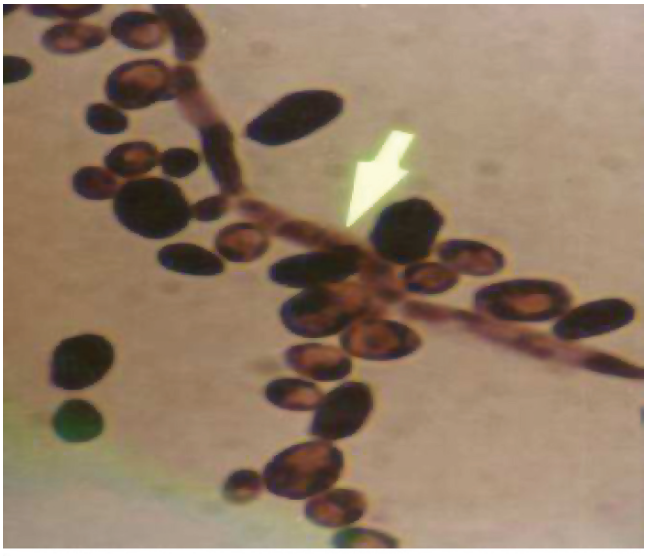
and occasional germ tube negative *C. albicans* has been reported (Odds, 1988). Morphological identification needs to be confirmed by chlamydospore formation. Chlamydospore, a resistance, resting stage of yeast, formation is a unique property specific to *C. albicans*. Corn meal agar (CMA) is a good media for testing this property. In a nutritionally deficient medium like CMA, *C. albicans* produces chlamydospores. This culture-based test is relatively rapid, cheap and easy to use. The chlamydospores are large circular structures with refractile thick walls, formed only in vitro (Fig. 11.4).

Inclusion of detergents (such as Tween 20) in the medium enhances the production of chlamydospores. The optimal temperature is 25°C–30°C, and low density of inoculum produces a better result. Ideal time of observation of CMA test is 48 h though after 24 h some strain can produce chlamydospores. CMA induces not only chlamydospores in *C. albicans* but also induce pseudo mycelium formation in isolates capable of this property. The use of CMA is therefore important not only to confirm identification of *C. albicans* but also for morphological examination of other species (Odds, 1988).

Biochemical characteristics of yeast by fermentation and assimilation of different sugars are also used to identify particular species but these are time consuming and some times produce variable results. Commercially made packages for identification of pathogenic Candida species have greatly enhanced the speed and ease in routine clinical laboratories. Of various systems, API 20 C is popular. Most of the kit rely mainly on physiological properties of the yeast. However many of the evaluator of these kits have stressed yeast morphology testing, which is unique in each species, should remain a significant part of any yeast identification and that identi-



a



b

Fig. 11.3 **(a)** Gram positive budding globular yeast cells characteristics of *C. albicans*. **(b)** Gram positive budding elongated yeast cell. Inter and intranodal bunch of blastospores (budding yeast cell) is characteristics of *C. tropicalis* Pseudo-mycelium (hyphae) designates elongated cells formed from blastospores (budding cells) which elongate, but do not break off from the mother cell. These filaments are very fragile and may break apart easily during smear preparation. On the other hand, true mycelium is formed by the elongation and branching of a germ-tube produced by the mother cell. Septae are formed along the length of the mycelium

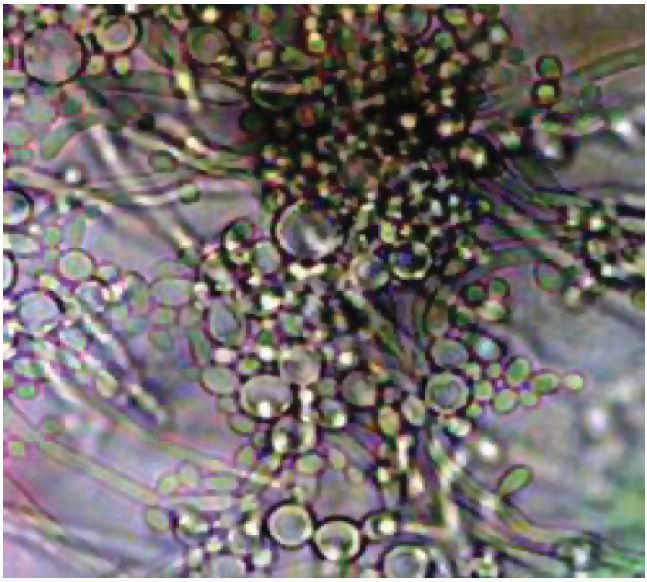


Fig. 11.4 Chlamydospores of *C. albicans* in CMA

fications based entirely on physiological properties can not be assumed to be correct (Odds, 1988).

Diagnosis:Specimens depend on the site of lesion. Usual specimens for investigation of candidosis are:

- Swabs/scraping from lesion/nail clippings
- Oesophageal brushing
- Blood for culture (hemoculture)
- Urine (suprapubic, especially in children)
- Other body fluids, where suspected
- CSF only in very rare cases
- Biopsy of tissue

Since Candida is a common and harmless commensal of mucous membrane and digestive tract of normal individual, demonstration of a few cells in a smear or isolation from specimens such as sputum, swabs from mucosal surfaces etc, have little significance. However, the presence of a large number of yeasts in a fresh specimen, in absence of any other known pathogenic organism, as well as repeated isolation of the same organism from the same site may have some diagnostic significance.

In the blood, urine (suprapubic or collected with sterile precautions), CSF and sample from closed inflammatory foci, the presence of Candida, whatever the species and the number of cells, is of pathogenic significance. It is wise, however,

to request repeat specimens, wherever possible, to rule out possible contamination from the skin when the specimen was obtained.

Quality control needs to be maintained at each step, starting from collection to the processing of samples in the laboratory till the final identification.

For example, to differentiate from colonization to the actual pathogenic role of particular yeast, multiple samples, specifically from the site that is normally not sterile, is advisable. For definitive diagnosis of OI, repeated demonstration and/or isolation of the same yeast from the same site of lesion or same yeast from multiple sites (depending on clinical presentation) are essential.

11.3 Cryptococcosis

Cryptococcosis, encountered world wide, is a serious, often fatal infection caused by the opportunistic yeast pathogen *Cryptococcus neoformans*, the only pathogenic species of the genus Cryptococcus. *Cryptococcus neoformans* is a cosmopolitan, free-living, saprophytic encapsulated yeast. It can survive in a variety of environmental niches. Source of infection is exogenous, mainly soil contaminated with bird dropping particularly pigeon droppings. Various large trees, especially *Eucalyptus camaldulensis*, another important source of this infection. Occasionally it has been isolated from healthy individuals

Depending on the structure of its polysaccharide capsule, *C. neoformans* has been typed in 5 serotypes (A, B, C, D and AD), and it exists in two varieties, var *neoformans* and var *gatti*. Recently a new variety has been suggested (*grubii*) for the serotype A. C *neoformans* is primarily a pulmonary pathogen, and infection generally begins through respiratory route with primary pulmonary invasion. In immune-competent individuals, it mainly remains as in apparent sub-clinical infection. In immune-suppressed patients, it spreads and occasionally becomes disseminated. *C. neoformans* has a predilection for the central nervous system (CNS). For many years, it was considered to be a rare disease; however, now it is recognized to be quite common, probably due to the fact that it is diagnosed more frequently.

Cryptococcosis is mostly chronic infection commonly seen in immunocompromised as well as immunocompetent patients, and sometimes also in association with other infections (Rippon, 1988; Kwon-Chung and Bennett. 1992; Mirdha et al., 1998). Chronic meningitis is the commonest presenting symptom (Diamond, 1990). It has gained increasing medical importance in recent years, particularly in the milieu of HIV/AIDS and it is predicted that it will be ‘Mycosis in future’ (Drouhet, 1997). Extra-pulmonary cryptococcosis is now regarded in an AIDS surveillance case definition (US Public Health Service, 1995). Cryptococcosis was first diagnosed in India in 1941 (Banerjee, et al., 2001(c))**.** It has since made its presence felt, more so in the AIDS era. Cases are diagnosed every year, though the actual annual number of cases in India varies (Banerjee et al., 2001(a), 2001(b) and Banerjee, 2005). Figure 11.5 shows the number of cases of cryptococcosis in AIIMS since 1985 as well as the cumulative index, in which the levels of annual occurrence

are clearly seen. Though occasionally diagnosed, increase in the number of cryptococcosis cases has been observed in AIIMS since 1993 (Banerjee et al., 1994), some times with spurt of infection (Banerjee et al., 1995). In three successive studies, expanding over a decade, we have observed that patients with cryptococcosis not only may present with various unusual clinical manifestation (Banerjee et al., 2001(c)) but also cryptococcosis can occur in substantial percentage (more than 40%) of individuals in whom there is no apparent immune-suppression (Banerjee et al., 2001(c); Banerjee 2005).

In this situation, two possibilities emerge; first, immune-suppression thresholds and parameters are not yet fully understood, and secondly, exact mechanisms of pathogenesis of cryptococcosis are not yet fully explained (Casadevall and Perfect, 1998). Related to these is the possibility of a strain variation occurring in the Indian clinical isolates (Banerjee et al., 2001(c)).

Concentrating on the factor of strain variation, in recent studies, we have found that unique strains of *C. neoformans* are circulating in the environment as different clusters (Jain et al., 2005). Some of these strains are widely distributed in nature. (Banerjee et al., 2005). Genetic analysis of sequential isolates from hospitalized patients with prolonged illness revealed microevolution taking place in particular strain (Jain et al., 2005). Further research is needed to ascertain association of microevolution of the infective strain with chronicity of disease as seen in cryptococcosis. Furthermore in vitro phase variation detected in both variety of clinical isolates (var *neoformans* and var *gattii*) as phenotypic switching (Fig. 11.6). In vivo experiments on switch variety corroborated the association with virulence

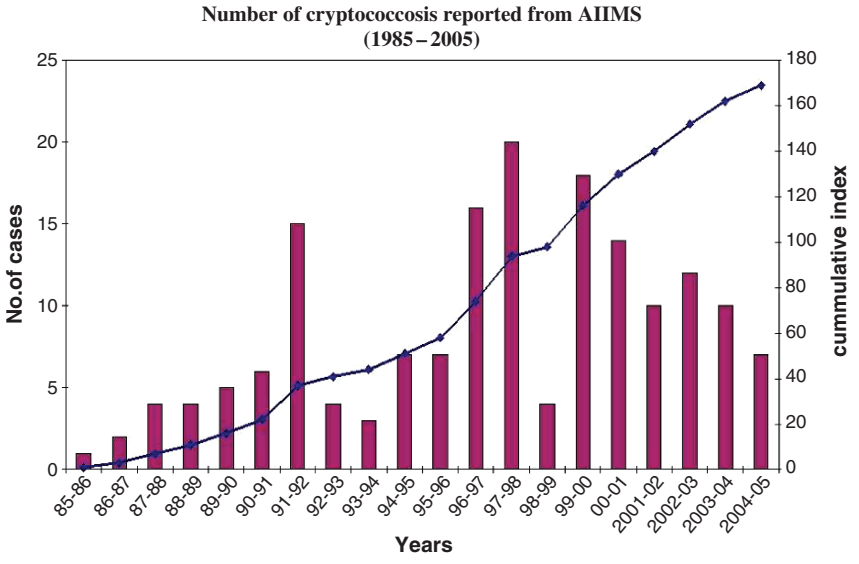


Fig. 11.5 Year wise distribution and cumulative index of laboratory proved Cryptococcosis at AIIMS between 1985–2005

and promotion of dissemination of infection (Jain et al., 2006). Well characterised polysaccharide capsule has been demonstrated in *C. neoformans* (Fig. 11.7) and shown to be associated with its virulence. It is composed of long, unbranched polymers of α-1,3-mannan with monosacharide branches of xylose and glucoronic acid. Capsule synthesis is mediated by a specific α GTP binding (G) protein encoded by the GPA1 gene in *C. neoformans* (Alspaugh et al., 1997). It is hypothesized that capsule protects the yeast from desiccation or reduce its ability to ingest and destroyed by soil amebae when in enviornment and prevents phagocytosis in side host's body, but there is no direct correlation between size of the capsule and virulence of the strains (Casadevall and Perfect, 1998).

Out of 416 clinical isolates studied in our laboratory, various thickness of large, medium and narrow capsulated strains have been demonstrated (Fig. 11.8) in clinical specimen by India Ink/nigrosin mount from both immnosuppressed and aparently immune competent patients.

Microcapsulated/acapsular strain considered as non pathogenic. Though rare, we have for the first time isolated micro-capsulated dwarf strain (Fig. 11.9) from endocardities following mitral valve prosthesis which has experimentally proved to be pathogenic strain (Banerjee et al., 1997).

Surprisingly we have found difference in cell size/capsule ratio in melanin positive (Mel+) and negative albino strain (Mel−) (Mandal et al., 2005) (Fig. 11.10a and b).

C. neoformans is relatively unique in its possession of an enzyme system (laccases) that allows it to metabolize a variety of catachol (such as dopa, dopamine, norephedrine and ephedrine) to a pigment melanin. (Polachek, 1991). Melanin

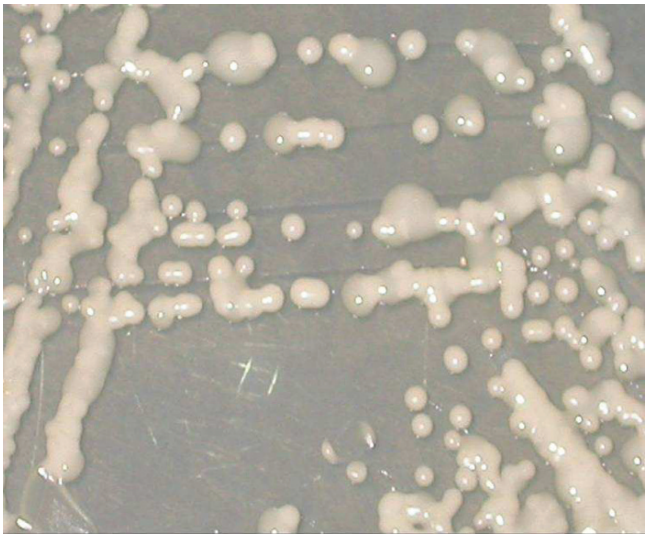


Fig. 11.6 Phenotypic switching of *C. neoformans* colony on SDA from smooth to mucoid

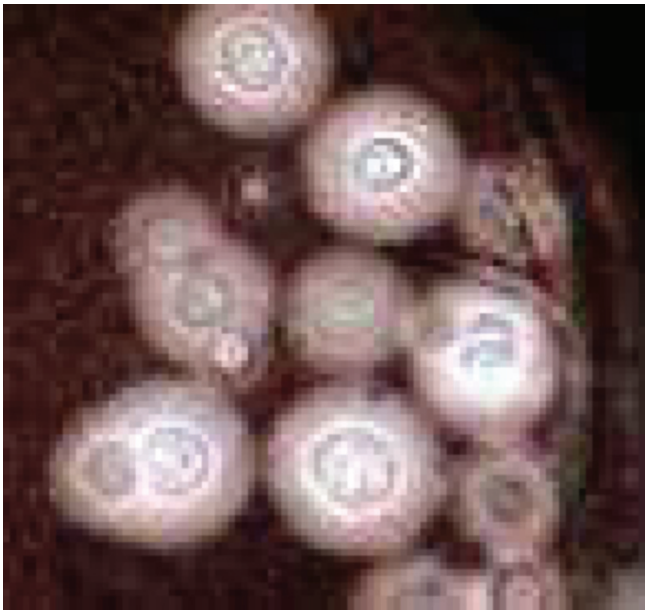


Fig. 11.7 India Ink mount of CSF showing encapsulated budding yeasts of *C. neoformans* in different stage of development

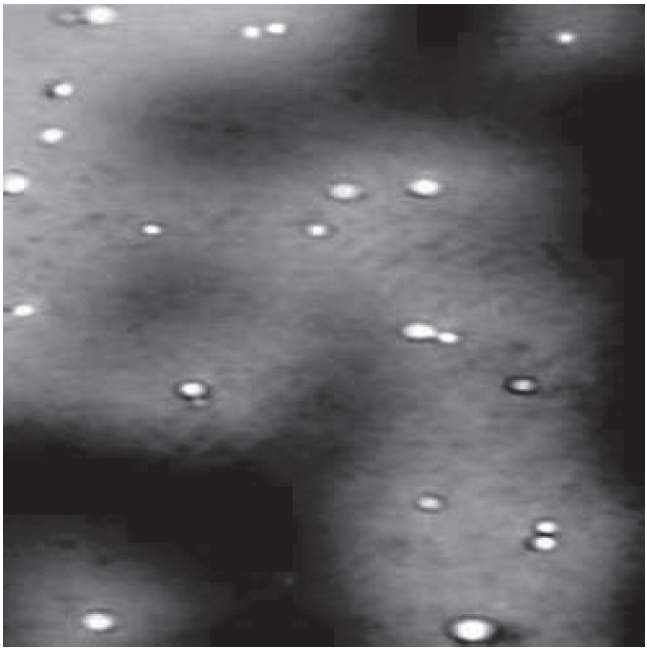


Fig. 11.8 India ink mount of acapsulated, dwarf strain of *C. neoformans* in microthrombus in a patient with cryptococcal endocarditis

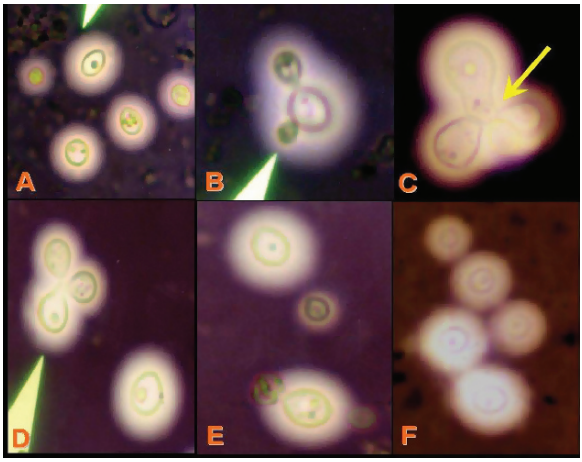


Fig. 11.9 India ink mount of CSF from different patients showing different dimension of capsules along with various cellular architecture. (**A**) and (**D**) depicts oval cells of *C. neoformans* var *gattii.* (**C**) denotes severe alteration of cellular architecture in a isolate from severely immunosuppressed host. (**E**) commonly seen in immune competent patients. (**B**) and (**F**) unusual form

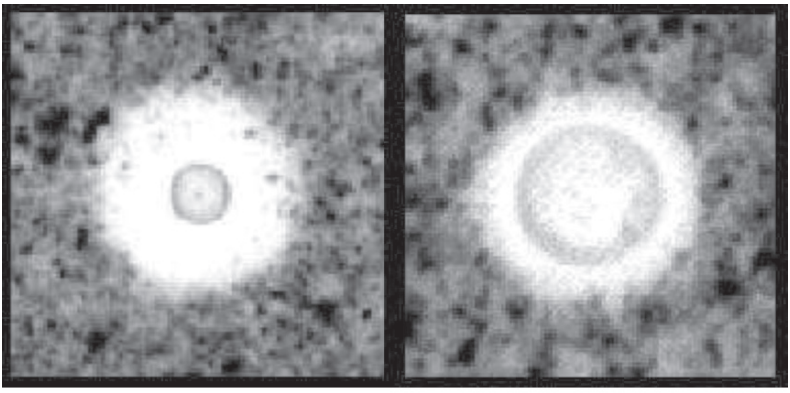


Fig. 11.10 Difference in cell size and capsule ratio in Melanin positive and Melanin negative isolates of *C. neoformans*

appears to be concentrated in the inner aspects of the cell wall of the yeast and acts as an antioxidant which protect the yeast against solar radiation and environmental oxidants as also in vivo protection against oxidative damage by professional phagocytes (Casadevall and Perfect, 1998).

Like capsule, melanin production is one of the virulent facator (Mel+) attributed to pathogenic strain of *C. neoformans* and melanin negative (Mel–) strains are considered as non-pathogenic. This property of *C. neoformans* has been used as a

diagnostic test for definitive identification of pathogenic Cryptococcus. Our experience revealed that melanin variable pathogenic strains are not infrequent (Fig. 11.11).

Optimum incubation time and temperature need to be provided to detect a stable melanin negative/variable strain. Recently we also demonstrated the pathogenic role of some melanine negative (Mel−) albino strain as an infective agent, causing serious meningitis in both HIV positive and negative patients either alone or in association with virulent melanine positive strain (Mandal et al., 2005). It is apparent that a single strain may represent a heterogeneous population of cell (Fig. 11.12) in which specific phenotypic clones can be selected. It has been further substantiated that Mel− strains had lacasse gene but due to some unknown reason failed to express it (Mandal et al., 2005). Cell size of Mel- strains was smaller compared to its counter part Mel+ strain but capsule size was significantly larger (Fig. 11.10), which further support its pathogenic potential. It is suggested that whenever unsual strains isolated from clinical specmen, detail investigations need to be conducted before dismisial of particular isolate as non pathogenic.

Identification of serotype/variety status of isolates of *Cryptococcus* not specifically relevant to diagnosis of disease but has significance not only in epidemiology, pathogenesis and clinical presentations but also on the therapeutic outcome. Serotype A, *var grubii*, is the most common serotype worldwide (Kwon-Chung and Bennett, 1984;Casadevall and Perfect, 1998), same as in India though 2% of our 45 isolate tested was untypable (Banerjee et al., 2004). In contrast to the conventional belief that var *gattii* (serotype B) does not infect HIV-positive patients and not present in India, there are few reports, both from north and south India, about the

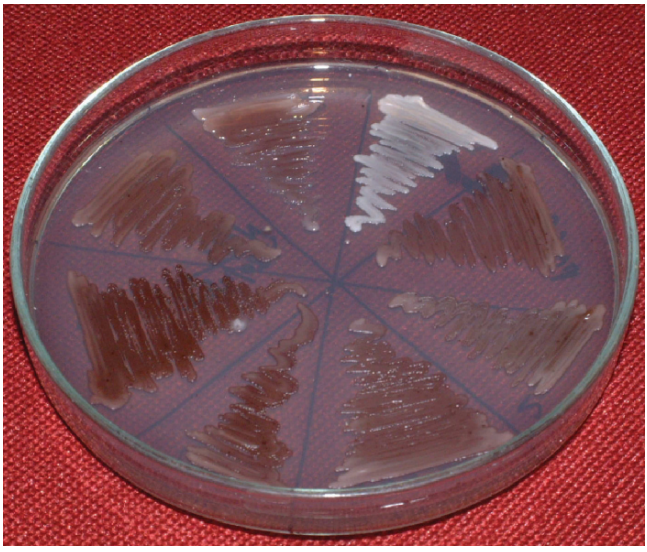


Fig. 11.11 Caffic acid agar showing melanin variable clinical isolates of *C. neoformans* along with non melanin producer yeast *C. albicans* (white colony) as control

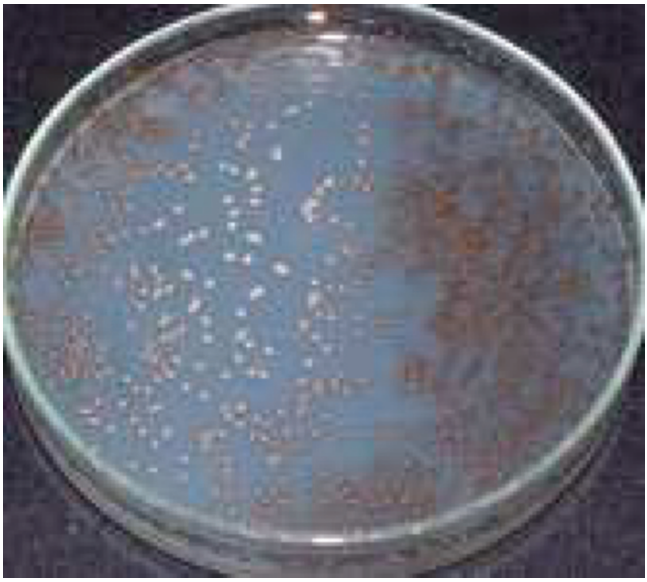


Fig. 11.12 A single CSF isolate on L Dopa agar showing heterogeneous population (strong and weak melanin positive and albino (white) population of *C. neoformans* cells

isolation of it in HIV infected patients (Banerjee et al., 2001a, 2004). This indicates the widespread reservoir of this agent in India, which is evidenced by a significant epidemiological study from the north (Chakrabarty et al., 1997). Identification of this variety of the isolate is particularly important, as it is quite often isolated from immunocompetent patients. This variety is relatively refractory to the treatment and prolonged course and /or increased dosage of antifungal may be necessary for therapy. Besides, sequelae of cryptococcosis is much more in *gattii* variety.

In vitro antimicrobial test is one of the integral part of a diagnostic set up in clinical microbiology. In contrast to the wide application of the in vitro antibacterial susceptibility test, development and adoption of in vitro antifungal test is still evolving. This test system is essential for therapeutic guidance in OPY in HIV disease, particularly in the case of therapy with fluconazole, widely used triazole, which is being used for prophylaxis of OPC and life long maintenance therapy in patients who have been treated for chronic meningitis with amphotericin B. There are reports of appearance of growing population of fluconazole resistant *C. albicans* in HIV disease, 6% per cent of our isolates from OPC in HIV positive patient showed in vitro resistant against fluconazole (Lattiff et al., 2004). On the other hand, 16% isolates of *Cryptococcus*, though not overly resistant, have higher inhibitory concentration (MIC) against fluconazole (Datta et al., 2003), many of these patients did hot have prior exposure to the drug. Reason of it unclear.

Recently transposon has been detected for the first time in some of our clinical isolates (Jain et al., 2005). Its role in acquisition of drug resistance need to be explored. Situation though is not alarming at this point, intensive research in these areas along with surveillance programs is, therefore, a prime necessity to forecast the advent of a resistant population.

Clinical Manifestation of Cryptococcosis: The clinical presentations of human cryptococcosis often varied and because of the variety of signs and symptoms, clinical evidence of infection without laboratory diagnosis can be difficult at times in both high and low risk patients. When disseminated from its primary location lung, it can lodge in any organ of the body and damage it, but brain becomes the organ with unique and still unexplained propensity for becoming a body site for clinical disease (Casadevall and Perfect, 1998). Therefore, most of the available data describe either lung or CNS manifestation of the infection.

CNS cryptococcosis: Onset of clinical symptoms often insidious, initially may linger for weeks or months as fever of unknown origin before classical manifestation of chronic meningitis sets in. Sever headache with minimal or no neck rigidity is one of the important feature of CNS cryptococcosis. Drowsiness and alteration in sensorium, with advancement of disease process. Cerebral cryptococcal granuloma is quite common.

Pulmonary cryptococcosis: Often presents with asymptomatic subclinical infection, usually detected during investigation to exclude other diseases. It is mainly diagnosed by extensive laboratory investigations and radiology.

Diagnosis: Specimen should be collected according to the symptom of the patient and suspected site of localization of infection.

Most common specimen is CSF; others are other body fluids, sputum, skin scrapping, biopsy tissues, prostatic fluid (in case of relapse) and blood for culture where indicated. If the initial demonstration/isolation is from an extra pulmonary site, attempts should be made to examine CSF irrespective of clinical presentation, to rule out asymptomatic meningeal involvement.

Diagnosis by conventional methods of direct demonstration of encapsulated budding yeast in the clinical specimen followed by successful culture. Large volume specimen increases the chances of microscopic demonstration and recovery of the infective yeast. Blood culture is helpful in diagnosis especially in the disseminated condition. However once cryptococcemia develops, fatality is almost certain even with specific antifungal therapy (Banerjee et al., 2004).

Most *C. neoformans* strains can be identified by morphological and biochemical characteristics. In its identification, certain features, such as encapsulation, growth at 37°C and production of melanin will presumptively identify the yeast as *C. neoformans.* Stain like India Ink and its various modifications – nigrosin is very useful, rapid and inexpensive diagnostic test for demonstration of *C. neoformans* in clinical specimen. It is a negative stain which demonstrates encapsulated budding yeast cells in various stages of development (Figs. 11.7 and 11.9). Its sensitivity varies depending on yeast load in a particular specimen. In CSF it can be observed when yeast concentration ranged between 10^3 to 10^4 CFU/ml. Sensitivity can be improved by centrifuging CSF (i.e. 500 rpm for 10 min) and using pellet for

staining. *C. neoformans* can be stained by Gram stain as gram variable budding yeast. Yeasts have been identified from various body sites and tissues with histological stain ranging from nonspecific Papanicolaou, hematoxylin and eosin, and acridine orange preparation to more specific fungal stains such as Calcofluor, which stain fungal chitin, or Gomori methenamine silver stain. Mucicarmine, periodic–Schiff, and alcian blue have been used to demonstrate capsular material in surrounding yeast in tissue. Immuno histochemistry not only can pinpoint specific localized site of lodgment of the yeast but also can identify specific soluble polysaccharide in the tissue even in absence of yeast cell.

In clinical specimens, yeast cell of *C. neoformans* are mostly globose in shape, although some may be oval to lemon shaped and var *gattii* cells may be actually be elliptical (Fig. 11.9). *C. neoformans* does not produce hyphae or pseudohyphae.

Biochemically *C. neoformans* is quite inert and does not ferment sugars. It can assimilate inositol but not nitrate. In its carbon assimilation profile, it will utilize galactose, maltose, galactitol and sucrose. However, it will not assimilate lactose or melibiose and its growth is strain variable with erythritol (Casadevall and Perfect, 1998).

There are a series of commercially available micro method systems employing modified conventional biochemical tests (API 20C, API 32C, Vitek Yeast Biochemical Card etc) which are being used for identification of *C. neoformans* in clinical laboratory in advanced countries but these tests, though reduce turn around time, are expensive. On the other hand conventional techniques though good and cost effective may not always offer the expected discriminatory power for early detection of infection and strain identification. It mainly depends upon the stage of the disease, suitable collection of appropriate specimen in adequate amount and proper processing in the laboratory.

Since the last decade there have been rapid development of different molecular techniques (Vilgalys and Hester, 1990; Huffnagle and Gander,1993; Mitchell et al., 1994; Hoper et al., 1993), which can rapidly distinguish *C. neoformans* from other yeast with in mixed sample or in tissue with 100% sensitivity and specificity. It seems some of these methods can be adopted directly in clinical specimen but besides being expensive, at this point of time it is uncertain when these molecular strategies will prove to supplement, complement or completely replace conventional method of diagnosis. (Casadevall and Perfect, 1998).

C. neoformans is not fastidious yeast and can grow on standard bacterial and fungal culture media. Sabouraud Dextrose Agar (SDA) mostly used in diagnostic service laboratory. Colonies may appear within 48–72 h of incubation, but may take a longer time depending on the fungal load and strain of *C. neoformans. C. neoformans* grows well at 25°C but incubation should be both at 25°C and 30°C to 37°C for clinical specimens. This range of temperature may actually speed up isolation, since some strains of var *gattii* may require prolonged incubation (5 to 7 days) and may not easily grow at higher temperature (35°C to 37°C). Thermo tolerance study conducted on 131 clinical isolates in our laboratory, 62% showed better growth in 30°C. Most isolates of *C. neoformans* are inhibited by cycloheximide under 25 μg/ml. Occasionally, nutritionally aberrant strains are reported, though specific

nutritional requirement of these strains remains unclear. It emphasizes that for some strains different media may be required for primary isolation. For instance, direct India Ink positive CSF/ histologically positive tissue which failed to grow, ideally may need to be cultured on several different media before yeast viability or non viability can be definitely determined. However in clinical practice quite often it is difficult to obtain large volume specimen, particularly from children and critically ill patients, for this purpose. Most of these culture negative cases are probably due to too few yeast at the site of infection to be detected. We have some success in this situation, by incubating a part of the sample at 25°C and reculture it after 24–48 h.

Canavanine-glycine-bromothymol blue (CGB) agar can be used to differentiate var *neoformans* from var *gattii.* Var *gattii* is resistant to canavanine and there will be good growth. It metabolizes glycine with production of ammonia which turns the medium blue. Var *neoformans* neither grows nor change the colour of the medium. Inoculum size standardization is very important in this test, and suitable positive and negative control need to included in each batch of test.

In general, colonies of *C. neoformans* are opaque, smooth or mucoid, soft in texture and creamy in colour which may turn tan or brown on prolonged incubation. There is variability among strains in their ability to appear mucoid. Mucilaginous character of colony is directly proportional to size of capsule around the yeast. *C. neoformans* can exhibit various morphological different forms of colony character especially after prolonged incubation. (Banerjee, 2005) Upon repeated subcultures (for instance, for maintenance of isolates) the colonies tend to become dry as the capsule size decreases.

For isolation of *C. neoformans* from heavily contaminated specimen like sputum in clinical practice or environmental sample, niger seed agar (Staib's medium) is an excellent selective medium. *C. neoformans* appears as brown colony due to its ability to break down caffic acid to melanin. Caffic acid agar is also available commercially and worked well in our hand. This medium can be used as a supplement as a primary culture medium for sputum, skin scrapping and urine in patients with HIV disease which increase the sensitivity for detection of *C. neoformans*, since these patients are frequently colonized with other yeasts (Staib et al.,1987; Denning et al.,1990). Comparative analysis of efficacy of bird seed with sunflower seed agar we have found that early appearance of colony and pigmentation is better in sunflower seed agar medium.

Phenotypic change of phase variation, from smooth to mucoid colony and vice versa has been observed, (Fig. 11.6) as phenotypic switch, in both var *neoformans* and var *gattii* (Jain et al., 2005) variety. The genetics of the switching mechanisms for certain phenotypes in the morphological changes of colonies and their relevance to the pathobiology of the yeast await further research.

Serology is an important adjunct of the indirect evidence of infection. Cryptococcal capsular polysaccharide antigen detection by latex agglutination is the test of choice with very high sensitivity and specificity. Antibody detection has prognostic rather than diagnostic value. Antibody becomes positive as the patient

recovers and antigen titer drops. However, it is doubtful that AIDS patients ever recover sufficiently so as to produce detectable antibodies.

A clinical service laboratory needs to be equipped with battery of tests including anti fungal susceptibility and ready for timely detection and identification of a particular unusual form of yeast, which may be pathogenic, for effective patient management.

11.4 Conclusions

Any yeast can be a pathogen in a particular situation and cause infection in an ICP. It is also to be borne in mind that being eukaryotic in nature, yeast infections, compared to other microbial infections like bacteria, virus etc, takes time to clinically manifest. Absence of pathogenomic signs and symptoms suggestive of classical manifestations of particular yeast infection, particularly the deep seated ones in ICP, often makes it difficult to recognize it, especially in the early stage of infection when yeast burden in the system is low, consequently delaying specific intervention. Confident diagnosis relies heavily on experience along with a combination of clinical, radiological, microbiological, histopathological and serological evidences. Though continuous addition of newer methods takes place, introduction of sophisticated equipment and techniques in day-to-day working of the laboratory invariably increases the cost of laboratory investigations. Therefore, at the primary level, the clinical mycologist has to fall back upon simple established standard procedures. No one single procedure may help in proper diagnosis, since each has its own limitations. Therefore, all these procedures (WHO, 2001) should be incorporated in a standard mycological laboratory as far as practicable.

OPY that can be diagnosed with reasonable accuracy by physical examination (oral candidosis) or by inexpensive laboratory techniques (*C neoformans* by India ink), may be documented more frequently than OPY requiring more cumbersome procedures of collection of specimen (oesophageal candidosis, VVC, endocarditis, endopthalmitis, prostatitis etc.) (Banerjee, 2005). Determining the spectrum of OPY and the changing pattern over the years, in a given region requires adequate surveillance and good local diagnostic services. However for definitive diagnosis of etiological role of these ‘opportunistic’ yeast pathogens strict criteria for ‘documented’, ‘possible’ and ‘probable’ yeast infection should be followed with adequate quality control. Communication with reference laboratory helps in confirmation of local diagnosis.

In spite of best effort to correlate a particular yeast as a pathogen, in nature constantly ‘new’ strains evolve or ‘old’ one change their character (phase variation, acquire resistance against antifungal etc.). Awareness of the local situation, documentation, proper preservation of isolates and timely communication of newly acquired data helps in understanding of pathobiology of the yeast and regional epidemiology. Endogenous infection as in Candidosis is difficult to control/or

eradicate. Early specific diagnosis followed by specific intervention in time can save the life of many patients. Nosocomial infection however can be reduced with good hospital infection control program. Situation is different in cryptococcosis. Since all Cryptococcus in the world are being maintained in environment, their capacity to evolve, acquire new genes and search new hosts is facilitated by proximity of host to environment. It can strike at an opportune time and periodically transmit to human being. Early detection and intervention can prevent the infection to spread to CNS and complication there after.

Follow up and monitoring of the patients infected with OPY is necessary especially in ICP, as not only relapse rate is quite high even after initial successful therapeutic outcome, some times spectrum of infective yeast changes during the course of therapy. (Foteder and Banerjee, 1996). Scenario of present status of OPY is expected to change in highly active antiretroviral therapy (HAART) era. Besides, presently good number of newer antifungals are available. Judicious use of these will help better patient management. Improvement, upgradation and networking of an effective data preservation system, specially that of the laboratory documented cases of uncommon yeast causing infection is essential. A high level of alertness is needed at both clinical and laboratory level and routine surveillance studies need to be undertaken.

References

Advani, S.H., Kochupillai, V., Lalitha, N., Shanta, S., Maitreyan, V., Nair, R., Banerjee, U., Kelkar, R., and Mukherjee, S. 1996. *J. Assoc. Phys Ind.* **44:** 764–73.

Aggarwal, O.P., Sharma, A.K., and Inddrayan, A. 1997. *HIV/AIDS Research in India,* Ministry of Health and Family Welfare, Government of India, New Delhi, pp. 713–720.

Almirante B., Rodríguez, D., Cuenca-Estrella, M., Almela, M., Sanchez, F., Ayats, J., Alonso-Tarres, C., Rodriguez-Tudela, J.L., and Pahissa, A. 2006. The Barcelona Candidemia Project Study Group. *J. Clin. Microbiol.* **44:** 1681–1685.

Alspaugh, J.A., Perfect, J.R., and Heitman, J. 1997. *Gene Dev.* **11:** 3206–3217.

Banerjee, U., Gupta, K., and Venugopal, P. 1997. *J. Med. Vet. Mycol.* **35:** 139–141.

Banerjee, U. 2005. *Indian J. Med. Res.* **121:** 395–406.

Banerjee, U., Chatterjee, B., Kapil, A., and Sethi, S. 1992. Abstract. XVI National Congress of Medical Microbiologists, New Delhi, India, P32a: 144.

Banerjee, U., Datta, K., and Casadavell, A. 2004. *Med. Mycol.* **42:** 181–186.

Banerjee, U., Dutta, K., Diwedi, M., and Sethi, S. 2001. *Nat. J. Infect. Dis.* **2:** 32–36.

Banerjee, U., Gupta, K., and Sethi, S. 1994. *Nat. Med. J. Ind.* **1:** 51–53.

Banerjee, U., Jain, N. and Prasad, K.N. 2005. Trends in Medical Mycology (TIMM), October 2005, Germany (Abstract).

Banerjee, U., Khadka, J.B., Sethi, S. and Gupta, K. 1995. *Ind. J. Med. Res.* **102:** 272–274.

Banerjee, U., Saha, D.C., and Sethi, S. 2001a. 25th Annual Congress of Indian Association of Medical Microbiologists, All India Institute of Medical Sciences New Delhi. Abstract No 251: p. 272.

Banerjee, U., Dutta, K., Majumdar, T., and Gupta, K. 2001b. *Med. Mycol.* **39:** 51–67.

Barnett, J.A., Payne, R.W., and Yarrow, D. 1983. *Yeasts: Characteristics and Identification*. Cambridge University Press, Cambridge.

Beynon, R.P., Bahl, V.K., and Prendergast, B.D. 2006. *BMJ* **333:** 334–339.

Burnie, J.P., Odds, F.C., Lee, W., Webster, C., and Williams, J.D. 1985. *BMJ* **290:** 746–748.

Casadevall, A. and Perfect, J.R. 1998. *Cryptococcus neoformans*. Washington DC: ASM Press, pp. 407–408.
Casadevall, A., and Pirofski, L.A. 2002. *Ann. Med.* **34:** 2–4.
Chakrabarty, A., Jatana, M., Kumar, P., Chatha, L., Kaushal, A., and Padhye, A.A. 1997. *J. Clin. Microbiol.* **35:** 3340–3342.
Chignell, A.H. 1992. *J. R. Soc. Med.* **85:** 721–724.
Colombo, A.L., Nucci, M., Salomao, R., Branchini, M.L., Richtmann, R., Derossi, A. and Wey, S.B. 1999. *Diagn. Microbiol. Infect. Dis.* **34:** 281–286.
Datta, K., Jain, N., Sethi, S., Rattan, A., Casadavell, A., and Banerjee, U. 2003. *J Antimicrob. Chemother.* **52:** 683–686.
Denning, D.W., Stevens, D.A., and Hamilton, J.R. 1990. *J.Clin. Microbiol.* **28:** 2565–2567.
Diamond, R.D. 1990. In: *Principles of infectious diseases*. (Eds. Mandell, G.L., Douglas, R.G. and Bennett, J.E.). New York: Churchill Livingstone, pp. 1980-–1989.
Drouhet, E.J. *Mycol. Med.* 1997. **7:** 10–27.
Fatkenheuer, G., Buchheidt, D., Cornely, O.A., Fuhr, H.G., Karthaus, M., Kisro J., Leithauser, M., Salwender, H., Sudhoff, T., Szelenyi, H., and Weissinger, F. 2003. *Ann. Hematol.* **82**(Suppl. 2): S149–157.
Fidel, P.L. Jr., Vazquez, J.A. and Sobel, J.D. 1999. *Clin. Microbiol. Rev.* **12:** 80–96.
Fotedar, R. and Banerjee, U., 1996. *J. Inf.* **3:** 243–245.
Goswami, R. Dadhwal, V., Tejswi, S., Datta, K., Paul, A., Haricharan, R.N., Banerjee, U., and Kochupillai, N.P. 2000. *J. Infect.* **41:** 162–166.
Goswami, D., Goswami, R., Banerjee, U., Dadhwal, V., Miglani, S., and Latiff, A.A. Kochupillai. 2006. *N.J. Infec.* **52:** 111–117.
Guiterrez, J. 2002. *J. Basic Microbiol*. **3:** 207–227.
Hall, W.J. 1980. *J. R. Soc. Med.* **73:** 567–569.
Handa, R., Banerjee, U., Gupta, K., Singh, M.K., Singh, H., and Wali, J.P. 1996. *J Assoc Phys Ind.* **44:** 348.
Hoper, R.L., Walden, P., Setterquist, S., and Highsmit, W.E. 1993. *J. Med. Vet. Mycol.* **31:** 65–67.
Huffnagle, K.E. and Gander, R.M. 1993. *J. Clin. Microbiol.* **31:** 419–421.
Jagarlamudi, R., Kumar, L., Kochupillai, V., Kapil, A., Banerjee, U., and Thulkar, S. 2000. *Med. Oncol.* **17:** 111–116.
Jain, N., Li L., Diane, M.C., Banerjee, U., Cook, E., Wang, X., and Fries, B.C. 2006. *Infect. Immunity* **74:** 896–903.
Jain, N., Wickes, B.L., Keller, S.M., Fu, J., Casadevall, A., Ragan, M.A., Banerjee, U., and Fries, B.C. *J. Clin. Microbiol.* 2005. **43:** 5733–5742.
Kojic, E.M., and Darouiche, R.O. 2004. *Clin. Microbiol. Rev.* **17:** 255–267.
Kralovicova, K., Spanik, S., Oravcova, E., Mrazova, M., Morova, E., Gulikova, V., Kukuckova, E., Koren, P., Pichna, P., Nogova, J., Kunova, A., Trupl, J., and Krcmery, V. Jr. 1997. *Scand. J. Infect. Dis.* **29:** 301–304.
Krcmery, V. and Barnes, A.J. 2002. *J. Hosp. Infect. 2002.* **50:** 243–260.
Kumarasamy, N., Solomon, S., Jayaker Paul, S.A., Venilla, R., and Amalraj, R.E. 1995. *Int. J. STD AIDS* **6:** 447–449.
Kwon-Chung, K.J., and Bennett, J.E. 1984. *Am. J. Epidemiol.* **120:** 123–130.
Kwon-Chung, K.J., and Bennett, J.E. 1992. *Medical Mycology*. Philadelphia: Lea and Fabiger, pp. 397–445.
Lattiff, A.A., Banerjee, U., Prasad, R., Biswas, A., Wig, N., Sharma, N., Haque, A., Gupta, N., Baquer, N.Z., and Mukhopadhyay, G. 2004. *J. Clin. Microbiol.* 1260-1262.
Leleu, G., Aegerter, P., and Guidet, B. 2002. *J. Crit. Care* **17:** 168–175.
Maenza, J.R., W. G., Merz, M. J., Romagnoli, J.C., Keruly, R.D., Moore, J.E., and Gallant. 1997. *Clin. Infect. Dis.* **24**: 1204–1207.
Mandal, P., Banerjee, U., Casadevall, A., and Nosanchuck, J.D. 2005. *J. Clin. Microbiol.* **43:** 4766–4772.
Marques, S.A., Robles, A.M., Tortorano, A.M., Tuculet, M.A., Negroni, R., and Mendes, R.P. 2000. *Med. Mycol.* –**38:** 269–279.

Mitchell, T.G., Freedman, E.Z., White, T.J., and Taylor, J.W. 1994. *J. Cin. Microbiol.* **32:** 253–255.
Mirdha, B.R., Banerjee, U., Sethi, S., Samantaray, J.C., and Malviya, A. 1993. *CARC Calling* **6:** 9–10.
Mirdha, B.R., Maheshwari, M.C., Sethi, S., Dan, G., and Banerjee, U. 1998a. *Ind. Paed.* **35:** 360–363.
Mirdha, B.R., Sethi, S., and Banerjee, U. 1998b. *Ind. J. Med. Res.* **107:** 91–93.
Musial, C.E., Cockerill, F.R., 3rd Roberts, G.D. 1988. *Clin. Microbiol. Rev.* **1:** 349–364.
Nguyen, M.H., Nguyen, M.L., Yu, V.L., Mc. Mahon, D., Keys, T.F., and Amidi, M. 1996. *Clin. Infect. Dis.* **22:** 262–267.
Nguyen, M.H., Peacock, J.E., Morris, A.J., Tanner, D.C., Nguyen, M.L., Snydman, D.R., Wagener, M., Rinaldi, M.G., and Yu, V.L. 1996. *Am. J. Med.* **100:** 617–623.
Odds, F.C. 1988. *Candida and Candidiasis: A Review and Bibliography,* 2Bailliere Tindall, Toronto, Ontario, Canada.
Peer, A.K., Hoosen, A.A., Seedat, M.A., van den Ende, J., and Omar, M.A. 1993. *S. Afr. Med. J.* **83:** 727–729.
Pfaller, M.A. 1996. Nosocomial Candidiasis. *Clin. Infect. Dis.* **22**(Suppl. 2): S89–S94.
Pfaller, M.A., Jones, R.N., Doern, G.V., Sader, H.S., Messer, S.A., Houston, A., Coffman, S., Hollis, R.J. and The SENTRY Participant Group 2000. *Antimicrob. Agents Chemother.* **44**: 747-751.
Polachek, I. 1991. *Int. J. Med. Microbiol.* **276:** 120–123.
Ramos, J.M., Cuenca-Estrella, M., Gutierrez, F., Elia, M., and Rodriguez-Tudela, J.L. 2004. *J. Clin. Microbiol.* **42**(5): 2341–2344.
Rastogi, A, Datta, K., Paul, A., Sethi, S., and Banerjee, U. 1999. *Nat. J. Inf. Dis.* **1:** 33–35.
Reed, B.D. 1992. *Obstet. Gynecol. Surv.* **47:** 551–560.
Rippon, J.W. 1988. *Medical Mycology: The Pathogenic Fungi and the Pathogenic Actinomycetes*. 3 The WB Saunders Co., Philadelphia, pp. 532–558.
Seidenfeld, S.M., Lemaistre, C.F., Setiawan, H., and Munford, R.S. 1982. *J. infect. Dis.* **146:** 569.
Sood, S., Majumdar, T., Chatterjee, B., Sethi, S., Dutta, K., and Banerjee, U. 1998. *Mycoses* **41:** 417–419.
Solomon, S., and Ganesh, A.K. 2002. *HIV in India, International AIDS Society – USA* **10:** 19–24.
Staib, F., Seibold, M., Antweiler, E., Forhlich, B., Weber, S., and Blisse, A. 1987. *Hyg. A.* **266:** 167–177.
Strinden, W.D., Helgerson, R.B., and Maki, D.G.. 1985. *Ann. Surg.* **202:** 653–658.
Tortorano, A.M., Caspani, L., Rigoni, A.L., Biraghi, E., Sicignano, A., and Viviani, M.A. *J. Hosp.* 2004. *Infect.* **57:** 8–13.
US Public Health Service/Infectious Disease Society of America. Guidelines for prevention of opportunistic infections in persons infected with the human immunodeficiency virus. 1995. *Morb. Mort. Week Rep*. **44**(RR-8): 1.
Vilgalys, R., and Hester, M. 1990. *J. Bacteriol.* **172:** 4238–4246.
Viscoli, C., Girmenia, C., Marinus, A., Collette, L., Martino, P., Vandercam, B., Doyen, C., Lebeau, B., Spence, D., Krcmery, V., Pauw, D.B., and Meunier, F. 1999. *Clin. Infect. Dis.* **28:** 1071–1079.
Wingard, J.R. 1995. *Clin. Infect. Dis.* **20:** 115–125.
World Health Organization. 2001. Guidelines on Standard Operating Procedure for Laboratory Diagnosis of HIV- Opportunistic Infections 2001. (Ed. Kumari, S.), New Delhi.
Yang, Y.L., Cheng, H.H., Ho, Y.A., Hsiao, C.F., and Lo, H.J. 2003. *J. Microbiol. Immunol. Infect.* **36:** 187–191.