

Laboratory manual for diagnosis of fungal opportunistic infections in HIV/AIDS patients

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Abbreviations

AIDS	acquired immunodeficiency syndrome
ARDS	acute respiratory distress syndrome
ART	antiretroviral therapy
BAL	bronchoalveolar lavage
BHI	brain heart infusion
BHIA	brain heart infusion agar
CFT	complement fixation test
CGB	canavanine glycine bromothymol
CMA	cornmeal agar
CSF	cerebrospinal fluid
CNS	central nervous system
CT	computed tomography
DMSO	dimethylsulfoxide
EIA	enzyme immunoassay
FNAC	fine needle aspiration cytology
GMS	Gomori's methenamine silver
GT	germ tube
H&E	hematoxylin eosin
HCl	hydrochloric acid
HIV	human immunodeficiency virus
IA	invasive aspergillosis
ID	immunodiffusion test
KOH	potassium hydroxide
LA	latex agglutination
LCB	lactophenol cotton blue
MIC	minimum inhibitory concentration

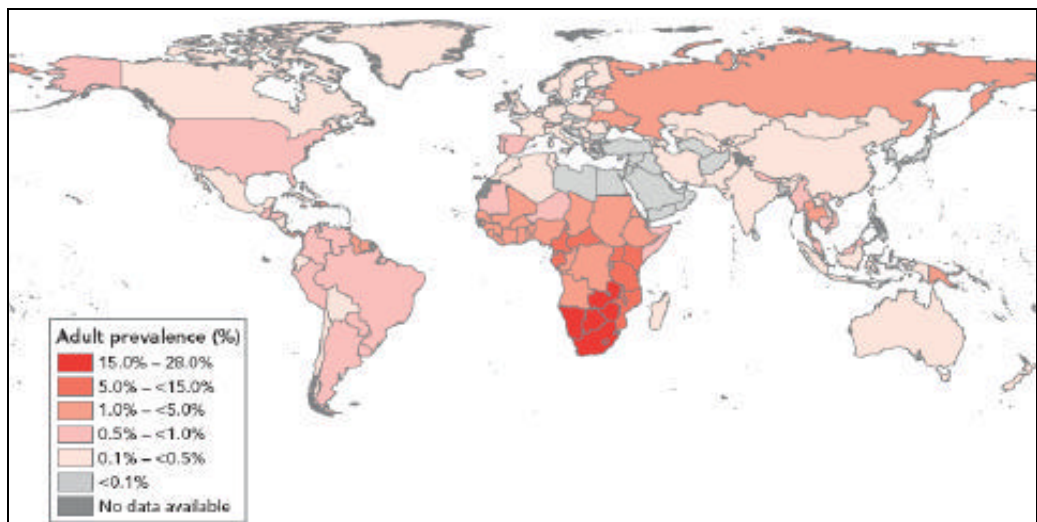
NSA	Niger seed agar
OI	opportunistic infection
PAS	periodic acid Schiff
PCP	pneumocystis carinii pneumonia
PCR	polymerase chain reaction
PLHA	people living with HIV and AIDS
PLHIV	people living with HIV
QC	quality control
SDA	Sabouraud dextrose agar
SEA	South-East Asia
YCB	yeast carbon base
YNB	yeast nitrogen base

Chapter 1: HIV/AIDS and fungal infections

Ever since its discovery, the human immunodeficiency virus (HIV) has emerged as a global disaster. Around the world acquired immunodeficiency syndrome (AIDS) has led to more than 20 million deaths. Over 33 million people are living with HIV today (Fig 1) and by 2010 it is estimated that more than 40 million children will have one or both parents dead from AIDS. People in productive age groups are predominantly affected by AIDS and hence in some countries the impact of AIDS has led to a major decrease in gross national product. To date the only tool available to ascertain the presence of HIV in an otherwise healthy-looking individual is a laboratory assay.

Fig. 1: A global view of HIV infection

33 million people [30–36 million] living with HIV, 2007



Source: UNAIDS, 2008 Report on the global AIDS epidemic

HIV/AIDS in the South-East Asia Region

The South-East Asia (SEA) Region bears the second highest burden of HIV/AIDS, with 3.6 million people estimated to be living with the virus. There is wide variation in the number of people living with HIV in countries in this Region (Table 1).

Table 1: People living with HIV (PLHA) in countries of South-East Asia Region

There is a wide variation in the number of PLHA from less than 100 in Maldives to 2.4 million in India

Country	Estimated number of people living with HIV (PLHA)	% of adult population infected with HIV
Bangladesh	12 000	<0.1
Bhutan	<500	<0.1
DPR Korea	NA	NA
India	2 400 000	0.3
Indonesia	270 000	0.2
Maldives	<100	<0.1
Myanmar	240 000	0.7
Nepal	70 000	0.5
Sri Lanka	3800	<0.1
Thailand	610 000	1.4
Timor-Leste	<100	<0.1

Source: Report on Global AIDS Epidemic, UNAIDS, 2008

HIV and opportunistic infections

HIV does not kill those who are infected directly. Instead, it weakens the body's ability to fight disease. Infections which are rarely seen in those with normal immune systems can be deadly to those with HIV.

People with HIV can get many infections (known as opportunistic infections, or OIs). Many of these illnesses are very serious and require treatment. Some can be prevented.

OIs are caused either by organisms of low or no virulence which are nonpathogenic in individuals with an intact immune system, or by known pathogens which present in a different way than usual in immunodeficient individuals, e.g. in the form of increased virulence, recurrence, multidrug resistance or atypical presentation.

The spectrum of OIs has been found to vary from continent to continent and region to region. With the unprecedented increase in the number of AIDS cases, OIs are also increasing. Several of these OIs are recognized as case-defining entities in HIV/AIDS patients (Table 2).

Table 2: WHO clinical staging of HIV/AIDS for adults and adolescents with confirmed HIV infection (fungal OIs highlighted)

Clinical Stage 1 Asymptomatic Persistent generalized lymphadenopathy
Clinical Stage 2 Moderate unexplained weight loss (<10% of presumed or measured body weight) Recurrent respiratory tract infections (sinusitis, tonsillitis, bronchitis, otitis media, pharyngitis) Herpes zoster Angular cheilitis Recurrent oral ulceration Papular pruritic eruptions Seborrhoeic dermatitis Fungal nail infections
Clinical Stage 3 Unexplained severe weight loss (>10% of presumed or measured body weight) Unexplained chronic diarrhoea for longer than one month Unexplained persistent fever (intermittent or constant for longer than one month) Persistent oral candidiasis Oral hairy leukoplakia Pulmonary tuberculosis Lymph node TB Severe bacterial infections (e.g. pneumonia, empyema, pyomyositis, bone or joint infection, meningitis, bacteraemia) Acute necrotizing ulcerative stomatitis, gingivitis or periodontitis Unexplained anaemia (<8 g/dl), neutropenia (< 0.5 x 10 ⁹ /L) and/or chronic thrombocytopenia (< 50 X 10 ⁹ /L ³)

Clinical Stage 4

HIV wasting syndrome

Pneumocystis pneumonia (caused by *Pneumocystis jiroveci*)

Recurrent severe bacterial pneumonia

Chronic herpes simplex infection (orolabial, genital or anorectal of more than one month's duration or visceral at any site)

Oesophageal candidiasis (or candidiasis of trachea, bronchi or lungs)

Extrapulmonary tuberculosis

Kaposi's sarcoma

Cytomegalovirus infection (retinitis or infection of other organs)

Central nervous system toxoplasmosis

HIV encephalopathy

Extrapulmonary cryptococcosis (including meningitis)

Disseminated non-tuberculous mycobacteria infection

Progressive multifocal leukoencephalopathy

Chronic cryptosporidiosis

Chronic isosporiasis

Disseminated mycosis (extrapulmonary histoplasmosis or coccidiomycosis)

Recurrent septicaemia (including non-typhoidal Salmonella)

Lymphoma (cerebral or B cell non-Hodgkin)

Invasive cervical carcinoma

Atypical disseminated leishmaniasis

Symptomatic HIV-associated nephropathy or HIV-associated cardiomyopathy

The early diagnosis of these infections is vital for better management and preventive measures. OIs can be bacterial, viral, parasitic or fungal, and it is in this context that the establishment and strengthening of existing mycology laboratories gains paramount importance. The ability to easily, cheaply and quickly diagnose these and other potential OIs is crucial.

Opportunistic fungal infections

Even though all fungal infections are "opportunistic", i.e. they cause disease in a host whose resistance is lowered, the true "opportunistic" fungal infections only infect those who are already sick or immunocompromised.

Opportunistic fungal infections such as mucocutaneous candidiasis, pneumocystosis, cryptococcosis and histoplasmosis are the most common AIDS-defining conditions in HIV-positive individuals. Other fungal infections like coccidioidomycosis and *Penicilliosis marneffe* are usually

seen in geographically restricted areas, the latter being reported frequently from northeast India. Blastomycosis and paracoccidioidomycosis have been reported to cause severe and disseminated disease in AIDS patients, though there has been no significant increase in the number of infections occurring in such patients. Unlike the above diseases, which occur primarily due to the defect in cell mediated immunity, aspergillosis and zygomycosis are now being increasingly encountered in advanced AIDS cases with neutropenia. Neutropenia in these patients also increases the risk of disseminated candidiasis, and invasive infections due to miscellaneous hyaline and dematiaceous (melanized) fungi. In Asia, paracoccidioidomycosis has only been reported from Japan, and only two authentic cases of coccidioidomycosis were reported from India (both were imported cases from an endemic area).

Antiretroviral therapy (ART) can lower the incidence of OIs in patients with AIDS. However, this therapy has not been available to most HIV-positive individuals due to its cost. Moreover, patients are not diagnosed in the early stages in a substantial number of cases. Hence there has been no let-up in incidence of the often fatal, invasive mycotic infections in the SEA Region, unlike the change in prevalence of these infections in developed countries.

CD₄ T lymphocytes and fungal infections

CD₄ counts, a useful prognostic marker in HIV/AIDS patients, also have critical levels below which certain invasive fungal infections start appearing frequently (Table 3).

Table 3: Relationship between CD4 T lymphocytes and fungal OIs

CD ₄ count	Opportunistic fungal infection
< 350 cells/ μ l	Mucocutaneous candidosis
< 200 cells/ μ l	Pneumocystosis
< 150 cells/ μ l	Histoplasmosis
< 100 cells/ μ l	Cryptococcosis and <i>Penicilliosis marneffe</i>
< 50 cells/ μ l	Aspergillosis and zygomycosis

Clinical criteria provide only a presumptive diagnosis of fungal infection. Usually there are few specific signs and symptoms indicating fungal infections. The lesions and radiological findings are sometimes characteristic enough (crescent sign and halo sign in invasive aspergillosis) to strongly suggest a fungal etiology but the accompanying immunosuppression frequently results in atypical presentations. In most circumstances, early diagnosis considerably increases the chance of successful treatment. Thus, it is important that the possibility of fungal infection be considered from the outset so that appropriate mycological investigations can be carried out.

Diagnostic mycology is also rapidly moving into the mainstream of clinical medicine as a result of the convergence of several independent developments. First, dramatic progress in antifungal therapeutics has increased the need for specific viral diagnosis. Second, the number of patients at risk of accruing opportunistic viral infections has expanded greatly as a result of the HIV/AIDS epidemic. Finally, modern management of HIV infection is providing a new paradigm for the integration of molecular techniques into management of chronic fungal infections. All these developments are not only increasing the use of diagnostic mycology techniques but have enabled the detection of a variety of fungal OIs. These guidelines are therefore intended to provide the laboratory techniques in diagnosis of fungal OIs at different levels of health laboratories.

Scope

This manual covers key aspects of diagnosing fungal infections. The manual describes the technique for processing the specimens and also can be of use to each laboratory in developing its own standard operating procedures. Although a variety of standard textbooks is available for these techniques, an effort has been made to consolidate relevant information in this manual.

Manual development process

The WHO Regional Office for South-East Asia commissioned the Department of Mycology, Postgraduate Institute of Medical Education and Research, Chandigarh, India (PGIMER) which is also the WHO Collaborating Centre on Fungal Infections of Medical Importance, to develop the first draft of the manual. The objectives were to assist

developing countries in establishing practical diagnostic facilities for fungal infections based upon a pragmatic approach and available scientific evidence. The draft manual was first reviewed by WHO staff and subsequently peer-reviewed by two mycologists. The manual was revised by experts in WHO CC in accordance with the reviewers' observations. The manual also provides information on the procurement of products that can be used in laboratories. However, this information is only suggestive, and WHO does not specifically endorse these products.

Manual development team

WHO wishes to acknowledge the support provided by various experts in drafting, reviewing and finalizing the manual (please see list in Annex 2).

Chapter 2: Common opportunistic fungal infections in HIV/AIDS

Candidiasis

Candidiasis is commonly an endogenous opportunistic infection. Occasionally exogenous acquisition has also been proven. Of the causative agents, the most common species is *Candida albicans*. Other non-*albicans* *Candida* species, especially *C. tropicalis* are increasingly being reported.

Candidiasis is the most common fungal infection found in HIV/AIDS patients. Extensive esophageal candidiasis is an AIDS-defining infection. But oral candidiasis, unless very extensive and causing symptoms unequivocally, is not diagnostic of AIDS. It is of prognostic value only as its presence indicates progression of immunodeficiency. Vulvovaginal candidiasis, though not unequivocally shown to occur more frequently in AIDS patients, nevertheless affects a considerable proportion of HIV-positive women with extensive disease. In advanced AIDS cases, with neutropenia and very low CD₄ counts, disseminated candidiasis is certainly a possibility.

Clinical considerations

Clinical manifestations include:

- oropharyngeal candidiasis – up to 90% of untreated, advanced HIV cases develop this disease, with more than 60% getting >1 episode per year.
- esophageal candidiasis – 10%-20% of HIV cases have esophageal candidiasis (the most common cause of esophageal disease in these patients)

- vaginal candidiasis – 27%-60% women of childbearing age develop this disease, and the rates are similar for HIV-infected and non-infected patients
- non-healing extensive skin ulcers (seen in Indian patients)
- intertriginous candidiasis*
- hospital-acquired UTI*
- superinfection of bacterial abscesses/infections in those treated for prolonged period with broad-spectrum antibiotics*
- candida lung infection (very rare)
- disseminated candidiasis (rare in HIV patients)

Diagnosis

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

- esophageal brushing
- blood for culture, central venous line tip
- urine (suprapubic, specially in children; or aseptic aspiration from Foley's) and other body fluids (CSF only in very rare cases)
- fine-needle aspiration samples
- biopsy samples



Yeast cells

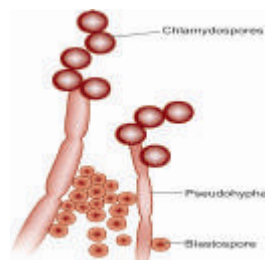


Candida albicans

* No special association with HIV infection, e.g. hospital-acquired UTI occurs in hospitalized patients whether or not they are HIV-infected.

Since candida is commensal, demonstration of a few cells or isolation from non-sterile specimens such as sputum, or swabs from mucosal surfaces, has no significance. However, the presence of a large number of organisms in a fresh specimen may have some diagnostic significance, especially in a symptomatic patient when other diseases have been ruled out.

In blood, urine (suprapubic or collected with aseptic precautions), cerebrospinal fluid (CSF) and sample from closed inflammatory foci, the presence of candida, with any number and any species 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. A central venous catheter may get colonized and serve as a portal for candidemia in hospitalized patients. The catheter should be removed in such patients and sent to the laboratory for culture isolation by rolling and flushing methods. Urinary catheter colonization may not however represent infection; these catheter tips are not recommended for microbiology processing, and instead properly and freshly aspirated urine through the catheter is recommended.



***Candida albicans* in culture**

Candida species are readily isolated on most laboratory media (including blood agar), of which the most commonly used medium is Sabouraud Dextrose Agar (SDA). Since antibacterials do not inhibit fungi, including candida, the use of media containing antibacterials is helpful in the isolation of candida, especially from specimens which are not sterile (e.g. skin, sputum and urine). Most strains grow well at 37°C or at room temperature (22-25°C).

Young colonies are white with a soft consistency; the surface and margins of the colonies are smooth, although rough surfaced strains have been described. In a wet mount, masses of budding cells and fragments of mycelium are seen. Gram stains of smears show gram-positive budding yeasts with or without pseudohyphae. Pseudohyphae are elongated cells formed from blastospores (budding cells). The daughter cell elongates, but

does not break off from the mother cell. On the other hand, true mycelium is formed by the elongation and branching of a germ-tube produced by the mother cell and formation of true septa. Identifying features in pseudohyphae are constrictions between cells, invariable presence of septa at points of branching and the smaller and rounded terminal cell. True hyphae on the other hand have no constrictions between two cells, may or may not have septa at points of branching and typically the subterminal cell is smaller than the terminal cell, which is usually cylindrical; true septa are normally refractive in nature.

Yeasts can also be cultivated from clinical specimens on the selective and differential medium called CHROMagar Candida, which uses a chromogenic mixture that allows selective isolation of yeasts and simultaneously identifies colonies of *C. albicans*, *C. tropicalis*, *C. glabrata* and *Candida krusei*.

This medium has proven to be useful for detection of mixed cultures of *Candida* sp. within a single specimen.

The rapid detection of *C. tropicalis* and *C. krusei*, which tend to be more resistant to antifungal agents, is important to optimize antifungal treatment especially for immunocompromised patients. Simple and easy detection of mixed yeast cultures is particularly useful to detect antifungal resistant strains that may be present in the sample as a minor population.

Serology (Antigen or antibody detection tests)

- *Antibody detection test* – no special use except in candida endocarditis.
- *Antigen detection test* – Mannan and Enolase antigens have been evaluated, but require further validation.

Metabolites and DNA detection

- *D-arabinitol (metabolite) detection* – D- /L-arabinitol ratio or D-arabinitol/creatinine ratio helps in indirect diagnosis, but requires sophisticated equipment. The test is yet to be validated for routine use.
- *PCR for detection of Candidal DNA in blood or body fluid* – This is under active research. Though many studies have demonstrated high sensitivity and early detection, the test is yet to be validated for routine use.

Cryptococcosis

The etiologic agent is *Cryptococcus neoformans*, the only pathogenic species of the genus *Cryptococcus*. There are five serotypes (A, B, C, D and AD), and it exists in three varieties, var. *grubii* (A), var. *gattii* (B & C) and var. *neoformans* (D). The current thinking on varieties, serotypes and pathogenesis of *Cryptococcus neoformans* is depicted in the box below:

***Cryptococcus neoformans* - varieties, serotypes and pathogenesis**

- The encapsulated basidiomycete *Cryptococcus neoformans* was recently divided into 2 species
 - *C. neoformans* (Cn) and
 - *C. gattii* (Cg).
- Cn consists of 2 varieties - *grubii* (CnVG) and *neoformans* (CnVN), which are opportunistic pathogens and predominantly infect immunocompromised persons
- CnVG is the major causative agent of cryptococcosis worldwide, except in central Europe, where CnVN infection is most prominent
- In contrast, Cg is a primary pathogen, which predominantly infects immunocompetent persons.
 - Cg was previously thought to be restricted to tropical and subtropical climates with a special ecologic niche on Eucalyptus trees.
 - However, the recent outbreak of Cg infection in healthy humans and animals in the temperate climate of Vancouver in Canada, and its isolation from several species of trees other than Eucalyptus have raised the strong possibility that this fungus might have broader geographic distribution
- The mechanisms underlying pathogenic and environmental differences between Cn and Cg are not known
- Within Cn species, CnVN infections are more likely to display skin involvement and to afflict older patients
- CnVG infections are reported to have a higher mortality rate
- In contrast, infections caused by Cg result in a lower mortality rate but are frequently complicated by neurologic sequelae and require surgery and prolonged therapy

Cryptococcosis generally begins with primary pulmonary invasion. In immunocompetent individuals, it remains as inapparent subclinical infection, but in immunosuppressed patients it spreads and occasionally becomes

disseminated. *C. neoformans* has a predilection for the central nervous system (CNS), and patients mostly present with symptoms of chronic meningitis. In the present era of AIDS pandemic, WHO has considered cryptococcal meningitis as one of the AIDS-defining infections. Incidence varies from 4%-7% in African patients to <1% in the developed world. Introduction of fluconazole and ART at reduced cost in developing countries like Thailand and Brazil has helped to reduce incidence in those countries. The effects are yet to be observed in other countries of the SEA Region.

Clinical considerations

CNS cryptococcosis/Disseminated cryptococcosis

Clinical manifestations include:

- onset is insidious; symptoms are present for weeks
- headache with minimal or no neck rigidity
- fever of unknown origin
- drowsiness and alteration in sensorium, with advancement of disease process
- chronic meningitis
- may result occasionally in cerebral cryptococcal granuloma
- May be associated with visual loss (rapid or slow), osteolytic bony lesions, secondary cutaneous cryptococcosis, cryptococemia and cryptococciuria – called disseminated cryptococcosis. In recent years a few cases of cryptococcal peritonitis have also been reported.

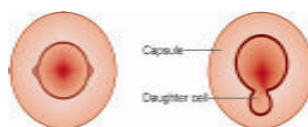
Pulmonary cryptococcosis

- Often asymptomatic.
- When symptomatic, patients have self-limiting pneumonitis, mild fever, rare pleural effusion, hilar adenopathy and calcification.

Diagnosis

Specimens should be collected according to the patient's symptoms. Most common specimen is CSF; other body fluids, sputum, bronchoalveolar lavage (BAL), bronchial washings, biopsy tissues, prostatic fluid (in case of relapse) and blood for culture are used where indicated. If the initial demonstration/isolation was from an extra-pulmonary site, attempts should be made to examine CSF irrespective of clinical presentation, to rule out asymptomatic meningeal involvement.

Diagnosis is by conventional methods as described above: direct demonstration and culture. Direct demonstration by India ink/Nigrosin wet mount is possible when the yeast cell count is more than 10^5 cells/ml of CSF. Since in HIV-positive patients the usual cell count is 10^7 - 10^9 cells/ml, direct microscopy is positive in 90% of cases. In tissue sections, mucicarmine stain and alcian blue stain are useful besides periodic acid Schiff (PAS) or methenamine silver-nitrate stain. Blood culture is helpful especially in the disseminated condition.



C. neoformans in CSF

On SDA, both at 25°C and 37°C, colonies may appear within 48 hours, but may take longer depending on the fungal load. If chloramphenicol is incorporated in media, delayed growth is observed. The colonies are soft and creamy in texture, or mucilaginous if considerable capsular material is present. In India ink mounts, *C. neoformans* appears as round budding yeasts 5-20µm in diameter surrounded by a prominent capsule (seen as a halo in India ink mounts). Rarely, acapsular, larger, smaller or short pseudohyphae-forming variants may be seen in samples. These yeasts are urease positive (Christensen's urea agar or broth containing 2.9% urea), nitrate negative, do not ferment any sugars and produce a chocolate-brown pigment on bird seed agar.

Serology

- **Antigen detection** – The latex agglutination test to detect cryptococcal polysaccharide capsular antigen using any of the commercial kits (Crypto-LA, MYCO-Immune, IMMY or CALAS) is a qualitative and semi-quantitative test for the detection of

capsular polysaccharide antigens of *C. neoformans* in CSF and serum. It is the test of choice, with very high sensitivity and specificity, and is considered one of the best serological tests for antigen detection in the field of infectious diseases.

- *Antibody detection* – not useful in immunocompromised patients like patients with AIDS.

***Pneumocystis jiroveci* infections**

Pneumocystis carinii was officially recognized as a fungus in 2001, and renamed as *Pneumocystis jiroveci*, though the disease continues to be referred as PCP (*Pneumocystis carinii* pneumonia). PCP is the most common AIDS-defining condition, though compared to the developed world it is less commonly reported from South-East Asia. It is nonetheless a major OI in HIV-infected individuals in the Region. The likelihood of developing PCP in AIDS patients increases as the CD₄ count falls below 200/μl. Thrush, persistent fever and presence of other AIDS-defining OIs are other independent risk factors for PCP in these patients.

Most healthy adults have been exposed to *Pneumocystis jiroveci* at some point of time in life, though asymptomatic carriage is quite common. The lifecycle of pneumocystis, though incompletely understood, contains at least two stages – the cyst and the trophozoite. The walls of the cyst forms take up the GMS and Toluidine Blue O stain, round-, cup- or typically “deflated-ball”-shaped. In Giemsa stain the trophozoites are also seen and the cysts show eight sporozoites within them.

Clinical considerations

- Clinical presentation is often insidious with slow but steady progression of fatigue, fever, chills, sweats and exertional dyspnoea. The features are characteristic of atypical pneumonia. Circumoral, peripheral and mucus membrane cyanosis may be apparent in extensive infection.
- Extrapulmonary pneumocystis has been reported in patients with advanced HIV disease, particularly in the setting of aerosolised pentamidine prophylaxis. Orbit, thyroid, skin, ears, adrenals, kidneys, bone marrow and lymph nodes are among the organs affected.

Diagnosis

- Among the biochemical tests, serum LDH is high.
- Pulmonary function tests and arterial blood gas analysis are very important adjuncts in the management of these cases. In fact, these are the tests used first to screen patients for PCP.
- Suggestive chest X-ray, high resolution computed tomography (CT) scan, diffusion capacity of the lung for carbon monoxide, and nuclear imaging studies using Ga₆₇ scan also aid in the diagnosis of these patients.
- Specimens include BAL, transbronchial biopsy, sputum and open lung biopsy. BAL and sputum should always be stained for acid-fast bacillus and other fungi also. Staining methods for *Pneumocystis jiroveci* include:
 - (1) Gomori's Methenamine Silver (GMS) stain
 - (2) Rapid GMS
 - (3) Toluidine Blue O stain
 - (4) Giemsa stain
 - (5) Diff Quick (Rapid Giemsa) stain
 - (6) Fluorescent antibody staining with monoclonal antibody: this is more specific and sometimes more sensitive when a low number of organisms is present in the specimen.
- Although polymerase chain reaction (PCR) technology has been studied as a diagnostic aid, further evaluation and validation are required before such tests can be applied widely.

Histoplasmosis

Histoplasmosis is an intracellular mycotic infection of the reticuloendothelial system, caused by inhalation of microconidia of the fungus *Histoplasma capsulatum*. Approximately 95% of cases of histoplasmosis are subclinical. Five percent of the cases have chronic progressive lung disease, chronic cutaneous or systemic disease or an acute fulminating fatal systemic disease. It is being increasingly reported in HIV/AIDS patients from endemic areas and the majority represents progressive disseminated disease.

Two varieties of *H. capsulatum* are recognized in human diseases, depending on the clinical disease: var. *capsulatum* and var. *duboisii* (the African type). *H. capsulatum* var. *duboisii* causes disease restricted to Africa and Madagascar, and no case has been described from South-East Asia. Histoplasmosis due to var. *capsulatum* has a worldwide distribution.

Clinical considerations

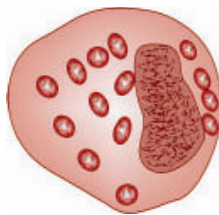
- All stages of this disease may mimic tuberculosis. In some clinical situations, it can be confused with sarcoidosis.
- Since this intracellular pathogen resides in macrophages, the ideal specimen for direct demonstration and culture is bone marrow. The organism is best grown when inoculated in the medium at the bedside immediately after collection from the patient.
- In patients with AIDS, histoplasmosis presents as a progressive disseminated infection in 95% of cases. The majority of such cases have CD4 counts <150 cells/ μ l and a median cell count of 50 cells/ μ l.
- Patients with disseminated disease usually present with fever, weight loss and malaise over a period of several weeks. In about half, respiratory symptoms are reported.
- Hepatosplenomegaly and generalized lymphadenopathy are encountered in 15%-30% of cases.
- A syndrome representing septicemia has been well documented. The patients usually present with hypotension, acute respiratory distress syndrome (ARDS), hepatic failure, renal failure and disseminated intravascular coagulation. It represents a later manifestation of disseminated histoplasmosis and can occur in 10%-15% cases of disseminated histoplasmosis in AIDS.
- Mucocutaneous involvement is very common in patients from South-East Asia, particularly India.
- Neurologic complications have been reported in up to 20% of cases. These include encephalopathy, lymphocytic meningitis and focal pulmonary lesions in the brain and spinal cord. Signs and symptoms include headache, fever, altered mental status and focal neurologic findings.

- Gastrointestinal and osteo-articular lesions are also found. Adrenal insufficiency may be a presentation of disseminated histoplasmosis.
- Patients who experience immunologic improvement following anti-retroviral therapy may demonstrate atypical pathology. This has been referred to as immune reconstitution inflammatory syndrome and is characterized by focal inflammatory histology, elevation of hepatic enzymes, hepatic abscesses, lymphadenopathy, arthritis, uveitis and intestinal obstruction.

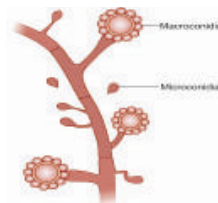
Diagnosis

- Specimens – Bone marrow, tissue or body fluid are usually sent to the laboratory. In disseminated infection, samples can be taken from blood, bone marrow, liver, skin lesion, or any other site of infection.
- Lysis centrifugation (Isolator Tube) system has been shown to be more sensitive than automated or conventional culture for growing *H. capsulatum* from blood.
- When sputum or BAL fluid is sent for culture, a selective medium containing ammonium hydroxide on the surface of agar to increase pH is helpful to inhibit the growth of commensals and allow the slow growing *H. capsulatum* to grow.
- For direct microscopy of smear from samples or tissue sections, routine 10% potassium hydroxide (KOH) wet mount or hematoxylin and eosin stains generally will not allow visualization of tiny yeasts. Smear or section should be stained with methenamine silver or PAS stain to best visualize *H. capsulatum*. Distinctive 2-4 µm, oval, narrow-based budding yeasts within macrophages, but also seen free in tissue sections, allow tentative diagnosis of histoplasmosis.
- Samples are plated on SDA and incubated at 25°C to allow growth of mycelial phase of *H. capsulatum*. It may take 6 weeks for growth of *H. capsulatum*, which appears as a white to tan mycelial growth.

- Two types of conidia are produced on hyphae: tuberculate macroconidia (8-15 μm in diameter) having characteristic projections on their surface; and smooth-walled microconidia (2-4 μm in diameter). Identification of macroconidia allows only presumptive diagnosis of histoplasmosis as *Sepedonium* spp. produce similar macroconidia. The following tests verify the identification of *H. capsulatum*:
 - Yeast conversion by repeated subcultures at 37°C on pine agar medium. This is a laborious and time-consuming process.



Yeast stage of *H. capsulatum*



***H. capsulatum* in culture**

- Exo-Antigen test can help in identification even in a contaminated culture, but it is also time-consuming and complicated.
- Commercially available chemiluminescent DNA probe (AccuProbe; Gen Probe, San Diego, USA) is used to identify *H. capsulatum* isolates in three hours. The test is highly specific though very rare false positives are reported.

H. capsulatum exhibits a thermal dimorphism by growing in living tissue or in culture at 37°C as budding yeast-like fungus, and in soil or cultures at temperatures below 30°C as a mould. On SDA at 25°C colonies are mostly slow-growing (2-3 weeks in many cases), white or buff brown, cottony with pale brown transverse folds. Sometimes colony types may be glabrous or verrucose in appearance.

Antigen detection - Detection of circulating polysaccharide antigen in urine and serum was originally developed as a solid phase radioimmunoassay. The assay was converted to more easily performed sandwich enzyme immunoassay (EIA) that uses *H. capsulatum* polysaccharide antigen, polyclonal rabbit anti-histoplasma immunoglobulin

conjugated to biotin, and horseradish peroxidase. Both assays are more sensitive in urine rather than serum. In AIDS patients, the antigen is detected in urine in 95% of patients and in serum in 85% of patients. The test can also help monitor response to therapy. There are few data on the usefulness of antigen assays for BAL fluid (detected in 70% of patients). False positive reactions are seen in patients who have other endemic mycoses (blastomycosis and paracoccidioidomycosis, but not with coccidioidomycosis). False positive reactions are also reported in patients with penicilliosis, which is prevalent in the South-East Asia region. The test is yet to be commercialized.

Antibody detection – It is not useful in AIDS patients, as they mount a poor antibody response.

Penicilliosis marneffei

The dimorphic fungus *Penicillium marneffei* causes the disease, which is an emerging systemic mycosis in AIDS patients. *Penicillium marneffei* is endemic in Thailand, Northeastern India (Manipur and Nagaland states), southern China, Hong Kong, Vietnam and Taiwan. Bamboo rats and soil are considered the reservoir of the disease though the agent has never been isolated from soil except near bamboo rat burrows.

Penicilliosis marneffei, after tuberculosis and cryptococcosis, is the third most common opportunistic infection in patients with AIDS in the South-East Asia Region, and is therefore considered an AIDS-defining illness. Persons affected by penicilliosis usually have AIDS with low CD₄ counts, typically <100/μl. The average CD₄ count at presentation is 63.5/μl.

Clinical considerations

Various types of manifestations include:

- fever of unknown origin, loss of weight, generalized lymphadenopathy, anemia
- hepatomegaly with or without splenomegaly
- pneumonitis: cough and dyspnea occur in about 50% of cases, sometimes with hemoptysis

- skin lesions - characteristic generalized papular eruptions, central umbilicated papules resembling those of molluscum contagiosum, or acne like lesions and folliculitis over face, trunk, and extremities
- pharyngeal and palatal lesions also can be seen
- subcutaneous nodules may be seen
- chest radiographic abnormalities typically manifest as diffuse reticulonodular infiltrates, and cavitations

Diagnosis

Specimen: Bone marrow aspirate, blood, lymph node biopsy, skin biopsy, sputum, BAL, pleural fluid, liver biopsies, CSF, pharyngeal or palatal ulcer, scrapings, urine, stool, kidney biopsy, pericardium, stomach or intestinal specimens.

- Giemsa, Wright, GMS or PAS stain shows characteristic intracellular (within neutrophils or tissue histiocytes) round to oval yeast-like cells, which may divide by crosswall formation. The crosswall formation can differentiate yeast cell of *P. marneffei* from those of *H. capsulatum*. Elongated sausage-shaped extracellular forms are also seen.
- Direct immuno-fluorescence test is the test of choice for specific diagnosis; this would be done at reference laboratories, since it requires a fluorescent microscope. Several types of antibodies have been used for detection of *P. marneffei* in paraffin embedded, formalin fixed tissue. Monoclonal antibody directed to *Aspergillus* galactomannan can be used to stain *P. marneffei* due to identical epitope between the two fungi (the difference in morphology helps to distinguish them). Rabbit antiglobulin against *P. marneffei* mycelial culture filtrate antigens is also proven to be specific for detection of *P. marneffei* in tissue section.
- Definitive diagnosis is based on culture isolation, which has a high sensitivity – bone marrow (100%), blood (76%) and skin biopsies (90%).

- *P. marneffei* exhibits thermal dimorphism by growing in living tissue or in culture at 37°C as a yeast-like fungus, and in culture at below 30°C as a mould. On SDA (without cycloheximide) at 25°C colonies are fast-growing, suedelike to downy, white with yellowish-green conidial heads. Colonies become grayish-pink to brown with age and produce a characteristic diffusible brownish-red to wine-red pigment. Conidiophores are hyaline, smooth-walled and bear terminal verticils or whorls of 3-5 metulae, each bearing 3-5 phialides. The conidiophores are described as biverticillate or irregularly monoverticillate. Conidia are globose to subglobose, 2-3 µm in diameter, smooth-walled and are produced in basipetal succession from the phialides.



Penicillium sp

- Other biverticillate, red-pigment-producing *Penicillium* species include *P. purpurogeum* and *P. rugulosa*, from which these can be definitely differentiated by demonstration of dimorphism (mould to yeast conversion is achieved by subculturing on brain heart infusion agar [BHIA] at 37°C).
- On BHIA at 37°C colonies are rough, glabrous, tan-colored and yeast-like. Microscopically, the yeast cells are spherical to ellipsoidal, 2-6 µm in diameter, and divide by fission, rather than by budding. Numerous short hyphal elements and elongated sausage-shaped forms are also seen. Tissue sections show small, oval to elliptical yeast-like cells, 3 µm in diameter, either packed within histiocytes or macrophages, or scattered throughout the tissue. Occasional, large, elongated, sausage-shaped cells, up to 8 µm long with distinctive septa may be present. Tissue sections need to be stained by GMS stain to clearly see the yeast-like cells, which are often difficult to observe in hematoxylin and eosin stain preparations.

- Several serological methods for detection of antibodies or antigens are attempted and produce conflicting results especially in AIDS patients. No standard kit is available to date. Commercially available Pastorex *Aspergillus* (Latex Agglutination test kit) or Platelia *Aspergillus* (EIA kit) for detection of *Aspergillus* antigen in serum or body fluid cross-react with *P. marneffei* and are positive in patients with penicilliosis marneffei at a low titre.
- A specific PCR assay is under evaluation and might be useful as an alternative test for rapid diagnosis of *P. marneffei* infection.

Invasive aspergillosis (IA)

Exposure to *Aspergillus* is universal, but aspergillosis is uncommon unless the host immune defense is compromised. The aspergilli producing infection are many but more than 95% of disease is caused by *A. fumigatus* and *A. flavus*; the latter is especially more common in the tropics. The disease usually occurs when phagocytic host defenses by granulocytes and macrophages are quantitatively or functionally suppressed. Despite the severe immunosuppression that results from advanced HIV infection, there are relatively few cases of aspergillosis in patients with HIV disease. The overall incidence varies from 1.1 to 3.5 episodes per 1,000 person-years. The incidence of aspergillosis in AIDS patients is significantly high in the following settings: age = 35 years old, men who have sex with men, white blood count <2,500 cells/ μ l, CD₄ count <100/ μ l, prior history of an OI, and prescribed medications associated with neutropenia.

In patients with AIDS, aspergillosis is usually restricted to the lungs with a variety of distinct manifestations, including thick-walled cavitary disease of the upper lobes, diffuse unilateral or bilateral infiltrates, ulcerative tracheobronchial disease, and obstructive bronchitis. The diagnosis is made often upon postmortem and relies on the histologic or microbiologic identification of the fungus in infected tissue.

Clinical considerations

- Pulmonary – most common site of involvement.
 - (1) Invasive pulmonary aspergillosis – fever, dyspnoea, cough, chest pain, hemoptysis, thick-walled cavities on chest X-ray, mainly in the upper lobes, pulmonary infiltrates – bilateral

diffuse or nodular; pseudo-membranous tracheo-bronchitis, extensive bronchial hemorrhage.

- (2) Noninvasive, obstructing bronchial aspergillosis – fungal plugs block the airways, leading to breathlessness, cough and chest pain. Chest X-ray shows hazy infiltrates, representing segmental or lobar atelectasis.
- Extrapulmonary aspergillosis – CNS aspergillosis is very uncommon in AIDS patients and may present as focal abscesses or hemorrhagic or mycotic aneurysms. In rare cases it may present as a focal neurologic deficit or intracerebral space-occupying lesion. The lesion usually extends from the sinuses; in fact it is merely an extension of the mycotic disease into the contiguous cranial cavity.
 - Very rarely, unusual manifestations in the AIDS patients include endocarditis and myocarditis, esophagitis, lymphadenitis, musculoskeletal and skin abscess, liver and spleen involvement, and renal and pancreatic abscesses.



Aspergillus

Diagnosis

For diagnosis of IA in patients with immunosuppression, the European Organization for Research and Treatment of Cancer and Mycoses Study Group have formulated consensus criteria considering host factors, clinical/radiological criteria and microbiological criteria. The disease can be considered definite, probable or possible aspergillosis. Definite aspergillosis is diagnosed when presence of fungi in tissue is documented; and probable aspergillosis when at least one host factor, one clinical/radiological feature and one microbiological criteria are fulfilled. In the absence of mycological criteria, the patient is labeled as having possible aspergillosis.

Specimens: Biopsy, fine needle aspiration cytology (FNAC), BAL, sputum, sinus mucosa.

- Because aspergilli are ubiquitous, growth on culture alone may not represent actual disease. Much controversy surrounds the specimens useful for diagnosis of IA in AIDS patients. A positive sputum or nasal secretion in an AIDS patient with consistent respiratory symptoms should merely invite suspicion. Probable diagnosis is established when BAL sample is positive for aspergilli, but definitive diagnosis is only established on observing acute angled, septate, 3-6 μm wide true hyphae in tissue samples followed by the growth of aspergilli in culture. Microscopy alone cannot distinguish between *Aspergillus* and other hyalohyphomycetes, most notably *Fusarium* and *Pseudallescheria* species.
- The fungus is rarely cultured from blood, CSF, bone marrow or other organs (brain, kidney and liver). In such a situation, the diagnosis is entertained only if the patient has a clinical history and presentation compatible with IA or if the fungus is isolated from another site.
- Aspergilli are identified on the basis of colony morphology (on Malt Extract Agar or Czapek Dox Agar at 37°C and 25°C) and microscopic examination (foot cells and vesicle are characteristic of the genus; colony morphology, size, colour on both media at two temperatures; and the microscopic arrangement of conidia; presence of sexual fruiting body; hülle cells, etc.).
- Monoclonal and polyclonal antibody staining of tissue sections has been used as an adjunct, but there are problems of poor specificity with these techniques.
- Enzyme-linked immunosorbent assay (ELISA) to detect galactomannan polysaccharides in serum of IA patients is a useful procedure for diagnosis of aspergillosis. Cross-reaction and lack of consistent results are major drawbacks of this assay. However the EIA technique is far better than the previous latex agglutination (LA) test available.
- β -1-3 Glucan Assay (G-test, Glucatell, Fungitell): This test helps in the diagnosis of invasive fungal infection other than

zygomycosis. However, it cannot identify the etiological agent of the disease. The test also requires further validation.

- PCR from BAL and blood samples is being standardized and validated in reference laboratories for the early detection of IA.
- Chest X-ray and CT scan are important adjuncts to diagnosis.

Zygomycosis

Though the disease is rare in AIDS patients, there has been an increase in the number of cases of zygomycosis in recent years, especially in tropical countries. Advanced AIDS with neutropenia is now recognized as a risk factor for acquiring zygomycosis, a disease caused by Zygomycetes belonging to the order Mucorales. *Rhizopus oryzae*, *Rhizopus rhizopodiformis*, *Rhizomucor pusillus*, *Absidia corymbifera*, *Apophysomyces elegans*, *Mucor circinelloides*, *Cokeromyces recurvatus* and *Saksenaea vasiformis* are the commonly recognized human pathogens causing this fatal disease. Fungi invade vascular tissues and cause extensive necrosis in these cases, and disseminated disease is common in the immunosuppressed. These fungi are characterized by wide (5-20 µm diameter), aseptate or sparsely septate, coenocytic hyphae with rightangled branching along with a predominantly necrotic and acute neutrophilic tissue reaction. In culture, these fungi show unrestricted cottony mycelia mostly white to grey and black in colour.

Clinical considerations

Zygomycosis may affect any or more than one organ complex and the disease is categorized as:

- (1) Rhino-orbito-cerebral (most common and carries a poor prognosis) – nasal congestion, dark-blood-tinged rhinorrhea, epistaxis, sinus tenderness, retro-orbital headache, fever, malaise, facial swelling, blurred vision, lacrimation, chemosis, proptosis, loss of vision. Examination of nasal cavities may reveal a black eschar on septum or turbinates.
- (2) Cutaneous – acute inflammation, tissue swelling, pus formation, progresses to necrotizing-fascitis-like lesion.

- (3) Pulmonary – hemoptysis, fever and cough – fatal and frequently diagnosed post mortem.
- (4) Gastrointestinal – necrotizing lesions.
- (5) Renal – though isolated renal disease is yet to be seen in patients with AIDS, the disease is prevalent in South-East Asia.
- (6) Cerebral – as a part of rhino-cerebral disease, or disseminated disease or isolated cerebral zygomycosis. Isolated disease presents as space-occupying lesion, encephalopathy or strokelike symptoms. Unlike in the west, where isolated disease mainly occurs among injecting drug users, in South-East Asia it occurs mainly in immunocompetent individuals or diabetics.
- (7) Disseminated – where more than one organ system is involved.

Diagnosis

Specimen – Biopsy, scrapings, aspirates

- Direct examination in KOH mount of sample from tissue, and histopathological examination of the same samples are the gold standard for diagnosis.
- Culture in SDA at 37°C and 25°C is sufficient to isolate the fungus. While processing, the tissue should not be crushed or ground, but rather cut into small pieces and inoculated. (The viability of the fungi is lost by crushing it.)
- Most fungi can be identified on routine lactophenol cotton blue (LCB) mounts; however, water agar may be required to induce sporulation, especially in *Apophysomyces elegans* and *Saksenaia vasiformis*.



Aseptate hyphae

Tissue demonstration of aseptate hyphae by a variety of staining techniques (H&E, GMS, KOH mount, PAS) is considered adequate for the diagnosis of zygomycosis. Culture is a useful adjunct to diagnosis. However, being ubiquitous fungi, growth in culture only does not conclusively prove the disease.

Radiographic findings of infection are often suggestive and also help in delineating the extent of disease. The agents are angioinvasive and often produce infarction in tissues. There are as yet no validated immunological or PCR-related diagnostic techniques available for zygomycosis.

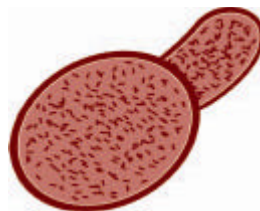
Blastomycosis

Blastomycosis, caused by a dimorphic fungus, *Blastomyces dermatitidis*, is a systemic fungal infection endemic in North America, but cases have been reported worldwide in Africa and the Middle East as well as South-East Asia. Frequently associated with point-source exposure, it occurs more commonly in men than in women, which may reflect a larger number of male occupational exposures. However, in more recent reports, occupational exposure accounts for a smaller proportion of cases, presumably as recreational exposures increase. Immunosuppressed patients typically develop infection following exposure to the organism, but reactivation may also occur.

Blastomycosis is uncommon among people infected with HIV and is not recognized as an AIDS-defining illness. In the setting of AIDS or other marked immune suppression, the disease is usually more severe with multiple-system involvement, including the CNS, and progresses rapidly to a fatal course.

Clinical considerations

- Acute disease typically presents as flu-like illness characterized by fever, malaise, fatigue, weight loss, and pulmonary involvement.



Yeast stage of *Blastomyces*

- Rarely, pneumonia with fever, chills, purulent sputum and hemoptysis may occur. ARDS may occur very rarely.
- Individuals with underlying lung disease may develop chronic pneumonia with symptoms lasting 2-6 months: weight loss, night sweats, fever, chest pain, and productive cough mimicking tuberculosis.
- Cutaneous blastomycosis is very common – lesions vary from nodules to verrucous lesions and may become ulcerative, mimicking squamous cell cancer and keratoacanthoma.
- Prostatitis, female genital tract infections, osteolytic lesions involving vertebrae, skull, ribs and long bone may also be seen.
- Disseminated disease has been found in the brain, skeletal system, prostate, myocardium, pericardium, sinuses, pituitary, and adrenal glands and also invades the reticuloendothelial system. Epidural or cranial abscesses as well as meningitis occur in up to 40% of patients with AIDS and chronic blastomycosis.

Diagnosis

Diagnosis depends on direct examination of tissue or the isolation of *Blastomyces dermatitidis* on culture.

Specimens - Sputum, BAL, transbronchial biopsy, pus, aspirates and biopsy from skin lesions, urine for antigen detection.

- Sputum as a specimen has diagnostic sensitivity of over 80% while samples obtained via bronchoscopy have > 90% positivity rates. Samples from cutaneous lesions yield the organism in most cases.
- Conversion from mould to yeast form and exoantigen testing are essential for confirming the identity as *B. dermatitidis*.
- Microscopically, the yeasts appear as round, budding, thick-walled cells (5-15 µm diameter) with a daughter cell forming a single bud that has a broad base and that may be found extracellular or within macrophages. The yeast form grows as a brown, wrinkled, folded colony at 37°C. In the mould form, *B. dermatitidis* has round to pyriform, 4 to 5 µm conidia attached directly to the hyphae or on short stalks.

- The yeast phase is characteristic, with yeast cells exhibiting unipolar, broad-based budding. The budding yeast cells, in both tissue and in culture, are described as “dumbbell-shaped”. Initially the colony appears yeast-like at room temperature and then develops hyphal projections, eventually becoming a fluffy white mould. The colonies are moderately slow growing, requiring up to three weeks’ incubation.
- Antigen detection in urine has been recently developed and is commercially available, being positive in 70%-80% of cases of disseminated disease and 100% of pulmonary cases. Crossreactivity with *Histoplasma capsulatum* is a problem. Serum antigen detection is positive in only 50% of cases.
- Antibody detection in AIDS cases is of limited utility because of the accompanying immunosuppression.
- Nucleic acid detection kits, both amplification and non-amplification based have been developed, but the tests still await validation.

Chapter 3: Overview of diagnostic mycology laboratory

Laboratory procedures

Laboratory procedures in diagnostic mycology are directed mainly towards:

- (1) Direct demonstration of the pathogenic fungi in clinical specimens (microscopy)
- (2) Successful isolation of pathogenic fungi (culture)
- (3) Supportive evidence of specific fungal infection (antigen, antibody, cell wall markers such as β 1-3 Glucan and metabolite detection). However, PCR for detection of fungal DNA is yet to be validated for routine diagnosis of any fungal infection.
- (4) Prediction of possible therapeutic outcome (antifungal susceptibility)
- (5) Tracing the source of infection (epidemiology)

Accurate diagnosis relies heavily on a combination of microbiological, histopathological and serological evidence. Since in recent years newer methods have been continuously added, introduction of sophisticated instruments in day-to-day working of the laboratory invariably increases the cost of laboratory investigations. Therefore, at the primary level, the mycologist has to fall back upon simple established standard procedures. No single procedure may help in definitive diagnosis, since each has its own limitations. All conventional procedures should therefore be incorporated in a standard mycological laboratory as far as practicable.

Prerequisites for laboratory diagnosis

For a proper diagnosis it is essential to have brief details of:

- (a) clinical history
- (b) history of any treatment with antimicrobials, cytotoxic or immunosuppressive drugs

- (c) the patient's residence (*Penicilliosis marneffeii* is endemic in South-East Asia, but only in Manipur and Nagaland states in India) and occupation (agricultural labourers engaged in soil-disturbing activities are predisposed to histoplasmosis and coccidioidomycosis, gardeners for sporotrichosis, etc.)
- (d) any history of travel or residence abroad (coccidioidomycosis in south-west USA, i.e. Arizona and California)
- (e) history of activities which result in exposure to soil, dust and organic debris
- (f) the source of the specimen
- (g) how and when the specimen was collected and transported

It is necessary that adequate amounts of appropriate and properly collected specimens are received in the laboratory. The procedures of mycological investigation that can be undertaken at the primary level are simple, and no elaborate equipment is required. For advanced techniques, specimens should be referred to better-equipped reference laboratories designated for this purpose.

Safety and training

With regard to protective measures against blood exposure, the laboratory safety officer should ensure that all staff members, particularly new staff, receive adequate instructions. These instructions should ensure that the safety aspects of new practices and the equipment used are understood and that standard practices are being followed.

A laboratory safety manual should be maintained and updated at least annually. Supervisory staff must ensure that their staff adhere to the documented safe working practices. Staff should formally report needle stick injuries or other episodes of exposure to blood or body fluids.

Safety considerations

Specimen collection	Universal precautions should be followed
Specimen transport/storage	Sealed plastic bag (preferably double-layered) with proper legible labelling
Specimen processing	Containment level II or III lab
Specimen disposal	All infected material should be treated as per biomedical waste management guidelines

Specimen collection

Optimum time of specimen collection	<ul style="list-style-type: none"> • Ideally as close to the onset of symptoms as feasible • Before initiation of antifungal therapy, where possible • Morning sample for inpatients
Correct specimen type	<ul style="list-style-type: none"> • Appropriate specimen according to the site of lesion, in adequate amount (as mentioned below), collected with sterile implements and precautions
Recommended quantity*	<ul style="list-style-type: none"> • CSF > 2 ml • Sterile body fluids (pleural, pericardial, synovial, ascitic fluid) > 10 ml • Lower respiratory (BAL, bronchoscopic washings/aspirate) > 1 ml (ensure it contains lower respiratory tract specimen) • Upper respiratory – oral swab/saline wash • Nasal sinuses – surgical removal of sinus contents • Urine (midstream) 10-20 ml (for catheter urine, clamp hub of Foley's catheter distally, clean hub sequentially with 70% alcohol, iodine and 70% alcohol, then aspirate collected urine from the hub with sterile needle and syringe) • Sputum 2-5 ml <ul style="list-style-type: none"> – Coughed-out sputum (not saliva) in a wide-mouthed sterile container; Q score is calculated from a gram stained smear and only representative samples are accepted – In case of dry cough, induced sputum in sterile container • Stool 1-5 g (rarely used, only to know colonization by Candida) • Vaginal Swab • Prostatic Fluid – Bladder emptied followed by prostatic massage • Blood 20 ml-10 ml in each bottle; for pediatric patients 4-10 ml divided equally between two bottles <ul style="list-style-type: none"> – Candidemia is known to be intermittent, so it is essential to obtain three samples to rule out yeast sepsis – Inoculated directly to biphasic blood culture bottle maintaining a ratio of 1:10 of blood to broth – Serum - additional 2-5 ml of blood to be put in clean, dry vials, preferably leak-proof screw-capped for serum separation • Subcutaneous sites <ul style="list-style-type: none"> – Abscess – Aspirate with sterile needle and syringe, if needed sample base of lesion and abscess wall (scrape, punch biopsy) – Open wound – Aspirate or swab deeply, especially base and margins – Tissue biopsy specimen – surgical collection, punch biopsies may be used for skin lesions • Repeated sampling from the same site (for authentic diagnosis of OI repeated demonstration/isolation of same organism from same site is essential)

* The yield increases with larger quantities of specimen

Specimen transport and storage

Time between specimen collection and transport	<ul style="list-style-type: none">• Specimens should be transported to the specific laboratory as soon as possible• Maximum time allowed for transport is 24 hours at room temperature from sterile site; specimen from non-sterile site may be transported at 4°C, if time for transport is > 1 hour
Storage	<ul style="list-style-type: none">• Specimen should be processed in the laboratory as soon as possible• Delay of more than four hours in processing of unrefrigerated specimens is undesirable. Where there is a delay in processing, specimens should be refrigerated except CSF and specimens for isolation of <i>Cryptococcus</i>• As a general principle, sterile samples should never be refrigerated, while samples expected to contain commensal should be refrigerated if there is delay

General techniques used in medical mycology

The techniques used in clinical mycology are in general similar to those used in clinical bacteriology. However, certain differences in the techniques should be noted.

Basic equipment required

- (1) A straight nichrome wire with a long handle – used for stabbing inoculation.
- (2) A bent (right angle) nichrome wire with long handle – used for handling the mycelial types of fungal cultures.
- (3) Pair of short stiff teasing needles – helpful in pulling apart dense masses of mycelium on the slide for better microscopic examination.
- (4) Scalpel with blades.

- (5) A pair of scissors, for cutting biopsy tissues into small pieces.
- (6) Pair of forceps.

Test tubes should be used in preference to petri dishes for primary culture of organism from clinical specimen, or transferring fungal cultures. Test tubes afford the following advantages:

- (a) easy handling
- (b) easy storage
- (c) less frequent breakage
- (d) greater protection to worker
- (e) less drying of medium

Cotton plugs in the test tubes for cultures are preferred over screw caps as they:

- (1) allow the surface of the slants to remain dry;
- (2) help better development of aerial mycelium and sporulation due to proper aerobic condition;
- (3) help in better pigment production;

Test tubes of fairly large diameter (18-25 x 150 mm) are preferable to narrow tubes. These allow a thick butt of agar, which will withstand drying during the several weeks of incubation often necessary for the growth of fungal cultures. At all times, for the protection of personnel and for securing and maintaining pure cultures, precise sterile techniques are to be employed.

It is preferable that all culture handling, including preparation of wet mounts, smears and slide cultures, is done in a separate inoculation chamber or hood fitted with ultraviolet light (to be switched on for 20 minutes between work). Microscopic observation should be done in an area well separated from the inoculation chamber.

Specimen processing

Proper documentation upon specimen receipt	<ul style="list-style-type: none"> Maintenance of laboratory records with lab number
Initial processing	<ul style="list-style-type: none"> Depends upon the specimen and suspected etiologic organism Blood culture - two bottles incubated at 37°C and 25°C (one each) CSF and other body fluids should be centrifuged in a separate sterile test tube or centrifuge tube at 2000-2500 rpm for 10 minutes, and supernatant poured back to the container of collection and stored for antigen detection. Pellet is used for smear and wet mount and for subsequent culture. Tissue – Mincing, grinding, or use of stomacher (do not grind if zygomycosis is suspected) Exudates, pus, drainage – washing, centrifugation and crushing of granules, if any Sputum: <ul style="list-style-type: none"> Sterile glass beads are added and vortexed briefly; for detection of <i>Actinomyces</i> and <i>Nocardia</i>, samples are plated onto Blood Agar and Modified Lowenstein Jensen Media at this point Equal volume of freshly prepared sodium citrate (2.94%) and 0.5 g% N-Acetyl L- Cysteine (or Sputolysin) added to specimen and vortexed again for 10-30 seconds depending upon the consistency of the specimen Dilute mixture in PBS by adding double the volume and centrifuge at 1 000 g for 15 minutes Use the sediment to prepare smears for direct microscopy and inoculate culture media
Direct microscopy	<p>Procedure employed depends upon specimen</p> <p><i>Wet mount:</i></p> <ul style="list-style-type: none"> KOH preparation India ink/Nigrosin wet mounts for screening <i>Cryptococcus</i> LCB wherever applicable <p><i>Stain/smears</i></p> <ul style="list-style-type: none"> Gram staining (for yeasts) Giemsa/Wright staining (for histoplasma, blastomyces and <i>Penicillium marneffe</i>); however PAS/GMS staining preferable Mucicarmine, Alcian Blue for <i>Cryptococcus</i> <p><i>Additional microscopic procedures</i></p> <ul style="list-style-type: none"> Direct fluorescence (Calcofluor White) where applicable Direct and indirect immunofluorescence where applicable
Histopathology	<ul style="list-style-type: none"> H&E stain PAS stain GMS stain Mucicarmine stain/Alcian Blue
Culture	Given in a separate chapter
Immunodiagnosis	Supernatant from centrifuged body fluids/clotted blood to be used for antigen detection or rarely for antibody detection, wherever applicable

Levels of laboratory for diagnosis and reporting

Level	Specimen	Process	Reporting of positives (stating if appropriate further report will be issued)	Comments
Minimal Level 1: Direct microscopy	CSF, sputum, BAL, pus, mucus membrane, tissue biopsy, serum, scrapings, peripheral blood	KOH mount Tube KOH for extended clearing of tissue samples	Same-day reporting presence of fungal filaments with characteristic branching (septate or aseptate, acute- angled or wide-angled branching and thin or wide hyphae) and yeasts	Clearing of specimen to make fungi more readily visible. Requires experience to distinguish artifacts
		India Ink/Nigrosin wet mount for <i>Cryptococcus</i>	Same-day reporting: Positive for encapsulated budding yeast compatible with <i>Cryptococcus</i>	False negative results may occur. Take care to distinguish artifacts (e.g. neutrophils).
		Gram staining: gram positive yeast cells with hyphae and pseudohyphae	Same-day reporting: Yeast cells seen, suggestive of fungal infection (except samples from respiratory tract contaminated with upper respiratory tract flora)	Mycelial fungi may not stain well, show only stippling, then very difficult to distinguish septate and aseptate
		Giemsa/Wright's stain: Intracellular yeast with occasional budding (narrow-based)	Same-day reporting: Intracellular yeast cells seen suggestive of <i>Histoplasma capsulatum</i>	<i>Candida glabrata</i> and <i>Penicillium marneffei</i> may be confused with histoplasma
		Intracellular yeast, oval or elongated with transverse septae	Same-day reporting: Intracellular yeast cells seen suggestive of <i>P. marneffei</i>	Take care to distinguish from histoplasma
		GMS/Toluidine Blue and Rapid Giemsa: characteristic cysts and Trophozoites of <i>P. jiroveci</i>	Same day reporting: Positive for <i>P. jiroveci</i>	Giemsa does not stain cysts of pneumocystis
Intermediate Level 2: (Level 1 + culture and serology for the common fungal infections)	Serum (if serology is applicable), CSF, urine, BAL	Antigen detection for presence of <i>C. neoformans</i> titre ≥ 8 confirmatory (adhere to kit manufacturer's instructions/interpretations)	Same-day reporting: Sample positive for cryptococcal antigen (with/without titre)	If titre is below 8, repeat test after 1 week
		Galactomannan assay for antigen detection of aspergillus	Same-day reporting: Sample positive for galactomannan antigen (mention cut-off index criterion 0.5 or 1.5)	Repeat samples may be required
		β -D Glucan Assay	Same-day reporting: Sample positive for Beta-D-Glucan	Not positive for zygomycosis; cannot distinguish between fungi

Level	Specimen	Process	Reporting of positives (stating if appropriate further report will be issued)	Comments
	Direct Microscopy	Immunofluorescent Staining for <i>P. jiroveci</i>	Same-day reporting: Positive for <i>P. jiroveci</i>	
	Culture: BAL, sputum, bronchial brushings, FNAC, rans-bronchial and open lung biopsy	Variety differentiation/serotyping of <i>C. neoformans</i> Animal pathogenicity (if needed) Histopathology	Communicate the relevant report to the doctor concerned as soon as possible	
Reference Level 3 : (Level 1 + Level 2)	1. All of above 2. Isolates for confirmation 3. Strain typing 4. PCR for diagnosis of fungal infections 5. Antifungal susceptibility testing	Antigen detection for presence of <i>H. capsulatum</i> *	Same-day reporting: Sample positive for <i>Histoplasma</i> antigen	
		Antigen detection for presence of <i>Blastomyces dermatitidis</i>	Same-day reporting: Sample positive for <i>Blastomyces</i> antigen	
		Antibody detection for presence of <i>H. capsulatum</i> . immunodiffusion test (ID) complement fixation test (CFT)	ID: Positive for H/M precipitin bands; result in three days. CFT: Same day result, CFT titre > 32 significant	Antibody detection rarely possible in AIDS patients
		Antigen detection for presence of <i>Penicillium marneffe</i>	Same-day reporting: Sample positive for <i>P. marneffe</i> antigen	Not commercially available

* At present available only in USA

Reporting should be done immediately upon a positive finding, and particularly in the case of demonstration of organism in normally sterile body fluids, the clinician concerned should be informed immediately by phone, if possible.

Chapter 4: Microscopy

Direct microscopic examination of the clinical material provides valuable information about the fungal aetiology. Various methods and staining techniques are available and in wide use. Some of the commonly used techniques are:

KOH mount preparation

- (1) Potassium hydroxide is a strong alkali used as a clearing agent to observe fungi in the specimen in a wet mount preparation.
- (2) 10%-20% KOH is usually used depending on the specimen; occasionally 40% is also used when not cleared by 10%-20% KOH.
- (3) Used for specimen such as sputum, pus, urine sediment, homogenate from biopsy tissue, nail, hair, etc., to clear cell debris.

Preparation of the mount

- (1) Take a clean grease-free glass slide.
- (2) Place a large drop of KOH solution with a Pasteur pipette.
- (3) Transfer small quantity of the specimen with a loop or the tip of a scalpel into the KOH drop.
- (4) Put a clean cover slip over the drop gently so that no air bubble is trapped.
- (5) Place the slide in a moist chamber, and keep at room temperature.
- (6) Tissue usually takes 20-30 minutes; sometimes overnight contact with KOH is useful for getting a positive result.
- (7) Clearing can be hastened by gentle heating of the slide, but it is best avoided.

Observation

- (1) Examine the clear specimen under low power (10X or 20X objective). Scan the entire cover slip from end to end in a zigzag fashion.
- (2) If any fungal elements are suspected, examine under high power (40X objective).
- (3) Reduce the light coming into the condenser while examining at high power.
- (4) Look for budding yeast cells, branching hyphae, type of branching, the colour, septation and thickness of hyphae.

Modification

For more distinction, stains like methylene blue or Parker blue-black fountain ink may be used along with KOH. This will impart a colored background and fungal elements, if present, will show as prominent refractile objects.

Advantages

Simple, economical and rapid.

Disadvantages

- Pus and sputum may contain artifacts, which may superficially resemble hyphal and budding forms of fungi. These artifacts may be produced by cotton or wool fibres, starch grains (in pleuritis) or cholesterol crystals.
- It gives an idea about the presence of hyphal element, but cannot distinguish different fungi.
- Preparation cannot be kept for too long; but drying can be prevented/prolonged by keeping the slides in a moist chamber (covered Petri dish with a wet filter paper on which a triangular glass rod is placed).

Precautions

- (1) The drop of KOH should not be so large that the cover slip floats.
- (2) If kept outside a moist chamber, the KOH dries and crystals form that restrict the visibility of the fungus.
- (3) After clearing, pressure is to be gently applied on the top of the cover slip with a fold of filter paper or the handle of a teasing needle. This ensures even spreading of the material onto the slide.
- (4) KOH should be kept in a closed container in small aliquots ready to use on the workbench.

Quality assurance

Fungal spores or hyphae may contaminate the KOH solution kept in the laboratory and may give false positive results. A negative control should therefore be put up every day.

India ink or nigrosin preparation for *C. neoformans*

- (1) The preparation should be made in the centre of a clean, grease-free glass slide.
- (2) Put 1 drop of India ink or Nigrosin on the centre of the slide. Too much stain makes the background too dark. (Upon examination, if the staining appears too dark, a small amount of water may be applied on the edge of the cover slip and the cover slip gently tapped: this will dilute the stain to some extent.)
- (3) Put 1 loopful of the specimen or preferably centrifuge sediment from the fluid specimen to be tested (e.g. CSF, spinal fluid, urine, and other body fluids) close to the drop of the stain.
- (4) Mix the two well with the loop, or preferably a sterile needle. The loop should be cooled before use, otherwise the stain tends to precipitate.

- (5) Hold the cover slip vertically such that one edge just touches the fluid on the slide. The fluid will spread along the edge by surface tension.
- (6) Keeping that edge in contact with the fluid surface, drop the cover slip gently on the fluid, so that no air bubble is trapped inside. If there are air bubbles, the surface of the cover slip may be gently tapped by the needlepoint, so as to move the bubbles towards the edge. But this should be avoided as far as possible.
- (7) Examine slide immediately under the microscope. Since the stain tends to dry fast in air, if immediate examination is not feasible, the slide should be kept in a moist chamber.
- (8) Scan the entire cover slip from end to end in a zigzag fashion. Encapsulated yeast cells (*Cryptococcus neoformans*) are seen under low power as luminous dots in an otherwise dark background. Under high power, the cells can also be seen, containing refractile bodies, and surrounded by the unstained thickness of the capsule. Characteristic pinched-off budding, when observed, is confirmatory for diagnosis.

Please note

- (a) Besides the classical budding-yeast form, various unusual forms can also be seen, including elongated forms that look like pseudohyphae; this is mainly due to a very high multiplication rate of the organism in HIV/AIDS patients.
- (b) In the very late stage in progressive AIDS, it may be difficult to differentiate the capsules of individual cells; the organism may remain enmeshed in a matting of the capsular material.
- (c) Micro- or non-capsulated strains of the organism are also reported on rare occasions. In such cases, gram stain of the sample is helpful in identification.
- (d) The edges of the cover slip should be specially examined. When placing the cover slip, the yeast cells tend to move towards the periphery along with the fluid. For this reason the common practice of draining the extra fluid from the sides should be avoided.

- (e) If the protein content of the CSF is too high, India ink sometimes may form floccules, which make it difficult to demonstrate the capsule.
- (f) In the case of Nigrosin stain, the preparation dries up quite quickly, which is a problem in hot climatic conditions, so quick examination is essential.
- (g) In case of HIV-positive patients, > 90% of cases may be positive by the India ink/Nigrosin test, whereas in non-HIV cases, < 60 % positivity is seen.

Precautions

- (1) The India ink or Nigrosin should be shaken well before every wet mount preparation.
- (2) The stain should be regularly checked for contamination by examining just the stain under a microscope.
- (3) False positive readings may occur with air bubbles or monocytes or neutrophils. Air bubbles, under high power, will be hollow and will not show the typical cell with characteristic nuclei. Monocytes and neutrophils have a crenated margin (and not the entire margin seen in cryptococcal cell) and will not show the characteristic refractive cell inclusions, and the luminous halo around the cell is not well demarcated.

Mayer's mucicarmine stain

This technique is useful in identifying *Cryptococcus neoformans* and *Rhinosporidium seeberi* from other fungi of similar size and shape when found in tissue samples.

Procedure:

- (1) Pass sections serially through xylene, absolute alcohol and 95% alcohol, and then wash with distilled water.
- (2) Stain with working solution of Weigert's Hematoxylin for 7 minutes.
- (3) Wash under tap water for 5-10 minutes.

- (4) Place in diluted mucicarmine for 30-60 minutes.
- (5) Rinse quickly in distilled water.
- (6) Dehydrate in 95% alcohol and absolute alcohol (2 changes of each)
- (7) Clear with 2 changes of xylene.
- (8) Mount in DPX and view under the microscope.

Observation

The mucopolysaccharides of the *Cryptococcus neoformans* capsule stain deep rose to red, nuclei are black while the other tissue elements stain yellow.

Periodic acid schiff (PAS) stain for tissue sections

Precautions

A slide of either skin or nail scrapings containing a dermatophyte should be stained along with slides of the specimen as positive control. Periodic acid may deteriorate and no longer oxidize the hydroxyl groups. This should be suspected when fungal elements on the control slide appear unstained. The periodic acid solution and the stock of periodic acid (a white powder) should be kept in dark bottles. The sodium metabisulphite solution is unstable. Deterioration of this reagent is suspected when the control slides show no evidence of having been subjected to a bleaching process, e.g. the background stains as intensely as the do the fungal elements.

Procedure

- (1) Fix slide by flaming.
- (2) Immerse in ethanol for 1 minute.
- (3) Place in 5% periodic acid for 5 minutes.
- (4) Wash gently in running tap water for 2 minutes.
- (5) Place in basic fuchsin for 2 minutes.
- (6) Wash gently in running tap water for 2 minutes.

- (7) Place in sodium metabisulphite (0.5%) for 3-5 minutes.
- (8) Wash gently in running tap water for 2 minutes.
- (9) Counter stain with dilute aqueous light green (0.2%) for 2 minutes.
- (10) Dehydrate in 70%, 80%, 95%, 100% ethanol and xylene, each for 2 minutes.
- (11) Mount the slide.

Observation

Fungi stain a bright pink-magenta or purple against a green background when light green is used as a counter stain.

Lactophenol cotton blue (LCB) mount preparation

This is used for staining and microscopic identification of fungi.

Procedure

- (1) Take a clean grease-free glass slide.
- (2) Put a large drop of LCB with a Pasteur pipette.
- (3) Transfer a small quantity of the culture to the drop.
- (4) Tease the culture (in case of a mould) well with teasing needles so as to get a uniform spread.
- (5) Put on a cover slip gently to avoid entrapment of air bubbles.
- (6) Examine under low- (10X) and high-power (40X) objective.
- (7) Observe the morphological features carefully.

Gram staining

- (1) Make a very thin smear of the material on a clean, grease-free glass slide.
- (2) Dry in air.
- (3) Fix the smear by flaming the slide.

- (4) Add gentian violet to cover the smear and leave undisturbed for 1 minute.
- (5) Drain off the gentian violet by tilting the slide and rinse in flowing tap water, taking care that the water flow does not fall directly on the smear.
- (6) Add Gram's iodine solution to cover the smear and leave for 1 minute.
- (7) Rinse with water in the same way as above.
- (8) Decolorise with acetone for about 1-3 seconds.
- (9) Quickly rinse again with water.
- (10) Counter stain with safranin for 30 seconds, and rinse in water.
- (11) Dry in air, and observe under oil immersion (100X).

Observation

Observe the Gram's reaction (positive or negative), size, shape and arrangement of elements.

Giemsa staining

- (1) Homogenize tissue section and make a thin uniform smear.
- (2) Flood the slide with methyl alcohol and leave for 3-5 minutes for fixation.
- (3) Add prepared Giemsa stain for 45 minutes.
- (4) Wash slide thoroughly with running tap water.
- (5) Blot dry with absorbent paper.
- (6) Observe under oil immersion lens.

Observation

Look for intracellular budding yeasts.

Wright stain

- (1) Make a uniform smear (peripheral blood, bone marrow *etc.*) on a clean, grease-free glass slide.
- (2) Cover the slide with freshly filtered Wright stain (it is important to cover the entire slide) and leave for 1-3 minutes.
- (3) Without removing the stain, pour on buffer solution (pH 6.4); surface tension will not allow the buffer to run off. Some workers prefer using tap water instead of the buffer solution.
- (4) Blow gently over the surface of the fluid to mix the buffer and the stain. Upon proper mixing, metallic green sheen (green scum) rises to the surface of the fluid. Leave for 3 minutes or longer (e.g. bone marrow takes longer to stain).
- (5) Wash the slide gently with flowing tap water, and wipe the bottom of the slide with a clean tissue.
- (6) Air-dry the slide and observe under the microscope.

Precautions

- (1) Since Wright stain is prepared in methanol, a separate fixation step is not required.
- (2) The timing of each step should be standardized in the laboratory for optimal colouration.
- (3) The staining of different components in a smear is dependent on the pH of the medium, which has to be maintained. Excess alkaline or acidic conditions may cause the colour to be too blue or red to be seen properly.
- (4) Unfiltered Wright stain may leave granular particles on the preparation.

Observation

Intracellular budding yeast, this is especially suitable for observation of intracellular *Histoplasma* and *P. marneffe*i in bone marrow or peripheral blood smears.

Gomori's methenamine silver stain (GMS)

GMS (Grocott's modification) delineates fungal elements sharply in black against a pale green background. It is especially useful as a histopathological tool and for the detection of *Pneumocystis jiroveci*.

Procedure

- (1) Dry the smear and then fix in absolute methanol for 5 minutes.
- (2) Wash in distilled water.
- (3) Dip slide in Coplin jar containing 4% chromic acid for 45 minutes.
- (4) Wash in distilled water.
- (5) Add 1% sodium/potassium metabisulphite for 1 to 2 minutes.
- (6) Wash in distilled water.
- (7) Dip slide in working solution of hexamine (which is preheated in a water bath to 56°C) for 1 hour (smear becomes dark brown).
- (8) Wash with distilled water, or if smear turns black wash with 0.1% FeCl₃.
- (9) Wash with 5% sodium thiosulphate for 2 minutes.
- (10) Wash with distilled water.
- (11) Wash with 1% light green solution for 1 minute.
- (12) Dry and view under oil immersion.

Observation

The control slide with fungal elements stains black; background stained green. *Pneumocystis jiroveci* cysts and fungi may appear very much alike. Look for various cyst forms, including those that show dark centres, cup-shaped crescents, and cysts with fold-like lines (resembling punched-in ping pong balls). If dark-staining organisms appear more oval, look carefully for budding forms, which may differentiate the organisms.

- (a) *P. jiroveci* cysts – 70% should have delicately staining walls, usually brown or gray. They will appear somewhat transparent with structures described as “parentheses” staining black; these curved structures are usually thick (much thicker than the cyst wall) rather than thin like a line drawing.
- (b) Fungi and actinomycetes – grey to black.
- (c) Glycogen, mucin and red blood cells – rose/dark grayish brown/gray.
- (d) Background is pale green.

Rapid giemsa stain (Diff-Quick or Giemsa Plus)

Precautions

This stain is never to be used alone. A cell wall staining method should always accompany it, because it is nearly impossible to differentiate organisms from cellular debris.

Procedure

- (1) Place 1 or 2 drops of red stain (solution 1) on specimen smear and control slide (normal blood film), hold for 10 seconds, and drain.
- (2) Add drops of blue stain (solution 2), hold for 10 seconds, drain, and rinse very briefly with deionized water.
- (3) After staining, air-dry.
- (4) Slides must be examined with oil or mounted with mounting medium.

Observation

Clumps of trophozoites in various sizes may be detected. In large clumps it may be difficult to differentiate individual organisms. Look at the organisms at the edges of the clump, and look for small, more dispersed clumps.

Immunofluorescent staining for *Pneumocystis jiroveci*

- (1) Strictly follow the manufacturer's directions on the package.
- (2) If negative control slide containing yeast cells exhibits fluorescence or specimen slide is equivocal, do not report specimen positive for *Pneumocystis jiroveci*.
- (3) Always confirm by cyst wall and organism stains in case of positive results.

Rapid methenamine silver stain

Procedure

- (1) Place plastic Coplin jar of 10% methenamine silver nitrate solution in oven at 95°C.
- (2) Fix in methanol for 5 minutes or by heating. If paraffin-fixed slide, rehydrate.
- (3) Wash in distilled water.
- (4) Dip slide in Coplin jar containing 10% chromic acid for 10 minutes.
- (5) Wash in distilled water for 5 seconds.
- (6) Add 1% sodium/potassium metabisulphite for 1 to 2 minutes.
- (7) Wash in distilled water, preferably hot.
- (8) Dip slide in 1.5% solution of methenamine silver nitrate at 95°C for 5-10 minutes. When sections become golden brown, remove control slide, wash under water and observe microscopically. When fungal elements are positive, further heating should not be done. Overheating may cause silver to precipitate. Solution is discarded after use.
- (9) Wash with hot distilled water and cool gradually to avoid cracking Coplin jar.
- (10) Wash with 1% gold chloride for 10 seconds.
- (11) Wash with distilled water.
- (12) Wash with 5% sodium thiosulphate for 2 minutes.
- (13) Light green working solution for 30 seconds.
- (14) Dry and view under oil immersion.

Toluidine Blue stain for *Pneumocystis jiroveci*

Procedure

- (1) Dry slide for 5 minutes.
- (2) Flood slide with sulphonation reagent (3:1 glacial acetic acid and sulphuric acid) for 10 minutes.

Note: Wear gloves and take care when handling this reagent.

- (3) Carefully wash off reagent and flush down sink.
- (4) Pour slide in a container and flush with running water for 5 minutes.
- (5) Flood slide with toluidine blue for 3 minutes.
- (6) Wash off and allow slide to dry.

Results

Pneumocystis jiroveci cysts of characteristic shape and size (3-5 µm diameter) are violet or purple against a bluish background.

Observations on microscopic examination

The table below provides a summary of the findings on microscopic examination of various clinical material and their diagnostic importance.

Sample	Finding	Special features	Fungus
Blood/Buffy coat (direct microscopy of blood is rarely done for detection of fungus)	Hyphae	Rare	<i>Fusarium</i> <i>Scedosporium</i> <i>Paecilomyces</i> <i>Aspergillus</i>
	Yeasts	Encapsulated	<i>Cryptococcus</i> <i>Rhodotorula</i>
		Un-encapsulated	<i>Candida</i> <i>Trichosporon</i> <i>Cryptococcus</i> (Un-encapsulated strains) <i>Blastoschizomyces</i> <i>Saccharomyces</i>

Sample	Finding		Special features	Fungus
Bone and Bone Marrow	Encapsulated yeasts			Cryptococcus
	Intracellular			<i>Histoplasma</i>
	Large, broad based buds (8-15 µm in diameter)			<i>Blastomyces</i>
Brain	Hyphae	Short	Oval cells, dark	<i>Exophiala</i> , <i>Cladosporium</i>
		Long	Aseptate	<i>Zygomycetes</i>
			Dark	<i>Pseudallescheria</i> , few neurotropic melanized fungi
			Hyaline	<i>Aspergillus</i>
	Yeasts	Encapsulated		<i>Cryptococcus</i>
		Un-encapsulated	Large, broad based buds (8-15 µm in diameter)	<i>Blastomyces</i>
			Extracellular ± Pseudohyphae	<i>Candida</i>
BAL, sputum, other respiratory specimens	Yeasts	Encapsulated		<i>Cryptococcus</i>
		Intracellular		<i>Histoplasma</i>
		Large, broad based buds (8-15 µm in diameter) "dumbbell-shaped"		Blastomyces
		Multiple buds, narrow base ("Carter's wheel" appearance)		<i>Paracoccidioides</i> (not detected in South-East Asia)
		Extra-cellular±Pseudohyphae		Candida
	Hyphae	Aseptate		<i>Zygomycetes</i>
		Septate		<i>Aspergillus</i>
				<i>Fusarium</i> <i>Geotrichum</i> <i>Paecilomyces</i> <i>Pseudallescheria</i>
CSF	Short hyphae	Rare		<i>Trichosporon</i> <i>Aspergillus</i> <i>Pseudallescheria</i>
	Yeasts	Encapsulated		<i>Cryptococcus</i> <i>Rhodotorula</i>
		Un-encapsulated		<i>Candida</i> <i>Cryptococcus</i> (Un-encapsulated strains)
		Intracellular		<i>Histoplasma</i>

Sample	Finding		Special features	Fungus
Muco-cutaneous Tissue	Pseudohyphae, yeast cells			Candida
Nasal Tissue, Paranasal Sinuses	Hyphae	Hyaline	Aseptate	Zygomycetes: Mucorales and Entomophthorales
			Septate	Aspergillus
		Melanized		Alternaria Exserohilum Curvularia Pseudallescheria Bipolaris
	Yeasts			Candida
Tissue	Spherules			Coccidioides
	Dark, short or long filaments			Exophiala Phialophora Wangiella
	Hyphae, hyaline	Septate		Aspergillus Scedosporium
		Aseptate		Zygomycetes
	Yeasts	Encapsulated		Cryptococcus
		Extra-cellular ± pseudohyphae		Candida
		Intracellular		Histoplasma
		Large, broad-based buds (8-15 µm in diameter)		Blastomyces
		elongated small yeasts ("cigar-shaped")		Sporothrix
	Melanized fungi			Phialophora Fonsecaea Cladosporium Rhinoctadiella

Preparation of stains

20% KOH – glycerol solution

- KOH – 20 g
- Glycerol – 20 ml
- Distilled water – 80 ml

Note

- (1) The relative amounts of KOH and distilled water must be adjusted according to the percentage of KOH to be used.
- (2) Addition of glycerol to KOH solution will prevent crystallization occurring in the solution, thus enhancing the shelf life of this reagent. It will also permit keeping the KOH preparation for a couple of days before it dries.

Sulphonation reagent

- Fresh reagent is made once a week.
- To 9 ml of glacial acetic acid in a bottle held in a cool water bath (10-15°C), slowly add 3 ml of concentrated sulphuric acid.
- Mix and leave in the water bath until the solution cools.

Toluidine blue

- Dissolve 0.3 g of toluidine blue O in 60 ml of distilled water.
- Add 2 ml of concentrated hydrochloric acid followed by 140 µl of absolute ethyl alcohol.

Lactophenol cotton blue (LCB)

LCB is used both as a mounting fluid and a stain. Lactic acid acts as a clearing agent and aids in preserving the fungal structures; phenol kills the organism and fixes it; glycerol prevents drying and cotton blue provides colour to the structure. It can be used alone or in conjunction with KOH.

- Lactic acid – 20 ml
- Phenol – 20 ml
- Glycerol – 40 ml
- Cotton blue (Poirier blue or Aniline blue) – 0.05 g
- Distilled water – 20 ml

Dissolve phenol in lactic acid, glycerol and distilled water, then add cotton blue and mix well.

Wright stain

Wright stain is a Romanowsky-type stain which contains methylene blue as the active ingredient. At the proper pH, the methyl groups are activated and react with charged components of the cell to produce coloration.

- Powdered Wright's stain – 9 g (0.3% w/v)
- Powdered Giemsa stain – 1 g (0.033% w/v)
- Glycerin – 90 ml (3% v/v)
- Absolute acetone-free methanol 2 910 ml (to make up the volume)

The components have to be mixed in a brown bottle and allowed to stand for 1 month before use. The stain must be stored at 4°C, otherwise the components may degrade.

Giemsa stain

- Giemsa stain, powdered (certified) 0.75 g
- Methyl alcohol, pure 65.0 ml
- Glycerol, pure 35.0 ml

Shake well in bottle with glass beads. Keep tightly closed at all times. Filter if necessary.

Methenamine silver nitrate staining reagents (rapid method)

10% chromic acid (chromium trioxide)

- Chromic acid 10.0 g
- Distilled water 100 ml

1% sodium metabisulphite

- Sodium bisulphite 1.0 g
- Distilled water 100 ml

5% silver nitrate

- Silver nitrate 5.0 g
- Distilled water 100 ml

Store in dark-coloured bottle in 4°C refrigerator; use as needed.

3% aqueous methenamine (hexamethylenetetramine)

- Methenamine 3.0 g
- Distilled water 100 ml

5% aqueous borax

- Borax (sodium borate) 5.0 g
- Distilled water 100 ml

1% aqueous gold chloride

- Gold chloride 15 grain vial
- Distilled water 100 ml

5% sodium thiosulphate (hypo) solution

- Sodium thiosulphate 5.0 g
- Distilled water 100 ml

Light green working solution

(a) 0.2% light green (stock solution)

- Light green, SF yellowish 0.2 g
- Distilled water 100 ml
- Glacial acetic acid 0.2 ml

(b) 10 ml of (a) is added to 50 ml distilled water

70% alcohol	}	use in plastic squirt bottles
95% alcohol		
Absolute alcohol		

Reagents for Mayer's mucicarmine stain

Weigert's iron hematoxylin

1. Solution A
 - Hematoxylin 1.0 g
 - Alcohol 95% 100 ml
2. Solution B
 - Ferric chloride, 29% aqueous solution 4.0 ml
 - Distilled water 95 ml
 - Hydrochloric acid, concentrated 1.0 ml
3. Working solution
 - Equal parts A and B prepared fresh.

Metanil yellow solution

- Metanil yellow 0.25 g
- Distilled water 100 ml
- Glacial acetic acid 0.25 ml

Mucicarmine stain

- Carmine 1.0 g
- Aluminum chloride, anhydrous 0.5 g
- Distilled water 2.0 ml

Mix stain in test tube. Heat in water bath for 2 minutes. Liquid becomes almost black and syrupy. Dilute with 100 ml of 50% alcohol and let stand for 24 hours. Filter. Dilute 1:4 with tap water for use. Expiration: 1 month.

Reagents for PAS stain

1. *Formal-ethanol mixture*

- | | |
|--------------------|-------|
| ➤ 40% formaldehyde | 10 ml |
| ➤ Absolute alcohol | 90 ml |

2. *Periodic acid, 5%*

- | | |
|-------------------|--------|
| ➤ Periodic acid | 5g |
| ➤ Distilled water | 100 ml |

3. *Basic fuchsin solution*

- | | |
|-------------------|-------|
| ➤ Basic fuchsin | 0.1 g |
| ➤ Ethanol | 5.0 g |
| ➤ Distilled water | 95 ml |

Mix ethanol and distilled water in a brown bottle; add basic fuchsin and mix by rotating.

4. *Sodium metabisulphite solution*

- | | |
|-------------------------|---------|
| ➤ Sodium metabisulphite | 1.0 g |
| ➤ HCl | 10.0 ml |
| ➤ Distilled water | 190 ml |

Add HCl to distilled water in a brown bottle. Then add sodium metabisulphite.

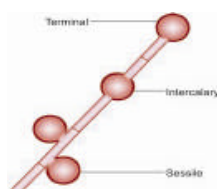
- Cool to 50°C and filter.
- Add 20ml N HCl (83 ml concentrated HCl/1000 ml distilled water). Cool to 25°C.
- Add sodium bisulphite, 1 g. Store in screw-top bottle in dark for 2 days.

- (d) Add activated charcoal, 0.5 g; shake intermittently for 1 hour.
 - (e) Filter.
 - (f) Store in dark-colored, tightly closed bottle in refrigerator (5 years); pour into a Coplin jar for use. Solution may be reused until it turns pink, at which time it must be discarded.
5. *Light green working solution (see reagents for methenamine silver nitrate stain)*
6. 70% alcohol }
7. 95% alcohol } *use in plastic squirt bottles*
8. *Absolute alcohol*
9. *Xylol (use from dropper bottle)*
10. *Mounting medium (Histoclad, Permount, or other mounting medium)*

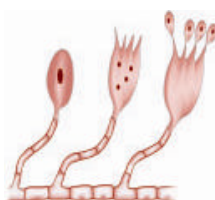
Chapter 5: Culture

Corn meal agar (Dalmau plate technique)

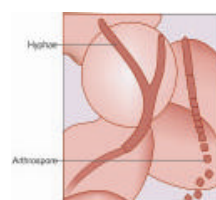
- (1) Prepare corn meal agar (CMA) containing 1% Tween 80 in a 90 mm plate. Divide the plate into 4 quadrants and label each quadrant.
- (2) Using a sterile straight wire, lightly touch a yeast colony and then make 2-3 streaks approximately 3.5-4 cm long and 1.2 cm apart.
- (3) Place a flame-sterilized and cooled 22 mm square cover glass over the streaks. This will provide partially an anaerobic environment at the margins of the cover slip.
- (4) Inoculate the plates at 25°C for 3-5 days.
- (5) Remove the lid of the petri dish and place the plate in the microscope stage and observe the edge of the cover glass using low-power objective (10X) first, and then high-power objectives (20X and 40X).
- (6) Morphological features like hyphae, pseudohyphae, blastospores, chlamydospores, basidiospores or sporangia are noted.
- (7) Tentative identification of *Candida spp.* may be made by tallying the features against the yeast species (see Table on page 29)



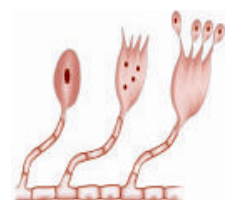
Chlamydospores



Basidiospores



Fungal hyphae in
KOH



Basidiospores

Morphology on CMA with Tween 80

Terminal chlamydoconidia	<i>C. albicans</i> , <i>C. dubliniensis</i>
Abundant pseudohyphae, pine forest arrangement, blastoconidia formed at or between septa	<i>C. tropicalis</i>
Elongated yeasts, abundant pseudohyphae (matchstick-like appearance)	<i>C. krusei</i>
Giant hyphae, blastospores at nodes	<i>C. parapsilosis</i>
Scant pseudohyphae with chains of blastoconidia	<i>C. guilliermondii</i>
Yeasts only	<i>C. glabrata</i> , <i>C. famata</i> , <i>Pichia anomala</i> , <i>P. augusta</i> , <i>Cryptococcus neoformans</i>
Short, distinctly curved pseudohyphae with occasional blastoconidia at septa	<i>C. lusitanae</i>
Arthroconidia with blastoconidia	Trichosporon spp.
Arthroconidia without blastoconidia	Geotrichum spp.

Culture media and conditions

Type of Specimen	Standard Media	Incubation			Reading of cultures	Target organism
		Temp	Atmosphere	Time		
All specimens ¹ except blood and bone marrow	Specimens are inoculated in the following media: <ul style="list-style-type: none"> • SDA with antibiotics – 2 sets • BHIA with antibiotics and cycloheximide – 1 set • Niger seed agar (NSA) where Cryptococcosis suspected - 2 sets • Selective medium⁴ with ammonium hydroxide on surface (for <i>Histoplasma</i>) 	1 SDA and BSA at 37°C	Aerobic	For at least 4 weeks before negative reported	Daily for first week, then twice weekly for the next 3-5 weeks	Any mycelial or yeast growth. Chocolate-brown colonies on NSA may indicate <i>C. neoformans</i> growth. Growth on ammonium hydroxide media - histoplasma
		1 SDA, BHIA, at 25°C NSA SM with NH ₄ OH (if inoculated)	Aerobic	Usually isolates appear in 48-96 hours; some strains may take longer time		

Type of Specimen	Standard Media	Incubation			Reading of cultures	Target organism
		Temp	Atmosphere	Time		
Blood Culture ³	Biphasic blood culture bottles containing BHIA and brain heart infusion (BHI) broth, with antibiotics – 2 bottles	1 bottle each at 37°C and 25°C	Aerobic	4-6 weeks	Daily. Subculture on SDA plates once every 3 days, till positive culture or discarded	Yeast, filamentous fungi
	Blood culture bottles as used for bacteriology	37°C	Aerobic	7 days. Keep sub-cultured plates for 48 hours.	At 6 hours, 24 hours, 48 hours and 7th day	Yeast (tiny white colonies)
Bone Marrow	Biphasic BHIA medium with 5% sheep blood and antibiotics	Vented bottle – 2 bottles 37°C and 25°C	Aerobic	4-6 weeks	Daily	Dimorphic Fungi ²

1. Discussed under each infection.
2. Dimorphic fungi (e.g. *H. capsulatum*, *P. marneffe*) – on primary isolation, mycelial form.
3. Other available methods for blood culture (Lysis centrifugation, Bactec, BacT/Alert, etc) are sensitive but costly, and require specific equipment and trained personnel.
4. Selective media for dimorphic fungi – yeast extract phosphate medium + ammonium hydroxide added to surface (for heavily contaminated samples like sputum and urine, in which *Histoplasma capsulatum* is the target organism).

Slide culture technique

Whenever it is difficult to identify moulds with tease mounts, slide cultures can be put up. Slide cultures provide a chance to make *in situ* observations of growing structures of a fungus, without causing much disturbance. Hence, they are the technique of choice for studying the developmental morphology of filamentous (sporulating) fungi. Nutritionally deficient media like CMA, potato dextrose agar, etc. are good for enhancing sporulation.

Procedure

A. Prepare the "Setup"

- In a 100 mm glass petridish, place a filter paper strip, V-shaped glass rod, a microscope slide and a cover slip.

- Autoclave the whole setup at 121°C for 15 minutes.
- Store the setups in a Petridish canister for a maximum period of 2 weeks.

B. Set up a slide culture

- Aseptically cut 1 cm square agar blocks from CMA or PDA.
- Transfer the agar block on to the slide in the setup.
- Transfer a very small amount of the mould to the 4 sides of the agar block.
- With sterile forceps, place the cover slip on the inoculated agar block.
- Add 1-1.5 ml of sterile water to the Petri dish; humid atmosphere does not allow the agar block to dry out.
- 5%-20% glycerine can be added to the sterile water to prevent condensation of moisture on the slide.
- Slide culture is ready to be taken down when mature conidia are observed; in general this takes 3-7 days. Longer incubation may be necessary if development of perfect state (sexual reproduction) is to be elucidated, or for those fungi which have slow growth.

C. "Taking down" the slide culture

- Take a small drop of mounting medium (LCB) on a microscope slide.
- With forceps, carefully remove the cover slip from the agar block. Do not push or pull the cover slip.
- Pass the cover slip through the blue portion of the flame very quickly. This will heat-fix the fungus and its spores (overheating can result in collapse of the hyphae).
- Carefully place the cover slip on the mounting medium so as to avoid trapping any air bubbles.

- Wipe off the excess of the mounting medium. Seal the edges of cover slip with nail polish.
- Second mount can also be prepared from the microscope slide of the slide culture setup by removing the agar block. Add a drop of lactophenol and a cover slip, seal with nail polish.

Preparation of culture media

Corn meal agar (CMA)

CMA is used with Tween 80 in distinguishing the different species of *Candida* and can be useful in slide cultures as it stimulates formation of chlamydospores.

- Corn meal – 40 g
 - Agar – 20 g
 - Tween – 80 10 ml
 - Distilled water – 1000 ml
- (1) Mix cornmeal well with 500 ml of water.
 - (2) Heat to 65°C for 1 hour.
 - (3) Filter through a gauze piece and filter paper till clear solution is obtained.
 - (4) Restore volume to 500 ml.
 - (5) Adjust the pH to 6.6–6.8.
 - (6) Add agar dissolved in 500 ml of water.
 - (7) Add Tween 80.
 - (8) Sterilize by autoclaving, and dispense into petri dishes or tubes.

For studying the morphology of yeasts, use 1/4 or 1/3 plate for each organism. Make 1 streak down the centre of the area (do not cut the agar) and 3 or 4 streaks across the first to dilute the inoculum. Cover with a 22 x 22 mm cover slip and incubate at room temperature for 3 days. Examine by

placing the plate without its lid on the microscope stage and using low-power (10X) and high-power (40X) objectives. The most characteristic morphology is often found near the edge of the cover slip (especially the terminal chlamydoconidia of *Candida albicans* or *Candida dubliniensis*). *Candida albicans* should always be included as a control for production of chlamydoconidia and blastoconidia.

Sabouraud dextrose agar (SDA) [Emmon's modification]

This modification differs from the original formula in that it has an approximately neutral pH and contains only 2% dextrose. The original medium (with high sugar, up to 40% and low pH of 5.4) devised by Raymond Sabouraud is more suitable for isolation and study of dermatophytes.

- Dextrose – 20 g
- Neopeptone – 10 g
- Agar – 15 g
- To be dissolved in 1 000 ml of distilled water.

The ingredients are mixed and dissolved by boiling and then autoclaved. This formulation is available in prepared or dehydrated form. The final pH is around 6.8 (near neutral). The prepared medium can be stored at 4°C.

SDA with antibiotics

To SDA while boiling and before autoclaving add cycloheximide* 500 mg dissolved in 10 ml of acetone and/or chloramphenicol 50 mg dissolved in 10 ml of 95% ethanol and gentamicin 20 mg in 10 ml of sterile double-distilled water.

* Also known as Actidione

Biphasic medium for blood culture

Agar

- BHIA (commercially available) – 52 g
- Distilled water – 1000 ml
- (1) Suspend 52 g of BHIA in 1 litre of distilled water; heat to boiling to dissolve.
- (2) Distribute 25 ml into 3-ounce bottles with screw-top caps fitted with rubber diaphragms.
- (3) Autoclave at 15 lb pressure (121°C) for 15 minutes.
- (4) Cool to room temperature, allowing agar to harden on side of bottle.

Broth (on the day after the agar is hardened)

- BHI (commercially available) – 37 g
- Distilled water – 1 litre
- (1) Suspend and dissolve 37 g of BHI infusion in 1 litre of water.
- (2) Autoclave at 15 lb pressure (121°C) for 15 minutes.
- (3) Add 30 ml to each bottle, aseptically.
- (4) Final pH: 7.4.

Canavanine glycine bromothymol blue agar (CGB)

Solution A

Glycine	10g	}	Adjust pH to 5.6 and filter sterilize
Potassium dihydrogen orthophosphate	1g		
Magnesium sulphate	1g		
L-canavanine sulphate	30mg		
Thiamine hydrochloride	1mg		
Distilled water	100ml		

Solution B

Bromothymol blue	0.4 g
Sodium hydroxide 0.01M	64ml
Distilled water	36ml

Add 2 ml of solution B to 88 ml of distilled water. Add 2 g of agar, autoclave at 121°C and 15 lbs pressure for 15 minutes, cool to about 55°C. Now add 10 ml of solution A, mix well and pour in tubes or plates.

Pine's agar medium for yeast phase *H. capsulatum*

Pine's citrate medium salt solution 1

➤ Potassium phosphate monobasic	8 g
➤ Ammonium sulphate	8 g
➤ Magnesium sulphate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$)	0.860 g
➤ Calcium chloride	0.080 g
➤ Zinc sulphate	0.050 g
➤ Water QS	1000 ml

Solution 2

➤ Ferrous sulphate, heptahydrous	5.7 g
➤ Magnesium chloride hexahydrous	0.8 g
➤ Sodium molybdate, dehydrate	0.15 g
➤ Concentrated hydrochloric acid	1.0 ml
➤ Water QS	1000 ml

Solution 3

➤ Casein hydrolysate	40 ml
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Solution 4

➤ Inositol	0.2 g
➤ Thiamine hydrochloride	0.2 g
➤ Calcium pantothenate	0.2 g
➤ Riboflavin	0.2 g
➤ Nicotinamide	0.1 g
➤ Biotin	0.01 g
➤ Water QS	1000 ml

Solution 5

➤ Hemin	0.2 g
➤ ETF water QS	1000 ml

Solution 6

➤ DL-thiotic acid	0.01 g
➤ Ethyl alcohol, 95%	10 ml

Solution 7

➤ Coenzyme A	0.01 g
➤ Water	10 ml

Pine's citrate medium, yeast phase, part 1

➤ Solution 1 (Salts)	250 ml
➤ Solution 2 (Minor elements)	10 ml
➤ Solution 3 (Caesin hydrolysate)	40 ml
➤ Solution 4 (Vitamins)	10 ml
➤ Solution 5 (Hemin)	10 ml
➤ Solution 6 (Thiotic acid)	1 ml

➤ Solution 7 (Coenzyme A)	1 ml
➤ Glucose	10 g
➤ α-keto glutaric acid	1 g
➤ L-Cysteine hydrochloride	1 g
➤ Glutathione (reduced)	0.5 g
➤ Asparagine	0.1 g
➤ L-Tryptophan	0.02 g
➤ Citric acid (for yeast phase only)	10 g
➤ Water QS	500 ml

Combine the 7 solutions in 80% of the water, then dissolve the remaining components. Adjust pH to 6.5 with 20% potassium hydroxide, QS to the full volume, and filter-sterilize.

Note: Citric acid is for yeast phase medium only.

This part is added to Pine's citrate medium, part 2.

Pine's citrate medium, yeast phase, part 2

➤ Oleic acid solution, agar:	0.1 ml
➤ Oleic acid	0.1 g
➤ ETF water	100 ml
➤ Starch solution, agar:	500 ml
➤ Starch, soluble	1 g
➤ ETF water	500 ml
➤ Agar	10 ml

Note: Filter-sterilize stock solutions.

For final medium: While part 2 is still hot, add part 1. Mix thoroughly and dispense aseptically.

Chapter 6: Special confirmatory tests

Rapid identification by germ tube (GT) test

The germ tube test is used for presumptive identification of *Candida albicans*. It is a rapid screening test wherein the production of germ tubes within 2 hours in contact with the serum is considered as indicative of *Candida albicans*. This test must be validated with a CMA test.

Procedure

- (1) Ensure that the test starts with a fresh growth from a pure culture.
- (2) Make a very light suspension of the test organism in 0.5 ml of sterile serum (pooled human serum or fetal calf serum). The optimum inoculum is 10^5 - 10^6 cells per ml.
- (3) Incubate at 37°C for exactly 2 hours.
- (4) Place 1 drop from the incubated serum on a slide with a cover slip. Observe under the microscope for production of GTs. These represent initiation of hyphal growth, arising directly from the yeast cell. They have parallel walls at their point of origin and are not constricted.
- (5) To record a positive, about 30% of the cells should show GT production.
- (6) Suitable controls should be kept with each test; a known strain of *Candida albicans* should be tested with each new batch of serum.

Note

- (1) The medium, inoculum size, temperature of incubation, concentration of simple carbohydrates and microaerobic conditions influence GT formation.
- (2) Increased concentration of inoculum causes a significant decrease in the percentage of cells forming GTs. Maximum percentage of GT formation occurs when 10^5 - 10^6 cells/ml are used as inoculum. As the concentration of cells increases, the percentage of GT formation decreases. A faintly turbid serum suspension is ideal for maximum GT development.
- (3) Approximately 95% of clinical isolates of *C. albicans* produce GTs when incubated in serum at 37°C for 2-3 hours.
- (4) A neutral pH (7.4) facilitates maximum development of germ tubes. Bacterial contamination may interfere with production of germ tubes.
- (5) Antimicrobial substances in the isolation medium may interfere with this test; human serum may contain inhibitory substances (such as ferritin) that suppress GT development.
- (6) Controls – *C. albicans* and *C. tropicalis* are run with each group of GT determinations to serve as positive and negative controls respectively.

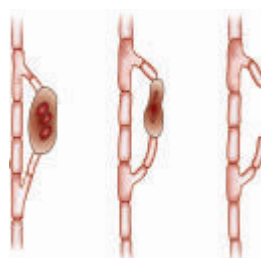
Ascospore demonstration test

Identification of yeast also involves determining whether or not the isolate has the ability to form ascospores. Some yeast will readily form ascospores on primary isolation medium whereas others require special media with limited nutrients (malt extract [5%] agar, acetate agar [0.5% NaCH₃COO.3H₂O, pH 6.5-7.0] and V8 juice agar).

Procedure

- Inoculate the yeast onto ascospore-producing agar plates.
- Incubate aerobically at 25°C.
- Examine the culture in 3-5 days and weekly thereafter for 3 weeks.

- Prepare wet mount of yeast in distilled water.
- Examine wet mount after oil immersion objective.
- Observe ascospore form, surface topography, size, colour, brims and number of ascospores per ascus.
- If not seen in wet mount, perform the modified acid-fast stain with 0.5% carbol fuchsin.



Ascospores

Ascospore morphology on malt extract agar (2-5 days growth)

1-4 hat-shaped ascospores in asci	<i>Pichia anomala</i> , <i>Pichia norwegensis</i> , <i>C. utilis</i> and <i>C. ciferri</i>
1-4 spherical or short ellipsoidal ascospores	<i>Saccharomyces cerevisiae</i>
1-2 /ascus, spherical with warts	<i>C. famata</i>
4/ascus, ellipsoidal with slimy sheath	<i>Blastoschizomyces capitatus</i>
1-2/ascus, spherical	<i>C. krusei</i>
1-4/ascus, spherical to hat-shaped, protuberances on 1 or 2 edges	<i>C. lipolytica</i>
1-4/ascus, clavate	<i>C. lusitaniae</i>

Canavanine glycine bromothymol (CGB) blue agar test

This test is used to differentiate between *C. neoformans* var. *neoformans* from *C. neoformans* var. *gattii*. The latter hydrolyses glycine to form ammonia to alter the pH of the medium towards alkalinity and thus change its colour to blue, while the former does not. Canavanine acts as a selective agent.

Procedure

- (1) Prepare CGB agar slants in tubes.
- (2) Inoculate surface of the slant using minimum inoculums of *C. neoformans* with a straight wire loop.
- (3) Incubate at 25°C for 1-5 days.
- (4) After 5 days, positive result is shown by colour change from greenish yellow (pH 5.8) to cobalt blue (pH 7.0).

Modified water agar technique to induce sporulation (Zygomycetes)

This technique has primarily been used to induce sporulation and help in identification of otherwise sparsely sporulating Zygomycetes – *Apophysomyces elegans* and *Saksenaia vasiformis*. It has been found that growth in deficient media like cornmeal-sucrose-glucose-yeast extract agar followed by growth on solidified water agar results in uniform sporulation of these two fungi. Padhye and Ajello modified this technique by incubating the agar block in a Petri dish containing sterile distilled water and 3 drops of 10% yeast extract at 37°C.

Procedure

- Grow the fungi as lawn culture on nutrient-deficient media like CMA, potato dextrose agar, cornmeal-sucrose-glucose-yeast extract agar, etc for 7 days at 30°C.
- Prepare 10% yeast extract solution; filter sterilized, and keep at 4°C.
- Pour 20 ml sterile distilled water onto a Petri plate.
- Cut 2 blocks of each isolate aseptically from the CMA and transfer to the water-containing plate.
- Add 3 drops of 10% yeast extract (kept at 4°C)
- Incubate at 37°C for 7 days.
- Prepare LCB mount and tease.
- Observe under microscope for typical vase-shaped sporangia of *S. vasiformis* and bell-shaped columella bearing sporangia of *A. elegans*

Sugar fermentation test

- Inoculate heavy growth onto sugar fermentation tubes containing the appropriate sugars and Durham's tubes.
- Incubate at 24°C up to 1 week. Examine tubes at 48 hours intervals for acid production (pink colour) and gas formation (in Durham's tubes).
- Production of gas indicates fermentation while only acid formation may indicate that the sugar has been assimilated. The reactions are read as A/G for each sugar separately.

Sugar/nitrate assimilation test (auxanographic method)

Disc impregnation: Pour plate auxanographic method of Wickerham and colleagues has withstood the test of time and is easier to perform than the liquid auxanographic technique, which was earlier erroneously claimed to be more sensitive and specific.

- Prepare yeast nitrogen base (YNB)/yeast carbon base (YCB), for nitrate test
- Prepare a yeast suspension from a 24-48-hours-old culture in 2 ml of YNB by adding heavy inoculum. Add this suspension to the 18 ml of molten agar (cooled to 45°C) and mix well in a sterile McCartney bottle (24 ml capacity). Pour the entire volume into a 90 ml Petri dish.
- Allow the media to solidify at room temperature.
- Now place the various carbohydrate-impregnated discs onto the surface of the agar plate.
- Incubate plates at 37°C for 3-4 days.
- Presence of growth around discs is considered positive for that particular carbohydrate.
- Growth around glucose disc is recorded first as it serves as a positive control.
- For the nitrate assimilation test, yeast suspension is prepared in YCB instead of YNB. In addition, a disc of peptone is used as positive control and one of KNO₃ as test.

Sugar fermentation test medium

- (1) Prepare liquid medium by dissolving the following in 1 litre distilled water.
 - Peptone 10 g
 - Sodium Chloride 5 g
 - Andrade's Indicator 0.05 ml
- (2) Sterilize at 121°C and 15 lbs pressure for 15 minutes.
- (3) Add filter-sterilized sugar at the concentration of 2% (20 g) to the medium aseptically.
- (4) Pour into small test tubes along with a single sterile Durham's tube in each and plug with cotton.

Sugar, potassium nitrate and peptone discs for carbohydrate and nitrate assimilation tests

Sugar discs can be obtained commercially or can be prepared as follows:

- Punch 6 mm diameter discs from Whatman no. 1 filter paper.
- Sterilize the discs by placing them in hot-air oven for 1 hour.
- Allow cooling, and then adding 1 drop of 10% filter-sterilized sugar solution to each disc.
- For the KNO₃ and peptone discs use a 3 g% solution
- Dry the discs in the 37°C incubator and store at 4°C in airtight container.

Q score Chart

Neutrophils	Epithelial cells		0	1-9	10-24	>25
			None	Few	Moderate	Many
			0	-1	-2	-3
	Q value		0	-1	-2	-3
0	None		3	0	0	0
1-9	Few		3	0	0	0
10-24	Moderate		3	1	0	0
>25	Many		3	2	1	0

Q0 & Q1

Q2

Q3

Not representative of lower respiratory tract secretions

Process; report with comment "Evidence of superficial contamination"

Proper deep sputum

Chapter 7: Serology

Antigen testing for specific diagnosis of cryptococcosis

Cryptococcal antigen detection in body fluids is a reliable and very sensitive technique for authentic diagnosis of cryptococcosis. Commercial kits are available with excellent sensitivity and specificity. Of the available methods of antigen detection, latex agglutination (LA) is simple, easy to perform, economical, and most popular.

- (1) LA titre of $\geq 1:8$ is indicative of active infection. In the case of HIV-positive patients with chronic meningitis, the titre in CSF may be extremely high, and antigen may also be detected in almost all body fluids. Antigen detection in urine may be a good non-invasive screening method.
- (2) If antigen titre increases progressively, it indicates an unsatisfactory prognosis. Conversely, if following therapy it decreases, the prognosis is considered good, though in the case of HIV-positive patients, it may take a long time to come down.
- (3) Antigen testing should go hand in hand with culture isolation, since low levels of antigen titre persist for a long time even after clearance of the organism from CSF.
- (4) In case of very high antigen concentration in the sample, the test may be falsely negative because of the prozone phenomenon. In such a case, dilution of the sample to at least 1:100 before repeating the test is recommended.
- (5) Occasional false positive reaction is also encountered, particularly in the presence of rheumatoid factor or infection with the yeast *Trichosporon* spp. False positive reactions generally have a low titre ($< 1:4$). To reduce false positivity, pretreatment of the sample with mercaptoethanol or pronase is

recommended. Pronase treatment also helps to dissolve the preformed antigen-antibody complexes in the sample. This is especially true when serum, instead of CSF, is to be tested.

Procedure

- (1) Serum and CSF specimen for cryptococcal antigen testing must be heat-inactivated at 56°C for a minimum of 30 minutes before processing.
- (2) Exact procedure to be followed should be according to the manufacturer's instructions.

Interpretation

Agglutination pattern should be observed and interpreted according to the manufacturer's instructions.

Commercial kits for detecting cryptococcal antigen

LA kits

1	Crypto-LA	International Biological Laboratories, Cranbury, NJ
2	Myco-Immune	American Micro Scan, Mahwah, NJ
3	IMMY	Immuno-mycologics, Norman, OK
4	CALAS	Meridian Diagnostics Inc., Cincinnati, OH

EIA kit

1	Premier	Meridian Diagnostics Inc., Cincinnati, OH
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Chapter 8: Antifungal susceptibility tests

Antifungal susceptibility testing has become important due to emerging antifungal resistant fungi causing infections in patients with AIDS.

Antifungal disk diffusion susceptibility testing of yeasts

Approved guideline M-44 A, CLSI, USA

The method described here is only for testing *Candida* species.

Medium: Mueller Hilton agar + 2% glucose and 0.5 µg methylene blue dye (GMB); pH 7.2-7.4. There should be no excess moisture on plates.

Storage of antifungal discs – at 4°C in the refrigerator.

Turbidity standard for inoculum preparation – 0.5 McFarland standard

Inoculum preparation

- (1) Streak onto SDA plate and incubate at 37°C to obtain a pure culture of *Candida* species.
- (2) Pick 5 colonies of approximately 1 mm diameter from a 24-hour culture and suspend in 5 ml of sterile normal saline.
- (3) Vortex for 15 seconds, adjust turbidity visually/spectrophotometrically at 530 nm to 0.5 McFarland standard (1×10^6 to 5×10^6 cells/ml) – this will produce semi-confluent growth with most *Candida* species.

Inoculation of test plates

- (1) Dip sterile cotton swab stick into suspension, rotate several times and press firmly against inside wall of the tube above fluid level to remove excess fluid.
- (2) Evenly streak over the entire agar surface 3 times, each time at an angle of 60°C, to ensure an even distribution of inoculum.

Application of disks to inoculated plates

- (1) Antimicrobial discs are dispensed onto the surface of an inoculated agar plate by means of a sterile forceps. They must be pressed down. The discs should be evenly distributed on the plate, no closer than 2.5 cm from centre to centre; 5 discs can be put on 1 plate.
- (2) Plates are inverted and incubated at 37°C within 15 minutes.

Reading

- (1) Read at 20-24 hours, when semi-confluent growth has formed.
- (2) In case of insufficient growth, read at 48 hours.
- (3) Zone of inhibition should be measured at the point where there is prominent reduction in growth.

Interpretation

Antifungal agents	Disc content	Zone diameter, (nearest whole in mm)		
		Resistant	Susceptible –dose dependent	Sensitive
Fluconazole	25 µg	< = 14	15-18	> = 19

Quality control (QC)

- (1) QC should be put up weekly.
- (2) Recommended QC strains include *C. albicans* ATCC 90028, *C. parapsilosis* ATCC 22019, *C. tropicalis* ATCC 750, and *C. krusei* ATCC 6258.
- (3) Recommended QC zone diameters are given in the Table below:

Antifungal agents	Disc content	<i>C. albicans</i> ATCC 90028	<i>C. parapsilosis</i> ATCC 22019	<i>C. tropicalis</i> ATCC 750	<i>C. krusei</i> ATCC 6258
Fluconazole	25 µg	28-39	22-33	26-37	-
Voriconazole	1 µg	31-42	28-37	-	16-25

CLSI micro-broth dilution method for yeasts, M-27-A2

Approved guideline M-27-A2, CLSI, USA

This method is used for testing yeasts that cause invasive infections, specifically *Candida* species and *Cryptococcus neoformans*. This method has not been used in studies of the yeast form of dimorphic fungi.

- RPMI 1640 with glutamine, without bicarbonate, buffered to pH 7.0 at 25°C with morpholinopropane sulphonic acid buffer (final concentration 0.165 mol/l) is used as medium to prepare antifungal agent dilution. For *Cryptococcus neoformans* yeast nitrogen broth may be used for better growth.
- Solvents and diluents for preparing stock solutions of antifungal agents.

Antifungal Agent	Solvent (full strength)	Diluent
Amphotericin B	Dimethylsulfoxide (DMSO)	Medium (RPMI 1640)
Ketoconazole	DMSO	Medium (RPMI 1640)
Itraconazole	DMSO	Medium (RPMI 1640)
Posaconazole	DMSO	Medium (RPMI 1640)
Ravuconazole	DMSO	Medium (RPMI 1640)
Voriconazole	DMSO	Medium (RPMI 1640)
Fluconazole	Water	Medium (RPMI 1640)
Flucytosine	Water	Medium (RPMI 1640)

- Serial two-fold dilution of antifungal agents (2x strength) in RPMI 1640 is prepared (0.1 ml of various antifungal concentrations in 12 x 75 mm tubes).

- These are dispensed in wells of a sterile disposable multi-well micro-dilution plate (96 U-shaped wells) from row 1 (highest strength 64 or 16 µg/ml) to row 10 (0.12 or 0.03 µg/ml; 100 µl each).
- Inoculum preparation – 5 colonies of ≥ 1 mm diameter from 24-hours-old cultures are suspended in normal saline and vortexed for 15 seconds. Cell concentration adjusted to 0.5 McFarland as above ($1-5 \times 10^6$ cells/ml). Working suspensions are made by 1:50 dilution followed by 1:20 dilution of the stock suspension with RPMI 1640 broth medium, which results in 1×10^3 to 5×10^3 cells/ml.
- Inoculum is added (100 µl) in a ratio of 1:1 to get desired final inoculum concentration 5×10^2 to 2.5×10^3 cells/ml.
- Quality control strains must always be put up in each batch. There must also be a growth control well for each isolate (no antifungal).
- Incubate at 35°C for 24 hours and observe for presence or absence of visible growth. For *C. neoformans* reading will be taken at 72 hours. Growth will be scored with a mirror as follows:
 - (1) Optically clear
 - (2) Slightly hazy
 - (3) Prominent decrease in turbidity
 - (4) Slight reduction in turbidity
 - (5) No turbidity
- For Amphotericin B, Itraconazole and the newer azoles, score of 0 is taken as MIC while for Flucytosine, Fluconazole and Ketoconazole Score of 2 is taken as end point.
- MIC values will be categorized as susceptible, susceptible dose dependent, intermediate and resistant, as per the following Table.

Antifungal agent	Susceptible (S)	Susceptible-dose dependant	Intermediate (I)	Resistant
Fluconazole	≤ 8	16-32	–	≥ 64
Flucytosine	≤ 4	–	8-16	≥ 32
Itraconazole	≤ 0.125	0.25-0.5	–	≥ 1
Voriconazole	≤ 1	2	–	≥ 4

CLSI micro-broth dilution method for filamentous fungi

Approved guideline M-38A, CLSI, USA

This method is used for testing filamentous fungi that cause invasive infections, including *Aspergillus* species, *Fusarium* species, *Rhizopus* species, *Pseudallescheria boydii*, and mycelial form of *Sporothrix schenckii*. Although other opportunistic melanized moulds have been evaluated, caution should be used in interpreting the MIC results for them. The method has not been used for the yeast forms of dimorphic fungi like *Blastomyces dermatitidis*, *Coccidioides immitis*, *Histoplasma capsulatum*, *Penicillium marneffei* or *S. schenckii*.

Procedure

Broth medium and buffers are similar to M-27-A2. Micro-titre plates similar to M-27A-A2 will be used for preparing drug dilutions. Growth-control well (no antifungal) will be incorporated for each isolate tested.

Inoculum preparation

- Fungi should be grown on potato dextrose agar for 7 days at 35°C. *Fusarium* should be incubated at 35°C for 18-72 hours and then at 25°C for 7 days.
- Cover the 7-days-old culture with approximately 1 ml sterile normal saline; prepare suspension by gently probing the colonies with the tip of a transferable pipette. Add 1 drop of Tween 20 to facilitate the preparation of aspergillus conidia. The resulting mixture of spores and hyphae is withdrawn and transferred to a sterile test tube. Heavy particles are allowed to settle for 3-5 minutes. The upper homogenous suspension is transferred to another sterile tube, cap tightened and vortexed for 15 seconds.

- The densities of conidia are adjusted to an optical density (OD) that all range from 0.09-0.11 (80%-82%) for *Aspergillus* species and *S. schenckii* and 0.15-0.17 (68%-70% transmittance) for *Fusarium* species, *P. boydii* and *Rhizopus* species.
- These suspensions are dissolved 1:50 in the standard medium (RPMI-1640). The end result corresponds to 0.5×10^4 to 5×10^4 CFU/ml.
- The rest of the steps are similar to M-27-A2.
- Incubation: *Rhizopus* species are examined at 24 hours, while the rest of the isolates at 48 hours except *P. boydii*, which is examined at 72 hours.
- Growth will be scored with a mirror as follows:
 - (1) Optically clear
 - (2) Slightly hazy
 - (3) Prominent decrease in turbidity
 - (4) Slight reduction in turbidity
 - (5) No turbidity
- For Amphotericin B, Itraconazole and the newer azoles, score of 0 is taken as MIC while for Flucytosine, Fluconazole and Ketoconazole Score of 2 is taken as end point.
- Recommended MIC results ($\mu\text{g/ml}$) for 2 QC (microdilution after 48 hours) and reference strains for broth dilution procedures are given in the following Table.

Antifungal agents	<i>C. parapsilosis</i> ATCC 22019 (QC)	<i>C. krusei</i> ATCC 6258 (QC)	<i>A. flavus</i> ATCC 204304 (reference)	<i>A. fumigatus</i> ATCC 204305 (reference)
Fluconazole	1-4	16-128	–	–
Voriconazole	0.03-0.25	0.12-1	0.5-4	–
Itraconazole	0.12-0.5	0.25-1	0.2-0.5	0.12-1
5FC	0.12-0.5	8-32	–	
Amphotericin B	0.5-4	1-4	0.5-4	0.5-2

Precaution

The manipulation of the culture should be performed in a Class IIA or IIB biological safety cabinet when risk of substantial splatter or aerosolization is present.

Etest for antifungal susceptibility of yeasts

Simple systems are available to assist laboratories with antifungal susceptibility testing, and one of them is Etest (® AB Biodisk), which is easy to incorporate into the routine laboratory and gives equivalent results to M27-A2. Etest is a reliable method for the susceptibility testing of *Candida* sp. and the 24 hours readings of Etest can serve as helpful preliminary results in most cases.

Etest can be used for antifungal susceptibility testing of yeasts such as *Candida albicans* and other *Candida* sp. and *Cryptococcus neoformans*. It provides the minimum inhibitory concentration (MIC mg/ml) of individual antifungal agents. Etest is based on a combination of the concepts of dilution and diffusion tests. Like dilution methods, Etest directly quantifies antifungal susceptibility in terms of discrete MIC values. Since the Etest strip has a predefined and continuous concentration gradient of the antifungal agent, the MIC values obtained can be more precise than values from conventional procedures based on discontinuous two-fold serial dilutions. Although processed like the disc diffusion test, the preformed and stable concentration gradient in Etest, differentiates the two methods clearly.

Etest consists of a thin, inert and non-porous plastic strip (5 x 60 mm). One side of the strip is calibrated with MIC values in mg/ml of a predefined and exponential gradient of the dried and stabilised antifungal agent immobilised on the other surface of the strip. The continuous gradient covers a concentration range corresponding to 15 two-fold dilutions in a conventional dilution procedure.

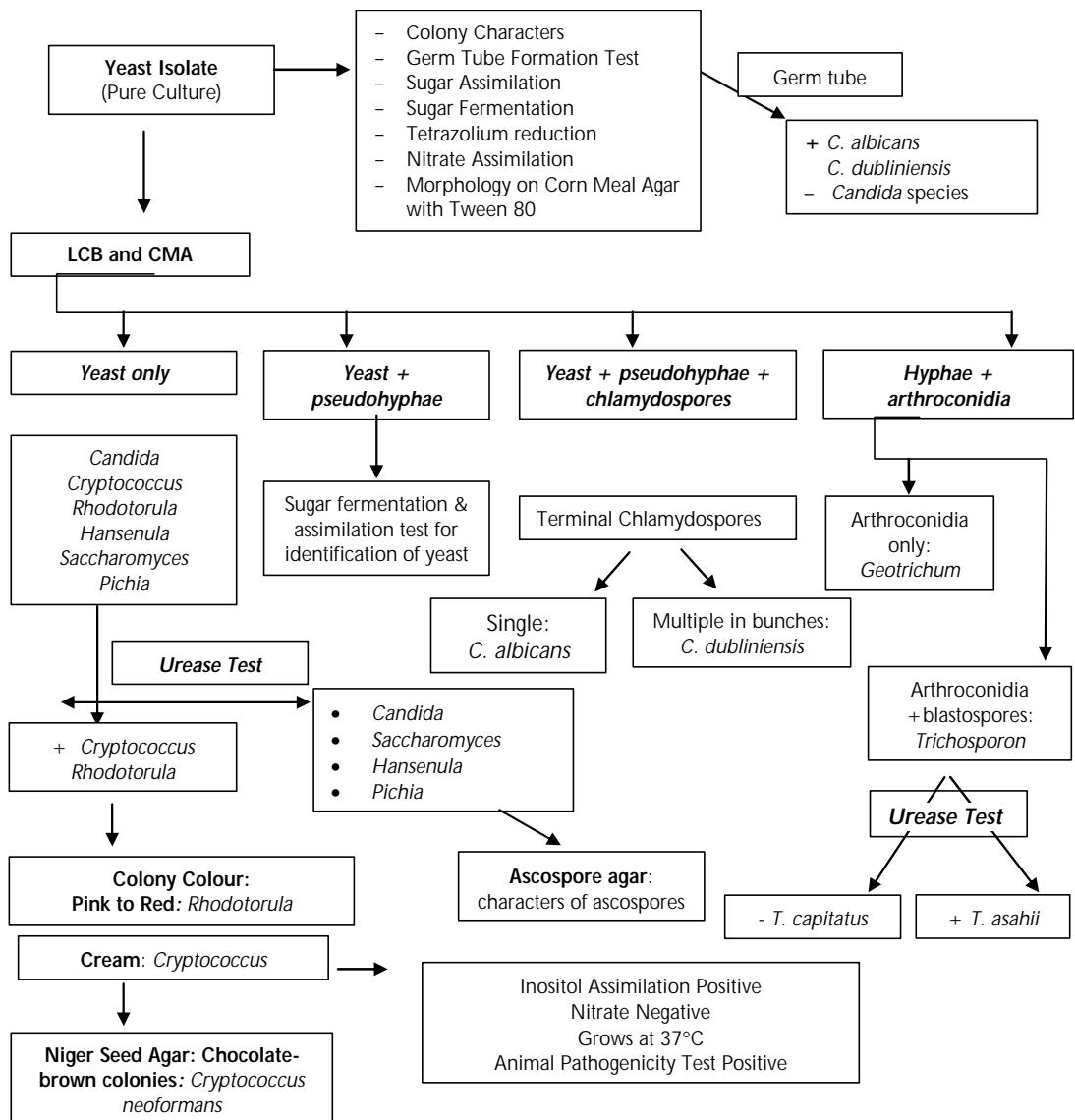
When applied onto an inoculated agar plate, there is an immediate release of the agent from the plastic surface into the agar matrix. A predefined, continuous and stable gradient of the drug concentrations is created directly underneath the strip. After incubation, whereby growth becomes visible, a symmetrical inhibition ellipse centred along the strip is seen. The zone edge intersects the strip at the MIC value.

Further reading

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Annex 1

Yeast Identification Scheme



Annex 2

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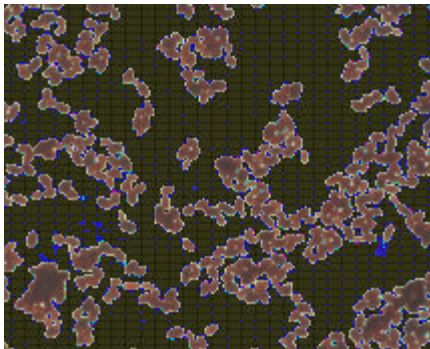
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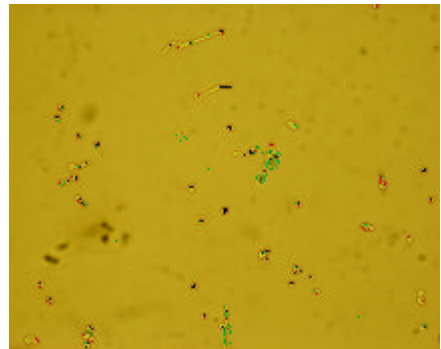
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Annex 3

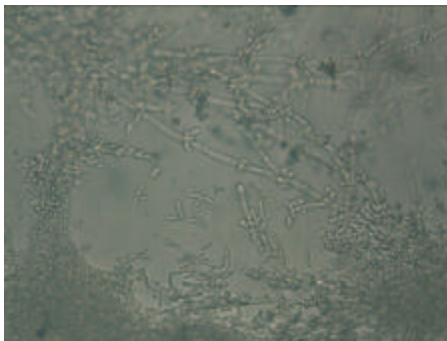
Photomicrographs of common fungal pathogens



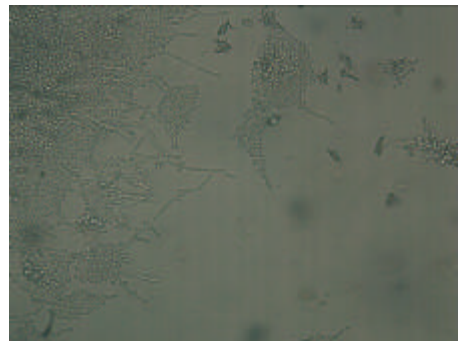
Gram stain: Gram positive oval budding yeast cells suggestive of *Candida* (1000x oil)¹



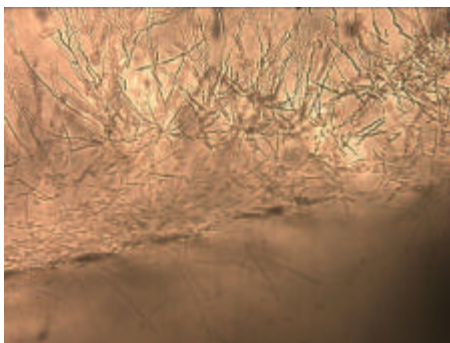
Germ tube test: *Candida albicans* producing germ tubes in serum after 2 hours incubation at 37°C (400x)¹



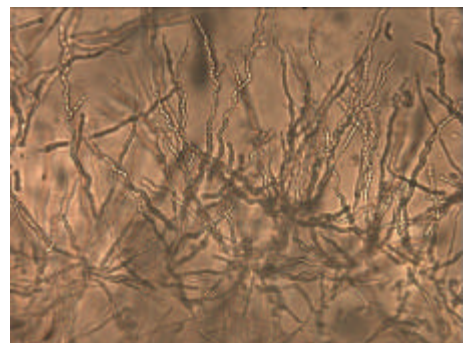
CMA: *Candida krusei* (400x)²



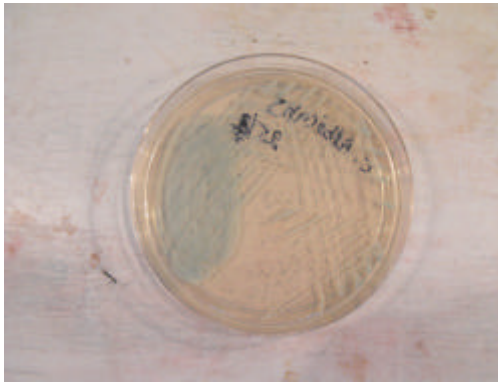
CMA: *Candida parapsilosis* (400x)²



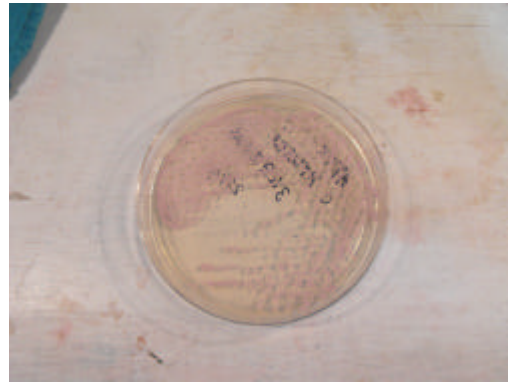
CMA: *Candida albicans* (200x)²



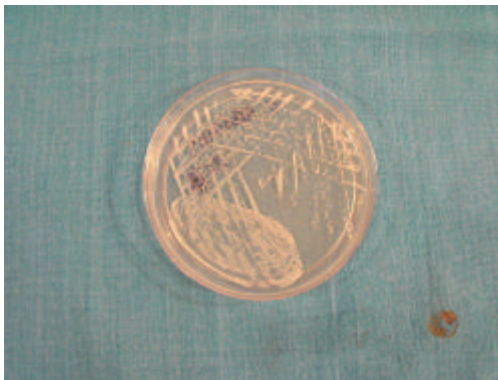
CMA: *Candida albicans* (400x)²



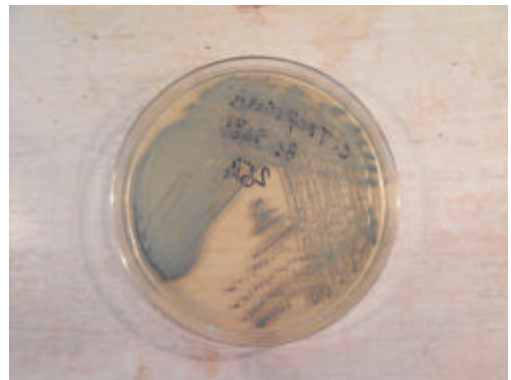
Chromagar plate: Growth of *Candida albicans* with a light green colour²



Chromagar plate: Growth of *Candida krusei* with a pink colour²



Chromagar plate: Growth of *Candida parapsilosis* (colourless)²



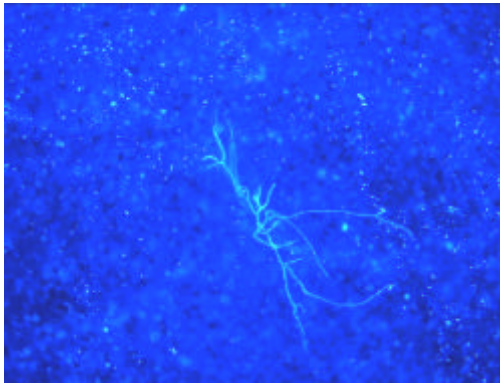
Chromagar plate: Growth of *Candida tropicalis* with a violet or purple colour²



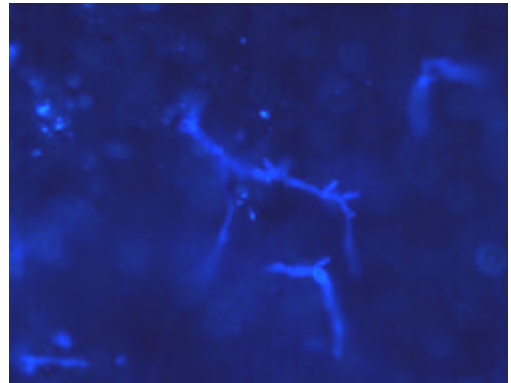
India Ink preparation:
Encapsulated yeasts (200x)¹



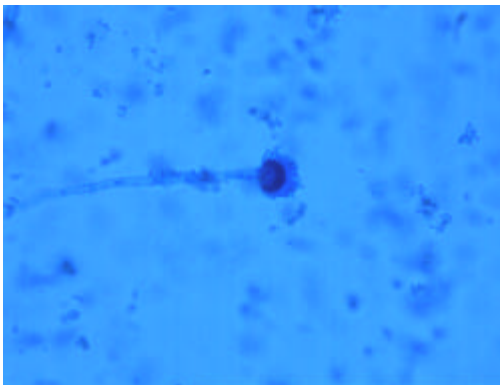
India Ink preparation:
Encapsulated yeasts (400x)¹



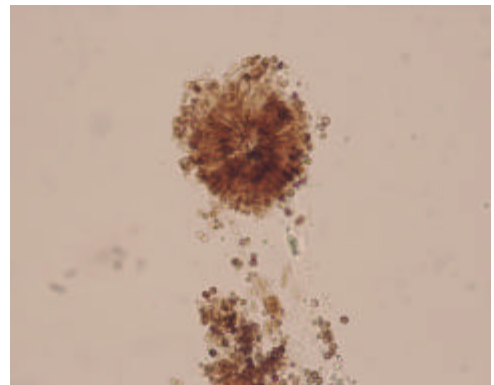
Calcofluor white: fungal hyphae²



Calcofluor white: septate fungal hyphae²



LCB mount: *Aspergillus fumigatus*²



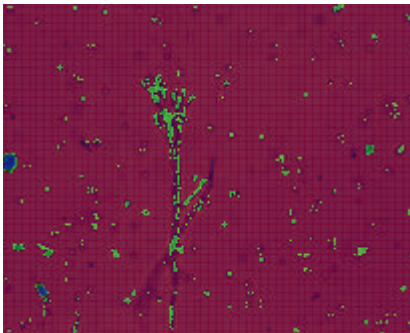
KOH mount: *Aspergillus* sp¹



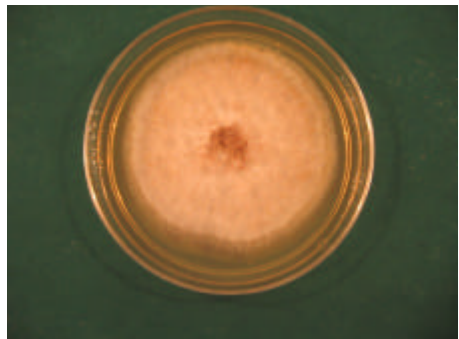
SDA: growth of *Aspergillus fumigatus*²



SDA: growth of *Aspergillus flavus* & *Aspergillus fumigatus*²



LCB mount: *Penicillium* sp showing septate branching hyphae and clustered phialides with conidia (400x)¹



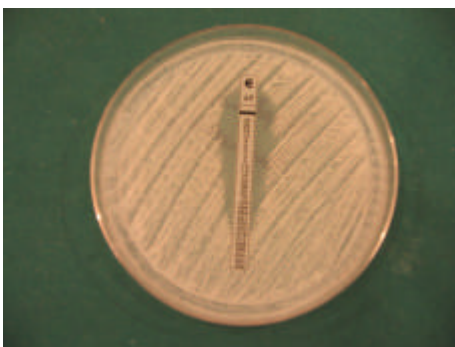
SDA: Growth of *Fusarium* sp²



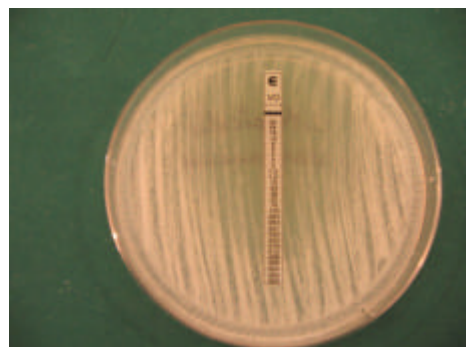
SDA plate with a Zygomycete (*Absidia Corymbifera*) growing to cover the whole plate, hence called a lid lifting fungus²



Growth of *Candida albicans* along with a Zygomycete (*Absidia corymbifera*) on SDA from an infant who presented with vomiting and a gangrenous bowel²



Anti fungal susceptibility for *Candida tropicalis* by Etest for Amphotericin B²



Anti fungal susceptibility for *Candida tropicalis* by Etest for Voriconazole (trailing effect, 24 hrs)²

Source:

¹Dr Sangeeta Joshi (Consultant Microbiologist, Manipal Hospital, Bangalore)

²Dr Iyer Ranganathan N (Senior Consultant, Microbiology and Infectious Diseases, Global Hospitals, Hyderabad)

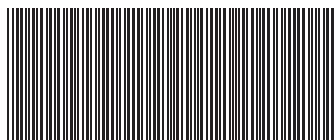


The human immunodeficiency virus per se does not kill the infected individuals. Instead, it weakens the body's ability to fight disease. Infections which are rarely seen in those with normal immune systems can be deadly to those with HIV. People with HIV can get many infections (known as opportunistic infections, or OIs). Many of these illnesses are very serious and require treatment. Some can be prevented. Of the several OIs that cause morbidity and mortality in HIV infected individuals those belonging to various genera of fungi have assumed great importance in recent past. Since these OIs were earlier considered as nonpathogenic, the diagnostic services for confirmation of their causative role need to be strengthened. This document is an endeavour in this direction and hopefully shall be useful in establishing early diagnosis of fungal OIs in HIV infected people thus assuring rapid institution of specific treatment.



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