Medical Mycology Laboratory Procedures

Editors

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The Fungal Kingdom: Essentials of Mycology

The increased incidence of fungal infections in past two or three decades has been overwhelming. Earlier, it was the pathogenic dimorphic fungi or the agents causing superficial fungal infections were known as pathogens for humans. However, starting from 1980s, the opportunistic fungi are recognized causing more infections especially in the immunocompromised host. More recently newer and less common fungal agents are being increasingly associated with infection in immuno-suppressed host.

Fungus was the first microorganism recognized because of the fruiting structure like mushroom, which are large enough to be seen without microscope. The word 'mycology', in fact, is derived from 'mykes', the Greek word for 'mushroom'. Historically, the discovery of the etiologic role played by fungi in disease marked the beginning of medical microbiology.The founder of the doctrine of pathogenic microbes was Agostino Bassi, a predecessor of Pasteur and Koch. In 1835 Bassi revealed a mould, *Beauvaria bassiana,* which caused devastating silkworm disease. It was quickly followed by the first discoveries of human diseases caused by fungi e.g. Favus by Remark and Schoenlein in 1837 and 1842 respectively, candidiasis by Gruby in 1842 and aspergillosis by Sluyter in 1847. These fundamental discoveries antedated the era of medical bacteriology ushered in by the monumental work of Pasteur and his contemporaries in the 1860's.

In the course of time more than 70,000 species of fungi have been recognized and described. It has been estimated that the total number of fungal species, known and as yet unknown, may be around 1.5 million. Only a few of the fungi pathogenic for human are sufficiently virulent to infect a healthy host. Most are relatively harmless unless they encounter an immunosuppressed patient, in which they invade readily and rapidly, sometimes with fatal consequences. A comprehensive list of all fungi that have been incriminated as opportunistic human pathogens may well exceed 400 species, even if the list is restricted solely to those species for which definitive evidence of infection is available. However, many of those have been encountered on very few occasion-often only once so that fewer than 100 fungal species approach the status of regular human pathogen. Morphology retains an important role in the identification of most fungi, particularly for the mycologist working in the laboratory. Although molecular and chemical techniques for identifying and classifying fungi are rapidly being developed and validated, this technology has not yet fully integrated into most of the laboratories. Morphology is more important for identification of moulds than for yeasts, but even with the latter morphology on the medium like corn meal agar is important in the identification process.

Morphology

Fungi are eukaryotes because the genome is organized in a nucleus surrounded by a membrane. This membrane is continuous with endoplasmic reticulum. Cellular division is accompanied by meiosis or mitosis. Cell organelles such as mitochondria, ribosome, vacuoles, lipid bodies and other storage inclusions are present. Fungi are chemo-heterotrophs with cell walls containing chitin and/or cellulose. They may be unicellular or multicellular, although there is a tendency for fungi to be multicellular and multinucleate. The colony of a fungus developed from a single conidia or spore is called 'thallus'.

Fungi pathogenic to man can be conveniently separated into two basic groups, moulds and yeasts. Moulds consist of those fungi that grow in a filamentous form, whereas yeasts are characterized by unicellular morphology that reproduces by budding. A number of fungi (*Histoplasma capsulatum, Blastomyces dermatitidis, Coccidioides immitis, Paracoccidioides brasiliensis, Sporothrix schenckii, Penicillium marneffei*) are dimorphic. That is, except for *C. immitis*, they can grow as a mould at room temperature (25-30^{o}C) and as yeast at 37^{o}C or in tissue. *C. immitis* grows as mould at room temperature

but as spherules producing endospores in tissue or on specialized media at 37°C.The list of dimorphic fungi causing human diseases including some rare species is presented in table -1.

The vegetative filament of a fungus is called a hypha. A number of these filaments are referred to as hyphae, and a large number of hyphae are known as mycelium. Hyphae are the actively growing assimilative phase of fungi. New growth occurs as linear elongation originating in a zone immediately behind the growing tip of the hyphae. As a result of linear elongation, the walls of the hyphae tend to be parallel. The diameter of the hyphae is a useful characteristic in distinguishing hyphae of the zygomycetes from other fungi. The zygomycetes typically develop hyphae that are sparsely septate, ribbon like, branching irregularly and having a diameter of 10-15 µm. Other hyphae are usually 1.5-3.5 µm in diameter. As the hypha develops, it gets divided into compartments or cells by the development of cross walls called septa. Such hypha is called as septate. The septa may be partial, complete or perforated. Zygomycetes have septa rarely, but once present those are complete. Rest other mycelial fungi have perforated (single or multiple) septa.

Hyphal cells may be quite variable in shape and size. Sometimes in unfavorable condition, a thick walled thallic conidium is formed (chlamydoconidia). It generally functions as resting spore. It is terminal as in the case in *Candida albicans, C. dubliniensis* or intercalary. The dermatophytes, as well as many other fungi, occasionally develop hyphae that contain cells having one end swollen at the distal portion. Such hyphae or racquet hyphae are not unique to any particular Genus or species. Another form of swollen hyphae produced by fungi, such as *Trichophyton schoenleinii*, is a favic chandelier. A favic chandelier is a cluster of repeatedly branching, swollen hyphae that have the overall appearance of a chandelier. These various forms of hyphae have no taxonomic value, but they occasionally help in identification of fungi.

In the yeast form of fungi, the somatic structures are globose, oval or elongated single cell. The cytoplasmic organelles and inclusion of these cells are not different from those of mycelial fungi. The somatic cells multiply by budding usually at the polar end, some by fission. Some spherical cells may produce buds simultaneously at several points. Sometimes the bud remains attached to the mother cell and continuously buds without separation to form chains of elongated cells called pseudohyphae. The confusion between true hyphae and pseudohyphae becomes most apparent when the cells of pseudohyphae are extremely elongated. The major differences between hyphae and pseudohyphae are set forth in the Table 2.

Taxonomy & Classification

Fungi reproduce by asexual and/or sexual method by formation of spores. Asexual spores are of two general types: sporangiospore and conidia. Sporangiospore are asexual spores produced with a containing structure (sporangium) and are characteristic of zygomycetes, such as *Rhizopus* spp., *Mucor* spp. Conidia are asexual spores that are borne naked, as evidenced in *Aspergillus* spp., *Penicillium* spp, dermatophytes. Sexual spores are of three types – zygospore, ascospores, basidiospore depending upon the group of fungi as shown in Table 3.

Table 1: Dimorphic fungi and diseases caused by them (Compiled by Prof. H S Randhawa)

Fungus	Disease	Natural habitat	Geographical distribution
Blastomyces dermatitidis	Blastomycosis	Poorly known, isolation from river and forest soil	North America (Mississippi & Ohio river valley) Canada, Africa, Asia
Coccidioides immitis	Coccidioidomycosis	Soil of arid regions, animal burrows	Arid regions of southwest USA, parts of Central & South America
Histoplasma capsulatum var. *capsulatum*	Histoplasmosis	Soil enriched with avian or bat dung	North America (Mississippi & Ohio river valley) rarely in Central and South America, Asia, Europe
H. capsulatum var *duboisii*	Histoplasmosis duboisii	Rarely isolated from soil	Central Africa, Madagascar
H. capsulatum var *farciminosum*	Histoplasmosis farcimiunosi	Unknown	Africa, Asia, Eastern Europe.
Paracoccidioides brasiliensis	Paracoccioidomycosis	Rarely isolated from soil	Central & South America
Sporothrix schenckii	Sporotrichosis	Dead or senescent vegetation	Cosmopolitan with higher prevalence in tropical & subtropical regions
Emmonsia crescens	Adiaspiromycosis	~118 animal species as host, rarely from soil.	Worldwide except Africa& Australia
Emmonsia parva	Adiaspiromycosis	~21 animal species as host, rarely from soil.	USA, Kenya, Europe, Australia.
Emmonsia pasteuriana	Name not given	Unknown	Solitary Italian case
Sporotrichum pruinosum	Name not given	Soil	Solitary report from India

Table 2: Differentiation between hyphae and pseudohyphae

Hyphae	Pseudohyphae
Growth results from hyphal apex by linear elongation and subsequent formation of septa	Growth results from a blowing-out process and subsequent appearance of basal constriction without separation
The terminal cell is typically longer than the preceding cell	The terminal cell is typically shorter or equal to the preceding cell
The terminal cell is cylindrical	The terminal cell is rounded
The walls are parallel with no invagination at the septa	The walls contain marked constriction at the septa
The septa are clearly visible and straight	The septa are often difficult to discern and are usually curved
Side branches are not constricted at the point of origin and the septum is some distance away from the main hypha	Side branches are constricted at their point of origin and there is a septum at the origin of the branch

Table 3: Classification of fungi

Division	Sexual spore type	Asexual spore type	Example of human pathogenic fungi
Zygomycota	Zygote (zygospore) – due to fusion of two gametangia (Figure –1)	Sporangiospore	*Rhizopus* sp, *Absidia* sp, *Rhizomucor* sp, *Mucor* sp, *Conidiobolus* sp, *Basidiobolus* sp
Ascomycota	Ascospores – Spore contained in a sac (Ascus) (Figure –2)	Conidium	Dermatophytes, *Aspergillus* sp, *H. capsulatum, B. dermatitidis*
Basidiomycota	Basidiospore – spore borne on the narrow stalk (sterigma) protruding from rounded structure called basidium (Figure –3)	Conidium	*Cryptococcus neoformans*
Deuteromycota (Fungi imperfecti)	Not known	Conidium or sporangiospore	*Candida albicans*, *Coccidioides immitis*, *Sporothrix schenckii*

Three more fungal divisions were recognized until recently. On the basis of molecular phylogenetic data, these groups are no longer accepted as fungi, while, in contrast, other organisms appear to have unexpected relationship to fungi. The Mycetozoa and the Mesomycetozoa are now the members of the kingdom Protista; the Oomycota belong to the kingdom Chromista. Because of their fungus like appearance they are indicated as 'Pseudofungi'. The causative agent of rhinosporidiosis – *Rhinosporidium seeberi* is now classified under Mesomycetozoa by molecular phylogenetic analysis. The protozoan genus *Microsporidium* is found to have a close affinity to the fungi. In the late 1980s, phylogenetic analyses based on the nuclear submit rRNA sequence alignments showed conclusively *Pneumocystis jiroveci* (earlier carinii) is a member of fungal kingdom. *P. jiroveci* was thought to represent

a single zoonotic species. However, it is now clear that the organism first identified, as '*P. carinii'* is actually a family of related organisms that exhibit mammalian host specificity.

Majority of the medically important fungi are in the group of Deuteromycota. The fungi in that group are segregated into Blastomycetes, Coelomycetes and Hyphomycetes. The Blastomycetes contain yeasts that reproduce by budding; the Coelomycetes contain moulds that produce conidia within a cavity of fungal tissue referred to as a conidioma (e.g. Pycnidium); and the Hyphomycetes contain moulds that produce conidia without fruiting structures or with synnema or that produce sterile hyphae. The hyphomycetes are often further divided as being either dematiaceous (darkly pigmented) or hyaline (non-pigmented), although these terms may be used to describe any fungus.

Table 4: Some of the fruiting structure that may help in identifying medically important fungi

Structure	Characteristics	Representative Genera
Sexual (Ascomycota) Cleistothecium	Spherical, completely closed ascocarp	*Pseudallescheria, Emericella*
Gymnothecium	Fruiting body composed of loosely interwoven hyphae	*Arthroderma, Ajellomyces*
Perithecium	Spherical or flask shaped ascoma with opening	*Chaetomium, Leptosphaeria*
Asexual Pycnidium	Globose to flask shaped asexual fruiting body with an inner lining of conidiogenous cell	*Hendersonula, Phoma, Pyrenochaeta*
Synnema	A group of erect conidiophores that are cemented together producing conidia at the apex and/or along the sides of upper portion	*Graphium*
Sporodochium	A cushion shaped stroma covered with conidiophores	*Fusarium, Epicoccum*

Table 5: Simplified classification of medically important fungi

Designation		Representative Genera
Division:	Zygomycota	*Rhizopus, Mucor, Rhizomucor, Absidia, Saksenaea, Apophysomyces*
Class:	Zygomycetes	
Order:	Mucorales	
Order:	Entomophthorales	*Basidiobolus, Conidiobolus*
Division:	Ascomycota	
Class:	Ascomycetes	
Order:	Endomycetales	*Saccharomyces, Pichia*
Order:	Onygenales	*Arthroderma, Ajellomyces*
Order:	Eurotiales	*Aspergillus, Penicillium*

Class:	Archiascomycetes	
Order:	Pneumocystidales	*Pneumocystis*
Division:	Basidiomycota	
Class:	Basidiomycetes	
Order:	Agaricales	*Amanita, Agaricus*
Order:	Filobasidiales	*Cryptococcus*
Division:	Deuteromycota	
Class:	Deuteromycetes	
Order:	Cryptococcales	*Candida, Cryptococcus, Trichosporon, Malassezia*
Order:	Moniliales	
Family:	Moniliaceae	*Epidermophyton, Coccidioides, Paracoccidioides, Sporothrix, Aspergillus*
Family:	Dematiaceae	*Phialophora, Fonsecaea, Exophiala, Wangiella, Cladophialophora, Bipolaris, Exserohilum, Alternaria*
Order:	Sphaeropsidales	*Phoma*

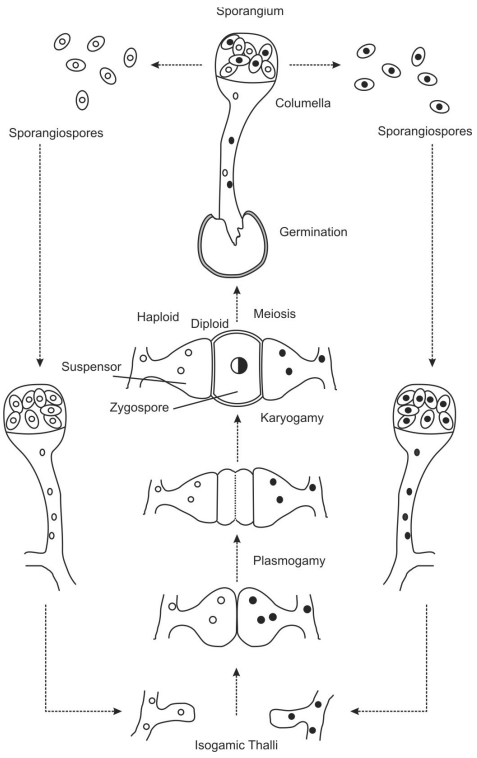


Fig. 1 : Zygospore formation

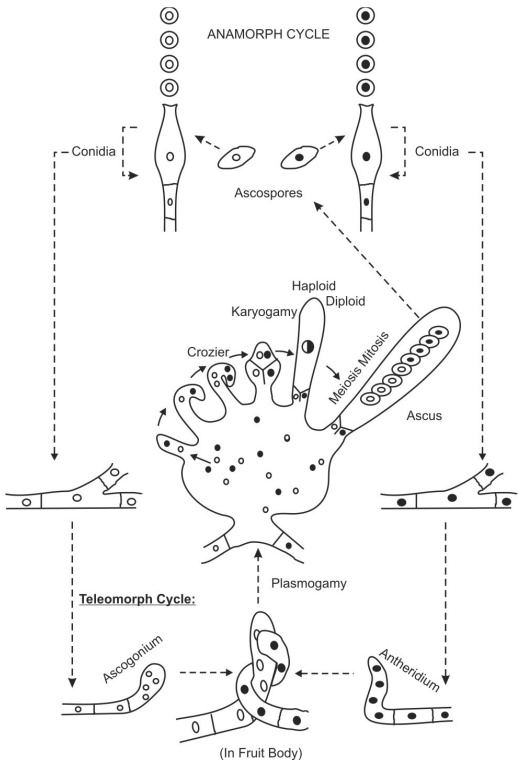


Fig. 2 : Ascospore formation

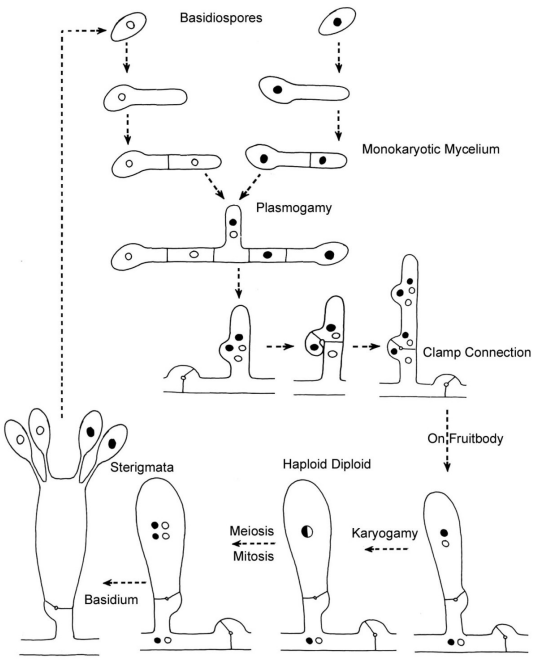


Fig -3 Basidiospore formation

Conidiogenesis

Understanding of conidiogenesis is important for identification of fungi. Fungi reproduce by either sexual or asexual means or both. Asexual reproduction is either an increase in the vegetative phase of the fungus or development of asexual propagules.

Asexual reproduction in the zygomycetes is characterized by the formation of a structure called sporangium. It is a sac like cell in which the entire internal contents are cleaved into spores. Each spore is called a sporangiospore and may have from one to several nuclei. Sporangiospores may be either randomly distributed in the sporangium *(e.g. Rhizopus arrhizus)* or in a row of merosporangium *(Syncephalastrum* sp.*)*. In *Cunninghamella* sp., the sporangium is reduced in size to one sporangiospore in each sporangium (sporangiolum). A sporangium develops on specialized hyphae called sporangiophore. A columella is a small domelike area at the apex of the sporangiophore. The term apophysis is applied to any swelling in the sporangiophore that is immediately below the columella.

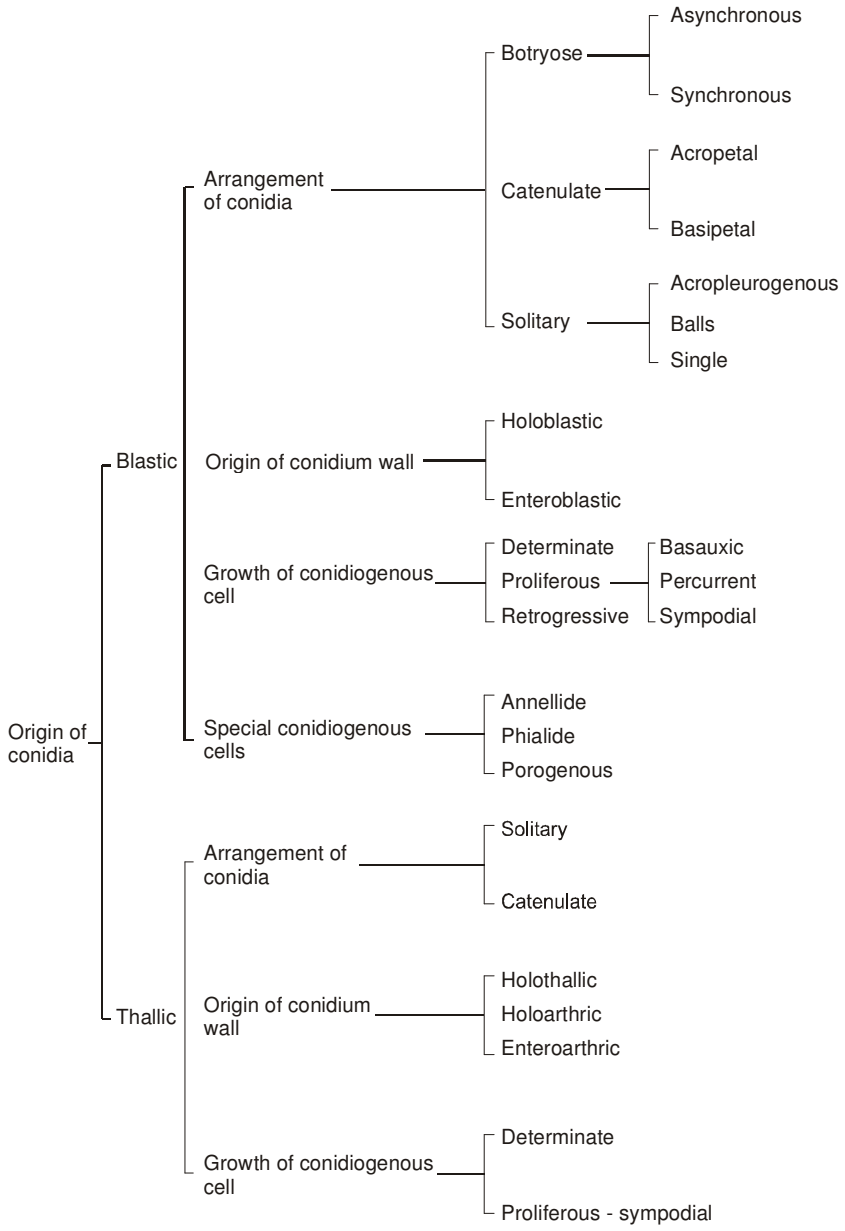
Most other mycelial fungi of medical interest form conidia upon specialized hyphae called conidiophores. A conidium (pl. conidia) is a non-motile, usually deciduous propagule that results from asexual reproduction. The term spore is restricted to those propagules that develop within a sporangium or via sexual reproduction (ascospore, basidiospore and zygospore). In contrast, the term conidium is used for propagules that originate by asexual means other than in a sporangium.

The cell that gives rise to the conidium is known as the conidiogenous cell, and the process of sequence of events that result in the new conidium is referred to as its mode of conidiogenesis (Fig-1).

Micronematous is a term to describe conidiophores that are morphologically similar to the vegetative hyphae; semimacronematus for conidiophores that are slightly different from the vegetative hyphae; and macronematous for conidiophores that are morphologically distinct from the vegetative hyphae.

Conidiophores may be either free or they may be united together to form a large upright structure called a synnema (e.g. *Graphium* sp). When conidiophores occur as a covering over a cushion – shaped mycelial mat, the entire structure is called a sporodochium. Some fungi develop their conidiophores on a tightly bound flat mat of mycelium known as acervulus. A pycnidium is a large, round to flask-shaped fruiting body. If the pycnidium has an opening, it is referred to as being ostiolate, Sterile spine liked structures are called setae (e.g. *Pyrenochaeta* sp.).

The major components of conidium development include the origin of conidium, conidial wall, type of conidiogenous cell, arrangement of conidia, and site of area that gives rise to the conidium.



Conidial Development

Conidia originate either by blastic or thallic conidiation (Fig-2). In blastic conidiation the young conidium initially begins to enlarge and is then separated from the parent cell by development of a septum (e.g. *Candida* sp.). In thallic conidiation, the conidium originates from the entire parent cell, since all of it becomes the conidium (e.g. *Geotrichum candidum*). The cell walls of the conidia and hyphae are multi-layered. When all the layers actively participate in the formation of the conidium, the origin of the conidial wall is either holoblastic (e.g. *Candida* sp.) (Fig -3) or holothallic (e.g. *Microsporum* sp.) (Fig - 4). In holoblastic development, the outer wall remains intact, whereas they break in enteroblastic development. Some conidia may develop through a channel or pore in the outer cell wall layer. Such sporogenous development results in poroconidia (e.g. *Drechslera* sp).

In thallic conidiogenesis, if the propagules fragment and then disarticulates from the parent hyphae, this is referred to as arthric development. Holoarthric development is characterized by having all of the cell wall layers involved in the formation of the conidial wall (e.g. *Geotrichum candidum*) (Fig-5). The term enteroarthric is used only when the inner cell wall layer participates in the formation of newly formed cell wall of the conidium (e.g. *Coccidioides immitis*) (Fig-6).

Conidiogenous cells may cease to grow in length just before or at the conidium formation. These are called determinate (e.g. *Helminthosporium* sp.) (Fig-7). In contrast, when the fungi produce conidiogenous cells or conidiophores that continue to increase in length, such cells are indeterminate or proliferous (e.g. *Ulocladium* sp.). The term retrogressive is applied when conidiogenous cells become shorter as they are converted into conidia (e.g. *Basipetospora rubra*) (Fig-8).

With respect to proliferous conidiogenous cells or conidiophores, the growth of the conidiophore may occur at the base (basauxic) or at the apical region (acroauxic). We are primarily concerned with acroauxic conidiophores. Sympoidal condiogenous cell represents a classic example of proliferous acroauxic growth. The coidiogenous cell grows and forms a conidium at its apex (Fig-9). A new growing point develops below and to one side of this developing conidium. A second conidium begins to form at the new apex (e.g. *Curvularia* sp). This type of conidiophore often has a characteristic bent knee appearance that is geniculate (e.g. *Drechslera* sp) (Fig-10).

A second type of conidiogenous cell that is of interest is the annellide. The first conidium is holoblastic in origin. This terminal conidium then breaks free. There is a proliferation of new growth through the area where the conidium was attached. A second conidium develops enteroblastically. Once the second conidium is released the entire process is repeated. Such continued growth through subsequent apices is referred to as percurrent. When each conidium breaks free, a tip of outer cell wall material is left behind. These rings or annellations may be regular or irregular around the tip of the annellide (e.g. *Scopulariopsis brevicaulis, Exophiala* sp.) (Fig-11). The conidia produced from annellides are called annelloconidia.

The phialide is usually elongate or flask shaped (Fig-12). The first conidium produced is holoblastic. After the conidium is released by rupture or dissolution there is no additional elongation of the conidiogenous cell. In species under the Genus *Phialophora* (Fig-13), a cup like extension of outer cell wall remains at the apex of the phialide when the first conidium is released. The collar of cell wall material is known as a collarette. All the subsequent conidia, after the first one, are produced enteroblastically. Phialides in contrast to annellides, typically have collorettes or apical thickening, do not increase in length with phialoconidium production, have a complete septum at the base of each conidium and leave one conidiogenous locus that given rise to many conidia. Phialoconidia develop in a basipetal manner from a fixed conidiogenous locus, with youngest conidium at the base of the chain and the oldest conidium at the tip.

Conidia may be formed either in any asynchronized or synchronized manner. Botryose is a term referred to describe conidia that are formed in clusters. Acropleurogenous is used to describe conidia that are arranged at the apex and around the fertile hypha.

Table-1: Conidiogenesis of some of the medically important fungi

Conidial group	Characters	Representative genera
Thallic development	A mode of conidial formation in which a conidium is formed by the transformation of an entire pre-existing hyphal cell or conidiogenous cell	
- Arthroconidia	A spore resulting from the fragmentation of a hypha at the septum	*Coccidioides, Geotrichum*
Blastic development	Conidial formation caused by the blowing out of fertile hyphae before being delimited by a septum	
-Blastoconidia	Holoblastic conidia that are produced solitarily, synchronously or in acrogenous chain. Blastoconidia are typically released by fission through septa	*Candida, Cryptococcus*, Other yeasts, *Cladophialophora*
-Phialoconidia	Conidia formation from tubular, often flask shaped conidiogenous cell (phialide) that does not increase in length	*Fusarium, Aspergillus, Malassezia, Phialophora, Penicillium, Lecythophora*
-Annelloconidia	Succession of conidia produced from a conidiogenous cells having multiple ring like scar around the tip, which are left by released conidia. The first conidia is holoblastic and remainder are enteroblastic, Apex of annelide increases in length as more conidia are produced	*Exophiala, Scedosporium, Scopulariopsis, Phaeoannellomyces*
-Poroconidia	Holoblastic conidium produced through a minute pore or channel in the cell wall of the conidiophore or conidiogenous cell	*Alternaria, Bipolaris, Curvularia, Drechslera, Exserohilum*
-Aleuroconidia	Thallic conidia that develop as an expanded end of an undifferentiated hyphae or on a short pedicel and is released by rupture of the supporting cell	*Microsporum, Epidermophyton*

Laboratory Diagnosis

The diagnosis of fungal disease is a multi-disciplinary approach requiring cooperation and collaboration of many people with diverse expertise. As most of fungi causing fungal disease are saprophytic in nature, close communication with physician is important to interpret of result of the laboratory. The demonstration of fungi in the tissue by histopathology is important to prove the invasive character of the saprobes. However, in large number of cases it is difficult to lacalize the site of lesion as invasive mycoses have no characteristic symptoms or signs. Increased awareness and imaging technique may improve the chance of localization of the site of infection. Even after possible localization, collection of biopsy or aspiration sample from deep tissue often faces difficulty as most patients are immunocompromised and thrombocytopenic. Due to the difficulty in sample collection, poor sensitivity and prolonged time required for conventional procedure, attention has drawn to the non-culture based diagnostic techniques. Though some of the serological or molecular techniques showed promising results for diagnosis, these test procedures are still to be validated for routine use.

Laboratory diagnosis of fungal diseases

Culture based (conventional)	**Non-culture based (molecular)**
Direct microscopy	Detection of fungal specific
Histopathology	-Antigen
Culture isolation	-Antibody
-Standard	-Cell wall component (chitins, 1-3 β-glucan)
-Lysis-centrifugation	-Metabolites
-Indirect (radiometric or non-radiometric)	-Nucleic acid
Identification of the isolate	

For conventional technique basic bacteriological techniques may be used when working with most of the fungi except cultures may be kept longer and provisions must be made to prevent media from excessive drying. This can be done by pouring more media in plates, sealing them with paraffin tape to prevent drying, incubating them in moist chambers or using media in tube. However, direct microscopic examination or histopathology of clinical samples is an important step in clinical mycology laboratory as this provides quick report to clinician (cultures take days or weeks), helps in interpretation of result as most fungi are saprophytic, proves the invasiveness of fungi and explains tissue reaction to fungi.

Microscopic studies used routinely include 10-20% KOH and PAS stained smears.. The Gram and acid-fast stains are used when looking for gram-positive filamentous bacteria. Giemsa or Wright's stain is often used for intracellular organism like *H. capsulatum*. An India ink preparation is used when looking for encapsulated organism like *C. neoformans*. Fluorescent brighteners (calcofluor white) used in conjunction with UV light source and the proper filter combination is useful in improving the sensitivity of direct microscopy. Optimal results are obtained using a 330 to 380nm excitation filter, a 420nm barrier filter. For identification of the fungus bright field optics and a magnification factor of about 500 x are sufficient to resolve the feature of medically important fungi mounted in lactophenol (for pigmented fungi) or lactophenol cotton blue (for unpigmented fungi). However, for critical evaluation of conidial ontogeny

higher magnification (1000 x) is required. Some structures, such as annellations (on annellides), collarettes (on phialides) are easier to distinguish in phase-contrast microscope. Yeasts can be manipulated in the laboratory like bacteria by looking into morphology and biochemical reaction. For identification of mould, morphology and conidial ontogeny are important. In more critical studies to determine ontogeny, it may be necessary to set up a slide culture.

In recent years techniques to improve timely diagnosis have focused on non-culture based methods like detection of pathogen specific antigens, antibodies, metabolites, cell wall components, nucleic acid sequences. Detection of Cryptococcal antigen for the diagnosis of cryptococcosis is probably the best antigen detection assay for the diagnosis of any infectious disease. Latex agglutination test or ELISA is commercially available (Murex, Meridian, Wampole, Immunomycologics) and they are reliable, being both sensitive and specific. They also assume a particular prognostic importance as it guides the clinicians towards monitoring the therapy. Cell wall mannan and mannoproteins have been considered for use as diagnostic markers of invasive candidiasis. A commercial ELISA (Platellia Aspergillus, Bio Rad, France) has been available for detection of *Aspergillus* antigen in serum, urine and broncho-alveolar lavage for diagnosis of invasive aspergillosis. Although the initial studies that assessed the performance characteristics of this assay reported high sensitivity and specificity, more recent studies show significant variation in performance. Detection of antifungal antibody is useful in diagnosis of systemic disease due true pathogenic fungi (histoplasmosis, coccidioidomycosis, blastomycosis etc) in immunocompetent hosts. In immunosuppressed patients, there is poor response for antibody development.

Detection of fungus specific metabolites has the potential to be a powerful tool for the diagnosis and management of fungal infections.however, the tests require sophistigated instruments and are expensive. Important metabolites detected include D-arabinitol, D-arabinitol/creatinine ratio, D-mannitol. The test is evaluated in limited number of laboratories and requires validation. (1-3) β-D-glucan is a characteristic cell wall component of all fungi except the zygomycetes. Other microbes and mammals lack this polysaccharide. Elevation of this glucan in blood, cerebrospinal fluid or other normally sterile body fluid may be used as a useful marker to diagnose deep mycoses or fungal sepsis. The test is mainly evaluated in Japan. Now, US firm have also developed similar kit (Fungitell, Glucatell). As chitin is an important component of fungal cell wall, detection of chitin by spectrophotometric technique has been exploited to diagnose invasive candidiasis or aspergillosis.

Maximum attention is now on development and standardization of nucleic acid detection procedure. The development of polymerase chain reaction (PCR) technology has led to rapid detection of fungal nucleic acid, improved sensitivity and specificity. However, refractory fungal cell wall and presence of inhibitors in blood and body fluids are the confounding factors. In addition, strict quality control measures are needed to exclude false positives, as potential environmental contamination due to saprophytic fungi is common. Though, considerable progress has been made in detection in fungal nucleic acid especially in invasive candidiasis and aspergillosis, none of the tests has come out of strict validation criteria.

Sample Collection, Transport and Processing

The principal goals of a sound clinical mycology laboratory are to isolate efficiently and to identify accurately the suspected etiological agents of fungal infection. Success depends much on the quality of clinical specimens sent to the laboratory.

The following points need to be emphasized: -

a) Appropriate sample/specimen collection

b) Prompt transportation

c) Correct processing of the specimen

d) Inoculation of specimens onto appropriate culture media and incubation at suitable temperature

Specimens should be collected aseptically in clean, sterile and properly sealed containers, delivered to the laboratory within 2 hours. The sample should be processed, and then inoculated to primary isolation media within a few hours of collection. Viability may decrease with prolonged specimen storage.

Swabs are not satisfactory since they allow drying of specimen and loss of viability; however, specimens from the environment or certain body sites such as the ear canal, nasopharynx, throat, vagina and cervix are not readily collected by other means. Swabs for collection of material from open wounds or draining lesions are frequently contaminated with environmental microorganisms.

All specimens sent to mycology laboratory must be clearly labeled with the patients' name, age, sex, and distinct patient identification number. The laboratory requisition form sent along with the specimen must have additional information like unit number, date and time of collection, source of specimen, antimicrobial therapy together with a brief relevant clinical history and the name of the attending physician.

Table 1: Frequently received samples in mycology laboratory

Specimen	Collection	Unacceptable specimen
I Superficial and cutaneous Mycoses		
a) Skin	–Lesions decontaminated with 70% alcohol –Small scales scraped off from the margin by blunt scalpel blade or glass slide. Strongly macerated skin between toes can be removed by forceps. –Collected on sterilized glass plate/paper envelope -Vesicles collected by gentle deroofing with needle.	Cotton swabs except moist skin areas
b) Hair	-Select the hair that fluoresce under a wood's lamp -Affected hair collected by removing them completely using epilation forceps/ scrapping with blunt scalpel blade. Specimen should include hair stubs, plugged follicles and skin scales	Clipped hair

	-Alternatively, brush is rubbed vigorously on the scalp. -Adhesive tape may also be used	
c) Nail	-Site cleaned with 70% alcohol -Clippings from nail (discolored, dystrophic or brittle) as far back as possible from the free edge -Full thickness of the nail to be included In superficial white onychomycosis collect scrapping from white spots discarding the upper most layer	Superficial scrapings, swabs
d) Mucous membrane (mouth, throat, vagina, rectum)	-Ideally scraping with a blunt scalpel/ spatula -Moist swabs to be collected in a sterile container	Dry swab
e) Ear	-Scrapings or moist swabs from ear canal -Ear washings	Dry swab
d) Ocular	-To be collected by ophthalmologist only -Scrapings from ulcerated, suppurative areas (scrapings form deeper layers of cornea preferable) -Specimen directly inoculated at the bedside or can be transported to the laboratory between two sterile glass slides (one for microscopy, and the other for culture) -Fluid specimen (aqueous/vitreous tap) collected in a sterile syringe	Swabs/samples collected by laboratory loop
II Subcutaneous mycoses		
a) Pus	-Aseptically with needle and syringe from undrained abscess -Pus expressed from abscess opened with scalpel / scrapings or crust collected from superficial lesion; transported to laboratory either in sterile container/ syringe and needle	Swab or materials from open wound
b) Biopsy	-Ulcerated lesions of the skin are biopsied -Place between two sterile gauze pads, sterile petridish/ tube (containing 2-3 ml of sterile normal saline/ brain heart infusion broth) -Tissue is collected from centre and edge of the lesion	Swabs, sample collected in thioglycolate broth or formol saline
c) Grains	-Collected by lifting the crust at the opening of a sinus. Curette the draining sinus to get grains -Grains frequently found underneath the pus or collected from the removed bandages -Aspirated from undrained sinuses	

III Systemic Mycoses		
a) Cerebrospinal fluid	-Obtained by lumbar puncture by a clinician / or by cisternal puncture ~ 3 ml in a sterile tube normally Occasionally 15-30 ml may be required	Insufficient quantity
b) Body fluids	-Sterile tube or in a heparinized syringe	Swabs
c) Bone marrow	-~ 0.2-0.3 ml collected in a sterile heparinized syringe from iliac crest/ sternum -Sterile cap is placed on heparinized syringe and transported immediately	Clotted bone marrow
d) Blood	-Using aseptic technique collect venous blood ~ 5-10 ml in yellow vacucontainer/syringe or in biphasic media containing brain heart infusion broth and agar; blood: broth ratio should be maintained at 1:10 -Multiple blood cultures at timed intervals to be collected. -BACTEC/lysis centrifugation technique may improve sensitivity	
e) Urine	-Early morning 25-50 ml of clean catch midstream urine specimen -Suprapubic aspirate, catheterized specimen -Collected in sterile container	24 hr collection is unacceptable
f) Faeces	-Not usually acceptable in the Mycology laboratory. -Sometime collected to access *Candida* carriage in G I tract as surveillance culture.	
g) Sputum	-5-10 ml; early morning prior to eating -Use mouth rinse and brush before collection. Induce sputum when unproductive -Collected in sterile wide mouthed container.	Saliva, nasal secretion, throat swab, 24 hour collection
h) Bronchial brush/washing/broncho-alveolar lavage	-Collected in sterile container using fiber optic bronchoscopes	Dried specimen
i) Lung biopsy	-Collected by bronchoscope, fluoroscope guided trans-thoracic needle aspiration or open lung biopsy -Best specimen is open lung biopsy but it is hazardous	
j) Serology	-Serum – 1-2ml with 1:100 parts merthiolate -3-5ml of spinal fluid with same concentration of merthiolate	Specimen collected after skin test with histoplasmin while performing serology for histoplasmosis

Environmental samples:

1. **Swabs**- Sterile swabs can be used to collect environmental specimens. Collect on an area of at least one square inch. At least two swabs per site are preferred. One swab will be used for direct mount and the other for culture. Store the swab at room temperature and deliver to the laboratory promptly.
2. **Soil and touch plates**- Collect in sterile container or zippered bag and deliver to the laboratory. Store at room temperature.
3. **Culture plates**- Once collected, plates should be sealed with tape and delivered to the laboratory immediately.
4. **Air sample** – by slit sampler

Transport of specimens:

1. Specimens should be transported in sterile, humidified, leak-proof container. Dermatological specimens, however, should be transported in a dry container. Transport medium should not be used unless the specimen can be easily and completely retrieved from the medium. Although fungi can be recovered at times from specimens submitted in anaerobic transport media, such media should be avoided.
2. Specimens should be processed and inoculated to primary isolation media as soon as possible after collection, ideally within few hours. Limited studies have shown significantly decreased viability for *Histoplasma capsulatum, Coccidioides immitis, Blastomyces dermatitidis, Rhizopus arrhizus* and *Aspergillus fumigatus* when stored at room temperature or in refrigerator. It should not be presumed that successful methods for storage of fungal cultures are suitable for temporary storage of clinical specimens that harbor relatively few fungal cells.
3. The effect of refrigeration on fungal specimens has not been well studied, but if processing is to be delayed for more than several hours, it is recommended that specimens be stored under refrigeration at 4^0C with the following exceptions: blood and cerebrospinal fluid are stored at 30-37^0C; dermatological specimens are stored at 15-30^0C.

Processing of specimen in the laboratory: -

- Improperly collected specimens like those collected in leaking containers /from inappropriate sites/ lacks the patient's details or specimen information should be rejected and notified to the concerned physician.
- Specimens should be first examined carefully. The examination allows for selection of the proper portion of the specimen that will likely contain the fungus. Caseous, purulent or bloody areas, and necrotic materials should be included for processing.
- Always insists on repeat sample in case the specimen is not suitable for processing .
- Specimens from cases of mycetoma are examined with the dissecting microscope for the presence of granules before processing.
- Punch biopsies should be examined carefully to ensure that they are divided vertically and not horizontally so that each layer of tissue is represented in each specimen.
- Clinical specimens must be processed as soon and as carefully as possible on the appropriate isolation media and temperature. Recovery of pathogens is necessary for their identification and evaluation against the antifungal agents.

- Most specimens suspected of having fungi other than dermatophytes should be handled according to practices outlined for Biosafety Level 2**.**

Direct inoculation:

- Many specimens (up to 0.5ml) can be directly inoculated to media
- Specimens like – abscess aspirate, bone marrow aspirates, cerebrospinal fluid, swabs, body fluid, hair, skin scraping, nail, bronchial washing or brushing etc. can be directly inoculated
- If the fluid has a clot or membranous material, mince with sterile scalpel and inoculate to media.
- 3-5 drops of fluid should be inoculated to each tube of media

Concentration:

- Large volume of fluids should be concentrated by centrifugation (1500-2000 X g for 5 minutes) before inoculation to isolation media as a means to enhance the detection and recovery of fungi.

Sputum:

Method: N-acetyl L- cysteine (NALC) treatment

Principle: NALC is mucolytic agent as it has the ability to split the disulfide bonds in the mucoprotein

Stock solution to be prepared:

1. 2.94% of Na-citrate in distilled water – autoclaved at 121^{o}C x 15 min
2. 0.5 g of NALC in 100 ml of Na-citrate (to be freshly prepared)
3. M/15 phosphate buffer (pH 6.8-7.1)

Procedure: -

Specimen to be vortexed with sterile glass beads

↓

Equal volume of Na-citrate and NALC to be added to the specimen; to be vortexed again for 10-30 sec

↓

Dilute mixture in the phosphate buffer by adding double the volume and centrifuge at 1000 g for 15 min

↓

Use sediment to prepare smears for direct microscopy and to inoculate on media for culture

Blood

Buffy coat – buffy coat preparation may be done for intracellular organism and can be stained either by PAS, Giemsa or Calcofluor

<u>Biphasic Medium</u>

- At least 3-5ml blood is cultured in biphasic medium (a ratio of 1:10 to 1:20 blood to broth is utilized)
- Two bottles can be utilized. One can be incubated at 25^{0}C and the other at 37^{0}C.
- After 48h, 5 days, and 7 days incubation, tilt the bottle in such fashion that fluid covers the whole agar surface; but does not reach the neck of the bottle. Keep the bottle in this position for one hour

<u>Broth culture (usually used for bacteria</u>)

- This can be used for isolation of yeast
- Blood is inoculated into a broth media (liquiod and bile)
- Subculture on solid media after 1, 2, 3, 7 days of incubation/ examine macroscopically for evidence of growth after similar incubation, make a smear and subculture on solid media.

- Incubate the solid media at least for **48 hours**
- Examine every day macroscopically for yeast growth (initially it may be very tiny, pin point colony**)**

Membrane filter technique

- Membrane filtration apparatus (0.45 □m) is used 50 ml of sterile triton-X isolation (0.2%) placed in the filter apparatus. After centrifuging and washing the filter, membrane is placed onto the SDA plate with the inoculated surface up. Seal the dish with parafilm and incubate at 30^{o}C

Lysis centrifugation system

- The Isolator (Wampole Laboratories, Cranbury, NJ, USA) or manual lysis centrifugation system appears to be most sensitive method for recovery of *Histoplasma capsulatum,* other dimorphic fungi and filamentous fungi
- Isolator specimen should remain at room temperature until processing, optimally within 16 hours of collection
- The sediment is streaked on varieties of media without cycloheximide
- On chocolate agar all fungi can grow well except *H. capsulatum*
- For *H. capsulatum* plates should be incubated at least for 3 weeks

Automated, continuously monitoring blood culture system

- ESP system (Trek Diagnostics, Westlake, Ohio, USA), Bac T/Alert system (Organon Teknika Corp, Durham, NC, USA) and Bactec system (Becton Dickinson Microbiology system, Sparks, MD, USA)
- 5ml of blood is sufficient
- The Bactec Myco/F Lytic medium can be used both for Mycobacterium and fungi

Body fluid, urine:

Filtration or centrifugation at 2000 X g for 10minutes

Exudates, pus, drainage*:*

Washing, centrifugation and crushing of granules

Cerebrospinal fluid

- No characteristic pathognomic feature seen
- Pressure - normal or increased.-Glucose - normal or slightly decreased (10-40 mg/dL)
- Protein - usually elevated, sometimes very high (50-1000 mg/dL)
- Alcohol content - rarely helpful
- Pleocytosis - rarely exceeds 300 (range 20-1000/mm^3)
- Predominant lymphocytes (*Cryptococcus, Histoplasma*)
- Predominant polymorphs (*Aspergillus, Candida*)Organisms
  - –Positive in *Cryptococcus* (~50% in India ink)*, Candida*
  - –Rarely in *Histoplasma, Aspergillus*
  - –Negative in intracerebral lesion
  - –Gram stain help in *Candida, Nocardia* & *Actinomyces*
- Difficult to isolate fungi from CSF except *Cryptococcus neoformans*
- *C. immitis, H. capsulatum, B. dermatitidis* infection occasionally positive
- *Candida, Aspergillus*, Zygomycetes – very low yield
- To improve isolation of fungi

- -Always culture 10-30ml of CSF
- -Centrifuge CSF - culture & microscopy of sediment; supernatant may be used for antigen detection
- -Repeat lumbar puncture at least 3 times
- -May collect sample even after start of therapy as fungi can be isolated till 3rd day of therapy
- -Consider cisternal puncture if lumbar puncture is negative

Catheter tip for isolation of fungi:

- External dressing carefully removed
- Alcohol used to remove blood, or antibiotic ointment from the site of catheter insertion
- Catheter is withdrawn through the skin
- Sterile forceps may be used to remove the catheter
- Short intra-vascular catheters: shaft cut off from the catheter hub with sterile scissor and dropped into a sterile vessel or cup (the catheter segment length should be 2.5 - 5 cm)
- Larger indwelling catheters: distal 5 cm should be aseptically collected
- Purulent exudates: if present at the catheter exit site, a sample should be collected for Gram stain and culture
- Catheter tip rolled across the surface of the culture plate at least four times
- The laboratory shall report the colony count for the catheter culture
- Sub-cultures of *Candida* from line tips stored at 4^0C and sent to central lab

Difficult situation in isolation of fungi from clinical specimen

Isolation of pathogenic fungi from tissue specimen mixed with yeasts/ bacteria: on certain occasions though we can demonstrate the fungal hyphae on direct microscopy, the isolation of fungi from those tissue will not be possible due to various reasons. Colonization/contamination with yeast or bacteria is one of the most common reasons. Fungi can be isolated from those specimens either by acidification of SD broth or using peptone glucose fluconazole agar.

(i) Acidification of Sabouraud dextrose broth

1. Wash the tissue specimen to be processed using sterile distilled water and cut into small bits.
2. Take 5 test tubes containing equal volume (5-10ml) of SDA broth and add one drop of 1N HCl to the first tube, 2 drops to the second, three drops to the third and 4 drops to the forth and 5 drops to the fifth.
3. To each test tube add a small bit of tissue and incubate at 30^0C for 24 hours.
4. Subculture 0.1 ml of broth into brain heart infusion agar plate. Incubate the plates at 37^0C and 30^0C.
5. Pick up the mycelia colonies, if any and subculture on fresh plate /tube.

(ii) Use of Peptone Glucose fluconazole agar for isolation of mycelia fungi from specimen contaminated with yeast

1. Homogenize sputum / BAL inoculated on peptone glucose fluconazole agar (Peptone 10 g, glucose 20 g, agar 20 g, Chloramphenicol 40 mg, gentamicin 25mg, fluconazole 5mg, distilled water 1000 ml, pH 6.8-7.0)
2. Incubate the media at 37^0C and 25^0C

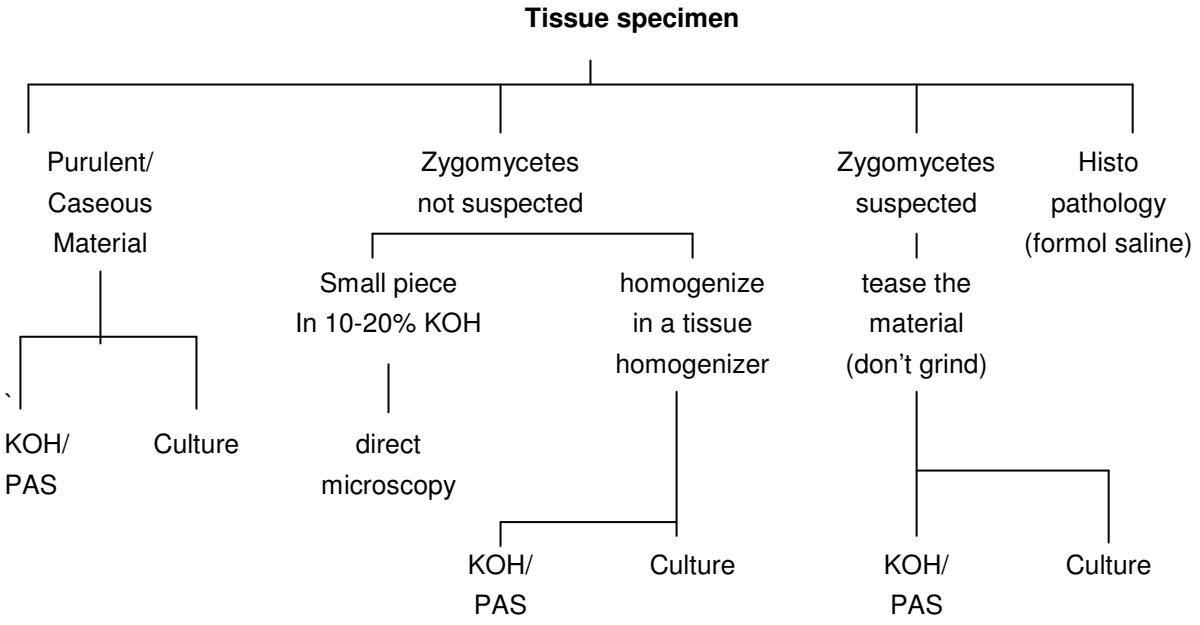


Fig. 1 : Processing of Tissue Specimens

Direct microscopy

Direct microscopy is of immense importance for mycological investigation of clinical specimens. Unlike bacteria, fungi may take pretty long time to grow in culture. Moreover, many fungi are saprophytic. Therefore, clinical situation should be correlated with direct microscopy findings and culture.

Direct Microscopy comprises of:

Preparation of reagents and stain – see Annexure-A

Wet mount	**Stains**
1) KOH (10-20%)	1) Giemsa
2) India ink/Nigrosin	2) Rapid Giemsa
3) Calcofluor white	3) Gomori's methenamine silver (GMS)
4) Methylene blue	4) Periodic acid schiff (PAS)
5) Fluorescent antibody	5) Grocott's
	6) Mayer's mucicarmine
	7) Masson Fontana
	8) Alcian blue
	9) Wright
	10) Toluidine blue

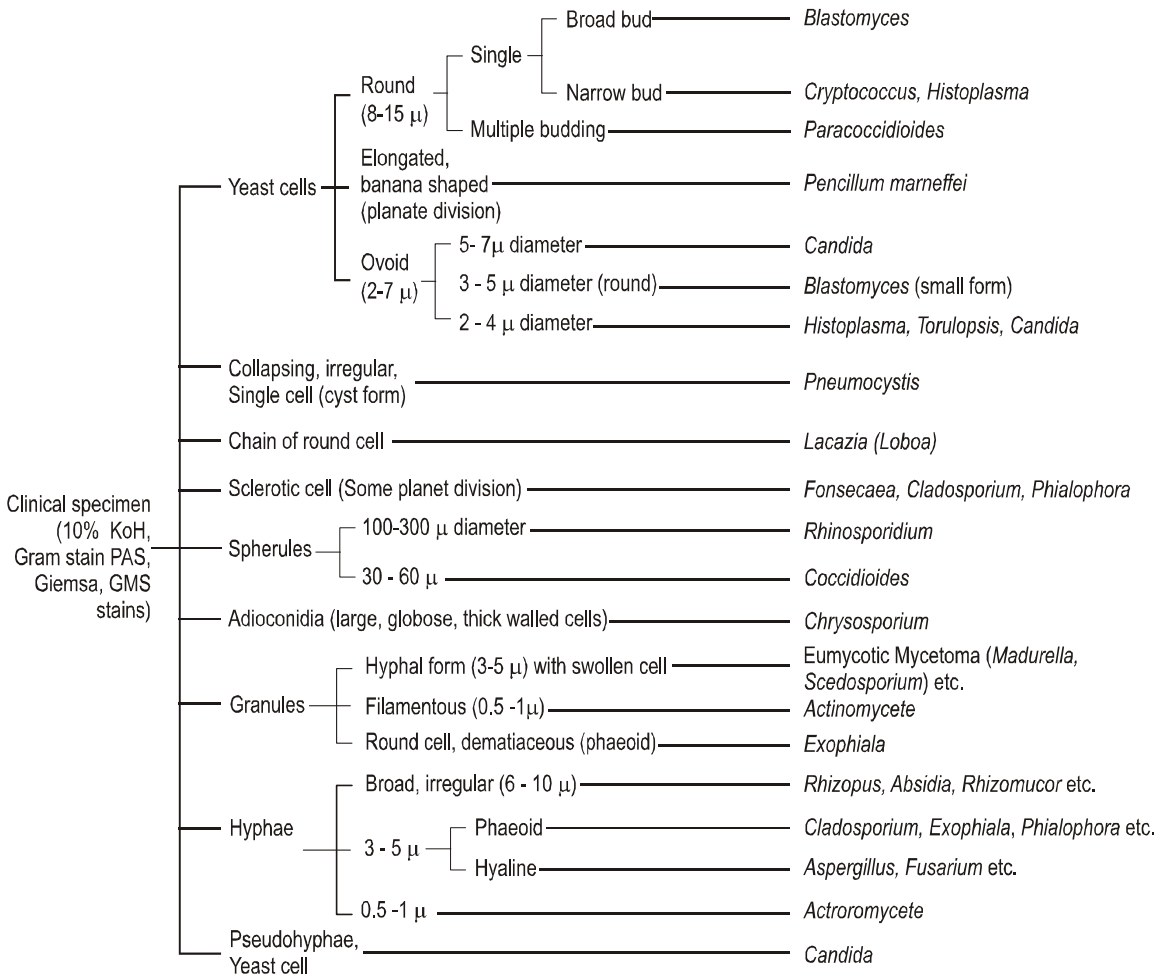


Fig 2 : Direct microscopical examination of clinical samples

1) 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% may be used when not cleared by 10-20% KOH.

(3) Used for specimen such as sputum, pus, urine sediment, homogenate from biopsy tissue 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 Pastuer 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 branching hyphae, type of branching, the colour, septation and thickness of hyphae, budding yeast cells.

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.

Advantage of KOH preparation

Simple, cheap and rapid

Disadvantage

- 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 fibers, starch grains (in pleuritis) or cholesterol crystals.
- It gives an idea about the presence of hyphal element, but cannot distinguish different fungi.
    (1) Preparation cannot be kept for too long; but drying can be prevented /prolonged by keeping the slides in a moist chamber.

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 restricts 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. So a negative control should be put up every day.

2. KOH-Calcofluor white (CFW) solution mixture:

Principle:

CFW stain may be used for direct examination of most specimens using fluorescent microscopy. The cell wall of the fungi (β-1,3 and β-1,4 polysaccharides, specifically cellulose & chitin of cell wall in fungi) binds the stain and fluorescent blue-white or apple green depending on the filter combination used. The CFW and its related compounds like Uvitex 2B and Blankophor are non-specific flourochromes, which are used in the textile industry and with the addition of KOH will enhance the visualization of fungal element in specimens for microscopic examination. Fungal elements appear bluish-white against a dark

background when excited with UV or blue-violet radiation. Optimal fluorescence occurs with UV excitation. A barrier filter 510, 520 or 530 nm should be used for eye protection. Typical *Pneumocystis jiroveci* cysts are 5-8 µm in diameter, round and uniform in size and they exhibit characteristic peripheral cyst wall staining with an intense internal double parenthesis like structure. Yeast cells are differentiated from *Pneumocystis jiroveci* by budding and intense internal staining. Human cellular materials and cotton fibers may exhibit non-specific fluorescence. KOH–CFW preparations may be preserved for several days at $4^{o}C$ in a humid chamber.

Procedure:

a) Place the material to be examined onto a clean glass slide

b) Add a drop of KOH-Calcofluor solution, or mix in equal volumes before processing.

c) Mix and place a cover glass over the material

d) If necessary, allow the KOH preparation to sit at room temperature ($25^{0}C$) for a few minutes until the material has been cleared. The slide may be warmed to speed the clearing process.

e) Observe the preparation by UV microscopy. Calcofluor may not stain strongly dematiaceous fungi. If such is suspected, the preparation should be examined by bright field microscope. *Candida glabrata* may fluoresce only very faintly. Elastin and collagen will also fluoresce, but with a yellow-green fluorescence.

Quality control:

1. Check the reagent prior to use, weekly, and with each new batch of calcofluor prepared.
2. Using an aqueous suspension of actively growing *Candida albicans*, the yeast cell walls will be bright green or blue white depending upon ultraviolet filters used.
3. Negative control consists of KOH and calcofluor combined.

3. India ink or Nigrosin preparation for identification of *C. neoformans*

(1) The preparation is to be made in the centre of a clean, grease-free, glass slide.

(2) Put one 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 little amount of water may be applied on the edge of the cover-slip and the cover-slip gently tapped. This dilutes the stain to some extent).

(3) Put one 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 on 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 practicable.

(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 (covered petri dish with a wet filter paper on which a triangular glass rod is placed).

(8) Scan the entire cover slip from end to end in a zigzag fashion. Encapsulated yeast (*Cryptococcus neoformans)* is 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.

It should be noted specially that

(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. While 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 case of Nigrosin stain the preparation dries up quite fast, 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 preparation.

(2) The stain should be regularly checked for contamination by checking only the stain under microscope.

(3) False positive readings may occur with air bubbles or monocytes or neutrophils. Air bubbles, under the 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.

4. Direct staining on vinyl plastic tapes:

Scotch brand tapes #681 and # 473 are recommended for use in stripping and staining for fungal infection of the horny epidermis. The tape with material, adhesive side up is placed on a slide and stained for one minute with Hucker Crystal violet, Loeffler methylene blue, or Giemsa solution. It is then rinsed with alcohol for a moment, dried, and mounted in permanent or temporary mounting media.

5. Gram's stain

Gram stain is usually a poor stain to use when examining a specimen for a fungus. Gram stain may be used when examining smears of *Candida, Malassezia*, and *Sporothrix* but should not be relied upon to demonstrate the yeasts of the other dimorphic fungi. All fungi are gram positive. It is most useful for demonstrating mycelial elements and budding yeasts cells in sputa, vaginal secretions, purulent material, gastric washing, lung aspirates and urine.

(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 directly fall on the smear.

(6) Add Gram iodine solution to cover the smear and leave for 1 minute.

(7) Rinse with water in the same way as above.

(8) Flood with acetone for about 30 seconds.

(9) Rinse again with water.

(10) Counter stain with safranin for 30 seconds, and rinse in water.

(11) Dry in air, and observe.

Observation

Gram reaction (positive or negative), size, shape and arrangement of elements should be observed under the oil immersion field.

6. Giemsa Stain:

This stain is used when intracellular structures are to be examined like the yeast cells of *Histoplasma capsulatum*. The intracellular cells of *H. capsulatum* stain light to dark blue and have a hyaline halo. The halo is not a capsule, rather a staining artifact. This stain can also be used to visualize trophozoite stage of *Pneumocystis jiroveci*. It is a compound stain formed by the interaction of methylene blue & eosin.

(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

Intracellular budding yeast.

7. Rapid Giemsa Stain (Diff-Quick or Giemsa Plus)

Precautions:

This stain is never to be used alone. Always a cell wall staining method should accompany, for 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.

8. Periodic acid Schiff (PAS) stain:

It is histopathological stain used to detect fungi in clinical specimens, especially yeast cells and hyphae in tissues. Fungi stain bright pink-magenta or purple against an orange background if picric acid is used as the counter stain or against a green background if light green is used. The procedure is a multi-step method combining hydrolysis and staining.

Principle: The periodic acid oxidizes, 1, 2 – glycol groupings of the fungus polysaccharides to aldehyde groups. The reactive aldehyde groups' combine with the basic fuschin in such a manner that it cannot be bleached out when treated with sodium metabisulphite.

Precaution:

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 should be kept in a dark bottle. The stock of Periodic acid (a white powder) should be kept in a dark bottle. The sodium metabisulphite solution is unstable. Deterioration of this reagent is suspected when the control slides show no evidence of fungi after having been subjected to a bleaching process e.g. 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 fuschin 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%) if 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.

Interpretation: Occasionally bacteria as well as polymorphonuclear neutrophils may retain the basic function but in neither case should there be any difficulty in differentiating these structures from mycotic elements. PAS stain used in clinical microbiology laboratory is a modification of Hotchkiss-McManus PAS stain used in the histopathology laboratory. The modification of this stain as used in the clinical laboratory

may occasionally stain some cocci and white cell granules as well as diphtheroid, *C. minutissimum*, Differentiation of fungus & tissues is enhanced when counter stained as in Gridley's modification.

9. Gomori's Methenamine Silver Stain (GMS):

Gomori's methenamine silver stain is perhaps the most useful stain for visualizing fungi in tissue. Fungal elements are sharply delineated in black against a pale green or yellow background. They are, however, specialized stains that are more often used in the histopathology laboratory rather than the microbiology laboratory. Grocott's modification of Gomori's methenamine silver stain is commonly used for the histopathological examination of de paraffinized tissue for fungi.It is especially useful as a histopathological tool and for the detection of *Pneumocystis jiroveci*.

Principle: The alcohol groups present in the cell wall of the fungus gets oxidized by the oxidizing agent (chromic acid) to aldehyde group. The latter acts as a reducing agent, which reduces silver nitrate to metallic silver, and in turn stains the fungal cell wall.

GMS (Grocott's modification) delineates fungal elements sharply in black against a pale green background.

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 56^0C) for one hour (smear becomes dark brown).
8. Wash with distilled water or if smear turns black, wash with 0.1% $FeCl_3$.
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 see 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 centers, cup shaped crescents, and cysts with fold like lines (look like 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 "parenthesis" staining black; these curved structures are usually thick (much thicker than the cyst wall).
B. Fungi and Actinomycetes – grey to black.
C. Glycogen, Mucin and red blood cells – rosy taupe to gray.
D. Background is pale green.

Rapid Methenamine Silver Stain

Procedure:

1. Place plastic Coplin jar of 10% Methenamine Silver Nitrate solution in oven at 95^0C.
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, 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 95^0C 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, 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 see under oil immersion.

10. 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. The muco-polysaccharides of the capsule stain deep rose to red, nuclei are black while the other tissue elements stain yellow.

Procedure:

1. Sections are serially passed through xylene, absolute alcohol, 95% alcohol and then washed 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 see under the microscope.

Observation

Cryptococcus neoformans stains rose red, nuclei black and tissue yellow.

11. 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 a metallic green sheen (green scum) rises to the surface of the fluid. Leave for three minutes or more (*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 timings of each step should be standardized in the laboratory for optimal coloration.

(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. marneffei* in bone marrow or peripheral blood smears.

12. Immuno-fluorescent Stain for *Pneumocystis jiroveci*

- Follow package directions (according to manufacturer strictly).
- If negative control slide containing yeast cells exhibits fluorescence or specimen slide is equivocal, do not report specimen positive for *Pneumocystis jiroveci*.
- Always confirm by cyst wall and organism stains in case of positive results.

13. Masson Fontana stain:

It was originally developed for demonstration of melanin granules in mammalian tissue. It is mainly used for the detection of dematiaceous fungi and to a lesser extent *C. neoformans* in histological sections. Fungal elements appear brown to brownish black against a reddish background.

Procedure

- Make a uniform smear (Centrifuged deposits of broncho-alveolar-lavage /sputum sample.) on a clean grease-free glass slide and fix it with methanol or heat.
- Add working silver solution on the smear
- Place the slide in the oven at 60 ^{0}C for one hour or in Microwave oven 20 power for 2 min.
- Check the stain under the microscope for the intensity and if required microwave for few more seconds or keep in oven for few more minutes
- Wash the slide with distilled water
- Add 0.1% gold chloride and allow to stand for 10 min.
- Wash with distilled water
- Add 5% hypo (sodium thiosulfate) and wait for 5 min

- Wash with tap water and then in distilled water

Results

Melanin positive – Black

Nucleii - Red

14. Alcian blue stain:

Alcian blue and the more commonly used mucicarmine stain are mucopolysaccharide stains. These stains are very useful for visualizing *C. neoformans* in histological sections of tissue. Capsular polysaccharide stains blue against a pink background.

Procedure

- Add 3% glacial acetic acid and allow to stand for 3 min
- Add alcian blur solution and allow to act for 30min at room temperature
- Wash with tap water and rinse with distilled water.

Results

Mucin – blue

Nuclei – reddish pink

15. Toluidine Blue stain for *Pneumocystis jiroveci*

It is an easy and rapid stain mainly used for the detection of cysts of *Pneumocystis jiroveci* from lung biopsy specimen imprints and broncho-alveolar-lavage specimens. The cysts appear reddish blue/dark purple against a light blue background. The cysts are usually clumped together, punched in and that gives it a crescent shape. However, trophozoites are not well delineated.

Procedure:

1. Dry slide for 5 minutes.
2. Flood slide with sulfation reagent for 10 minutes. Stir the reagents in between (at least twice in 10 min). Wear gloves and take care when handling this reagent.
3. Remove the slide from sulfation reagent with forceps and carefully wash off reagent and flush down sink.
4. Pour slide in a container and flush with running water for 5 minutes.
5. Place the slide in the Coplin jar containing Toluidine Blue for 3 minutes.
6. Dip the slide in and out of 95% ethyl alcohol (in two jars) for approximately 10 seconds.
7. Then dip the slide in and out of xyelene until clean.
8. Fix the slides with DPX mount or Paramount when the slide is wet with xyelene
9. Observe under the microscope.

Results

Pneumocystis jiroveci cysts appears lavender with of characteristic shape and size (3-5µm diameter).

16. Buffy coat for *Histoplasma capsulatum*:

Hematological smears may be used for examining specimens for *H. capsulatum*, which usually occurs as intracellular yeast. Fluids, exudates, or buffy coats should be spread evenly over a slide and

impression smears may be prepared from tissues. Stain according to directions. Diff-Quick, Wright stain or Giemsa stain may be used.

17. Modified Kinyoun Acid Fast Stain for *Nocardia*:

Principle: *Nocardia* species posses the charaterstics to resist decolorization with acid alcohol.

Procedure:

a) Make a thin smear of the specimen to be stained; fix in methanol or heat. A positive control smear (*Nocardia asteroides*) and a negative control smear (*Streptomyces* sp.) must be included.

b) Flood the smear with Kinyoun carbol fuschin;

c) All to stand for 5 minutes, no heat

d) Rinse with water

e) Flood the smear with 50% ethanol and pour off until excess carbol fuschin is removed

f) Rinse with tap water

g) Decolorize with 0.5% aqueous sulfuric acid for 3 minutes

h) Rinse with tap water

i) Flood with 1% aqueous methylene blue

j) Allow to stand for 1 minute

k) Rinse with tapwater

l) Blot or air dry

m) Examine under oil immersion

Culture techniques

Materials from suspected cases of mycotic infection should be cultured, even though direct examination of the material fails to reveal the presence of a fungus. The use of a variety of culture media at incubation temperatures of 25^{o}C & 37^{o}C is likely to increase the recovery of fungal pathogens. Further, the inhibitory growth patterns of fungi on these various media may aid in their identification. Cycloheximide/Actidione can inhibit a large number of saprobes/opportunistic fungi as well as certain medically important fungi.

No one specific media or combination of media is adequate for all specimens. However, Sabouraud dextrose agar and Brain heart infusion agar are commonly used primary isolation media for primary isolation of fungi. The inoculated media should be incubated at 25 – 30^{0}C. Media is dispensed into containers such as 25 x 150 mm screw cap tubes or 100 mm petridishes. Petri plates offer the advantages of a large surface area for isolation and dilution of inhibitory substances present in the specimen. It must be poured thick with at least 25 ml of medium to resist dehydration during incubation. However, as plates are vented, they are more likely to get contaminated. Therefore, plates may be placed in gas permeable sterile bags or sealed with gas permeable tapes to avoid this disadvantage. The lid must be taped at two points to prevent accidental opening of the plate. All inoculated media should be read every day for a week following incubation and twice weekly thereafter. Plates must be opened only within the biological safety cabinet to prevent contamination of the plate and exposure of personnel to potentially dangerous fungi or *Mycobacterium* spp. Media in tubes have a smaller surface area but offer maximum safety and resistance to dehydration and contamination.

Fungi develop varying characteristics on different media, so it is important to describe the characteristics on a standard medium such as Sabouraud Dextrose Agar (SDA), virtually all the medically

important fungi are normally described by their appearance on SDA, which is one reason this medium continues to be used as the primary isolation medium.

Gross morphology:

Points to be noted:

a) Growth of the fungus (characteristic colony morphology) –

   i) Within 2-5 days – rapid grower

   ii) Within 6-10 days – intermediate grower

   iii) Within 2-3 weeks – slow grower

   iv) The growth rates varies with the type of media and incubating conditions

b) Surface –

   - Flat
   - Hemispherical
   - Raised
   - Folded
   - Verrucose
   - Cerbriform

c) Texture

   - Yeast like
   - Glabrous
   - Powdery
   - Granular
   - Velvety
   - Cottony

Texture of the colony depends upon: -

- Type of media
- Incubation temperature
- Age of the colony

In general, the more granular the texture of the colony the more abundant is the spores.

d) Pigment

Pigmentation of many fungi tends to be variable even within the same strain. The pigmentation on the surface of colony is usually governed by the pigmentation of conidia or spores and is usually not evident until spore formation is significant. The pigmentation on the reverse of the colony is formed by soluble pigments in the medium and pigmentation of the vegetative hyphae.

Tissue Reactions in Fungal Infections

A diagnosis of fungal infections requires a multidisciplinary approach with a high index of suspicion. The diagnosis becomes more fruitful when an aggressive approach is taken in obtaining tissues for mycological and histopathological examination. A diverse morphological pattern seen in fungal infections makes the histological diagnosis more difficult. Hence, one has to be well versed with the histological pattern. In addition, the pattern may mimic other diseases like tuberculosis, necrotizing inflammation or any vascular event like infarct. Both tuberculosis and fungal infections can show granulomatous inflammation. Vascular involvement by fungal infections can result in bland infarct which can simulate any atherosclerotic or thrombotic vascular event.

The factors responsible for varied histologic reactions include host immunity, the presence of intracellular or extra-cellular organism, acute or chronic nature of illness and the presence or absence of tissue macrophages in organs.

Clinically, the fungal infections can mimic tumour, abscess or infarct depending on the type of infections. The different setting of fungal infections include immunocompetent individuals, patients who are subjected to invasive techniques or received multiple infusions and a large number of immune-compromised individual i.e AIDS, Renal transplant recipients, steroid therapy in different clinical settings and liver and renal failure.

The histological techniques used in the diagnosis of fungal infections include a good quality Haematoxylin and Eosin stained slides supplemented by several special stains like PAS, Grocott methanamine silver, Mucicarmine and Diastase PAS with Alcian blue. These stains are used for the detection of fungi. PAS is helpful for the detection of *Candidal* yeast and pseudo-hyphae, cryptococci, *Histoplasma*. Mucicarmine is useful for the detection of Cryptococci. Moreover, a number of Immunohistochemistry are being used for the detection of different fungi.

The most common histopathological features seen in fungal infections are the finding of granulomatous pathology in chronic invasive fungal infections which can mimic tuberculosis, sarcoidosis or foreign body granulomatous reactions. This type of histologic reactions is seen in immuno-competent individuals like in chronic invasive granulomatous fungal rhinosinusitis. The granulomas are composed of collection of epithelioid cells with Langhans' and foreign body type of giant cells surrounded by chronic inflammatory cells. These granulomas are embedded in a fibrous stroma and can mimic a mass lesion clinically; particularly within the central nervous system.

The other histologic features include suppurative granulomatous inflammations like in chromoblastomycosis. This is characterised by collections of neutrophils surrounded by epitheloid cells with giant cells containing fungal structures. The overlying skin or mucus membrane may show extensive hyperplasia mimicking carcinoma.

Sometimes the reactions can be purulent such as acute candidiasis

A large area of bland infarct or necrotizing inflammation is seen classically in acute invasive fulminant fungal infections like rhinosinusitis caused by Zygomycosis. This is characterised by the presence of bland necrosis with vascular involvement. Extensive necrotizing inflammation can be seen. This pathological feature can be confused with vasculitis like Wegener's granulomatosis in lung or in paranasal sinuses. A large number of fungal hyphae are seen in these conditions. PAS stain may not be so helpful as it stains *Zygomycetes* poorly.

In cryptococcal meningitis, no significant inflammatory reactions are seen in the meninges. The absence of inflammatory reactions may be due to these capsulated fungi.

The non-invasive variety like the fungal ball in nasal sinuses or in cavities of lung like bronchiectasis or tubercular cavity can also be seen. In this variety, an entangled mass of fungal balls are seen in a cavity surrounded by extensive inflammatory cell reactions

The other non-invasive variety which is supposedly due to so-called allergic reaction to sparse cavitary fungi, like allergic broncho-pulmonary aspergillosis and allergic fungal rhinosinusitis may complicate the picture. Here, sparse fungal hyphae are seen inside allergic mucin which contains pleny of eosinophils and Charcot layden-crystals. Special stains are always needed to identify the fungal hyphae.

Moreover, many isolated fungal infections are being recognised in our autopsy material like isolated bilateral renal and gastrointestinal zygomycosis.

The identification of fungal hyphae may be helpful with the use of special stains but a mycological examination is always needed to characterise the specific species.

Hence, the diverse histological reactions has to be recognised to make a comprehensive diagnosis because it can change the clinical management of the patient. A mere finding of fungal hyphae may indicate both invasive and non-invasive fungal conditions. Among the invasive there is need to distinguish the chronic granulomatous fungal infections from acute fulminant necrotizing variety. The surrounding tissue reactions are also needed to clinch an accurate diagnosis. Sometimes, there is evidence of both dominant non-invasive and focal invasive component. In addition, there is both acute necrotizing and chronic granulomatous component.

Tissue reactions in fungal diseases

Fungi	**Tissue reaction**	**Morphology of the fungi**
1. *Candida* species	Pyogenic reaction- Acute commonly, chronic occasionally	Budding oval yeast (3-5µm), pseudohyphae, occasional septate hyphae
2. *Aspergillus* species	(a) Acute inflammation, often with vascular invasion and infarction (b) Chronic inflammation, granuloma formation as in chronic fungal rhinosinusitis	Septate hyphae (3-8 µm) with acute angled branching, parallel wall; fruiting head in fungal ball
3. Zygomycetes	Acute inflammation, often with vascular invasion and tissue and infarction	Broad (8-20 µm), aseptate (or sparsely septate), ribbon like, occasionally distorted with unparallel wall
4. *Cryptococcus* species	Caseating granuloma, histocytic granuloma in immunocompetent patients; minimal or no inflammatory reaction (gelatinous appearance) In immunocompromised patients; rarely non-necrotizing granuloma 'sarcoid' type	Spherical yeast cells (2-10 µm) with single bud and having capsule; stain positively with mucicarmine and melanin stains.
5. Sporothrix schenckii	Mixed pyogenic and granulomatous inflammation	Spherical, oval or cigar-shaped yeasts (2-10 µm) with single bud; often not visible in tissue
6. Agents causing chromoblastomycosis	Chronic pyogenic inflammation, epitheloid cell nodule and granuloma, pseudoepithellomatous hyperplasia	Dark colored thick walled spherical cells (5-15 µm with planet division

7. Agents causing phaeohyphomycosis	Mixed pyogenic and granulomatous inflammation	Brown pigmented hyphae (2-6 µm) often constrictions at prominent septation
8. Histoplasma capsulatum	Caseating or non-caseating mature granuloma; chronic inflammatory reactions; histiocytic granuloma; rarely granuloma 'sarcoid' type	Round to oval yeast with single bud , intracellular (macrophages)
9. Blastomyces dermatitidis	Mixed suppurative and granulomatous inflammation; occasionally pseudoepethellomatous hyperplasia	Spherical multinuclear yeasts (8-15 µm) with thick (double contour) walls, and single broad-based buds
10. Coccidioides immitus	Caseating or fibrocaseous granuloma; mixed pyogenic and granulomatous reaction; rarely pseudoepithellomatous hyperplasia	Spherules (20-200 µm) with or without endospores (2-5 µm)
11. Paracoccidioides brasiliensis	Mixed pyogenic and granulomatous reaction; rarely pseudoepithellomatous hyperplasia	Large globose yeast (5-60 µm) with multiple buds attached by narrow necks ('mariner's wheel')
12. Pneumocystis *jiroveci*	Mononuclear cell infiltrate often minimal; rarely granulomatous	Round, oval or cup-shaped cysts (5-6 µm) detected on silver stain
13. Rhinosporidium seeberi	Mixed pyogenic inflammatory reaction; microabscesses are frequent; occasionally granulomatous; occasionally eosinophilis are present in large numbers	Mature sporangia (350 µm) with spores (7-9 µm)
14. *Lacazia loboi* (*Loboa loboi*)	Extensive hyaline fibrosis interspaced with masses of histiocytes and giant cells	Chains of uniform yeast (7-12 µm)

Yeast Identification

Yeasts are heterogeneous fungi that superficially appear as homogenous. Yeasts grow as unicellular form and divide by budding, fission or a combination of both. The true yeasts are those fungi that reproduce sexually, developing ascospores or basidiospores. In contrast, yeast- like fungi or imperfect fungi reproduces only by asexual means. The various yeasts are distinguished from each other based upon a combination of morphological and biochemical criteria. Morphology and the methods of asexual reproduction are primarily used to identify Genera, whereas biochemical tests are used to differentiate the various species.

Approaches for the identification of the yeasts includes:

1. Culture characteristics- Colony color, shape and texture.
2. Asexual structures -
    - Shape and size of cells
    - Type of budding - unipolar (*Malassezia)*, bipolar *(Hansaniaspora kloekera*) multipolar (*Candida*), fission (*Schizosaccharomyces*)
    - Presence or absence of arthroconidia, blastoconidia, ballistoconidia, clamp connections, germ tubes, hyphae, pseudohyphae, sporangia or sporangiospores.
3. Sexual structures- arrangement, cell wall, ornamentation, number, shape and size of ascospores or basidiospores.
4. Physiological studies-
    - Sugar fermentation
    - Sugar assimilation
    - Nitrogen utilization
    - Urea hydrolysis
    - Temperature studies
    - Gelatin liquefaction

Identification Procedures:

1. <u>Isolation techniques for mixed cultures</u>

Before proceeding for the identification of the yeast it is necessary to confirm the purity of the yeast because initially isolated yeast may be contaminated or in mixed culture.

Purification of the yeasts can be done by the following techniques.

<u>A. For bacterial contamination</u>

i) Isolation on Sabouraud dextrose agar(SDA)

- Suspend a colony of the yeast in sterile distilled water.
- Inoculate a loopful of suspension on SDA plate.
- Incubate at 25^0 C for 48 hours.
- Prepare a wet mount from a isolated colony and verify the purity of the colony.
- If the colonies are not pure further step are necessary (step ii).

ii) Isolation on SDA agar plus chloramphenicol and gentamicin / SDA plus penicillin and streptomycin / BHI plus 10% blood plus chloramphenicol and gentamicin.

- Suspend a colony of the yeast in sterile distilled water.
- Inoculate a loopful of suspension on any one of the above media.
- Incubate at 25^0 C for 48 hours.
- Prepare a wet mount from an isolated colony and verify the purity of the colony.
- If the colonies are not pure further step are necessary (step iii)

iii) Acidification of SDA broth

- Suspend a colony of the yeast in sterile distilled water.
- To each of the 4 tubes containing 10ml of the SDA broth, add one drop of 1 N HCl to the first tube 2 drops to the second tube, three drops to the third tube and 4 drops to the fourth tube.
- Add 0.5 ml of yeast suspension to each tube.
- Incubate at 25^0C for 24 hours. Check for the purity.

<u>B. For mixed yeasts</u>

- Suspend a colony of the yeast in sterile distilled water
- Streak a loopful of the suspension onto a SDA agar plate.
- Incubate at 25^0C for 48 hours.
- Check for the purity. If the yeasts are not pure the procedure must be repeated. Note some strains may have both rough and smooth colony types in pure culture.

<u>C. For mould contamination</u>

i) Isolation on yeast malt agar(YM)

- Suspend a small portion of yeast-mould colony in a tube containing sterile distilled water
- Inoculate a loopful of the suspension on YM agar plate.
- Incubate the plates at 25^0 C for 4-6 days.
- Check for the purity of the colony. If not pure, further steps are necessary (Step ii).

ii) Isolation in yeast malt broth

- Suspend a small portion of the yeast -mould isolate to a tube containing 10 ml of broth.
- Incubate the YM broth at 25-30^0C for 48 hours. Using a capillary pipette, remove a small portion of sediment carefully without disturbing the mycelial pellicle.
- Inoculate the sediment on YM agar.
- Incubate at 25^0 C for 4-6 days.
- Check for the purity of the culture by examining the wet mount. If the culture are not pure further steps are necessary (Step iii).

iii) Isolation in shake culture

- Streak a small portion of the yeast -mould isolate into YM broth in an Erlenmeyer flask (approx. 100-ml).
- Incubate the flask in a rotary shaker at 30^0C for 4-6 days.

- Remove a small amount of the sediment with a sterile capillary pipette taking care not to take the mycelial balls by accident.
- Inoculate the sediment onto YM agar
- Incubate at 25-30^{0}C for 4-7 days and prepare a wet mount using a small portion of the yeast colony.

2. Direct mounts

Direct mounts are made to study yeast microscopic morphology and to determine the purity of the isolates. Direct mounts are made using Lactophenol Cotton Blue (LCB).

3. Germ-tube test

Germ tube test is used for presumptive identification of *Candida albicans*. It is a rapid screening test where the production of germ tubes within two hours in contact with the serum is considered as indicative of *Candida albicans*. This test has to be validated with Corn Meal Agar (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 37^0 C for exactly two hours.

(4) Place one drop from the incubated serum on a slide with a cover slip. Observe under the microscope for production of germ tubes. Germ tubes 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 germ tube 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:

(2) The medium, inoculum size, temperature of incubation, concentration of simple carbohydrates and microaerobic conditions influence GT formation.

(3) Increased concentration of inoculum causes a significant decrease in the percentage of cells forming germ tubes. Maximum percentage of GT formation occurs when 10^5 to 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.

(4) Approximately 95% of clinical isolates of *C. albicans* produce germ tubes when incubated in serum at 370C for 2-3 hr.

(5) A neutral pH (7.4) facilitates maximum development of germ tubes. Bacterial contamination may interfere with production of germ tubes.

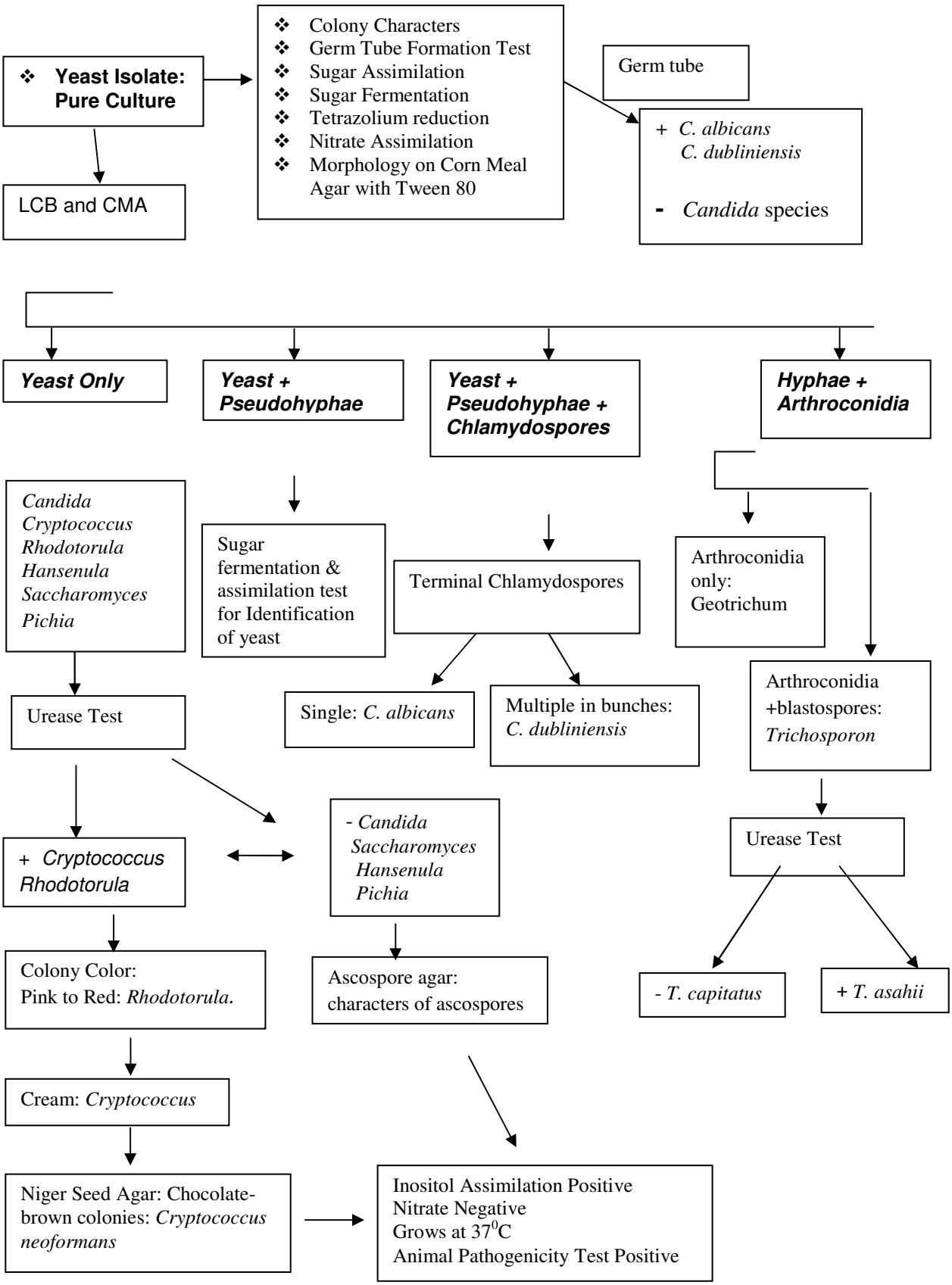
(6) Antimicrobial substances in the isolation medium may interfere with this test; human serum may contain inhibitory substances (such as ferritin) that suppress GT development.

(7) Controls – *C. albicans* and *C. tropicalis* are run with each group of GT determinations to serve as positive and negative controls respectively.

4. Morphological characters on Corn Meal Agar (Dalmau plate)

- Prepare Cornmeal agar containing 1% Tween 80 in a 90mm plate. Divide the plate into 4 quadrants and label each quadrant.
- Using a sterile needle or straight wire, lightly touch the yeast colony and then make 2-3 streaks of approximately 3.5 - 4cm long and 1.2 cm apart.
- Place a flame sterilized and cooled 22mm square cover glass over the control part of the streak. This will provide partially anaerobic environment at the margins of the cover slip.
- Incubate the plates at 25°C for 3-5 days.
- Remove the lid of the petri plate 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 objective (40X).
- Morphological features like hyphae, pseudohyphae, blastospores, ascospores, chlamydospores, basidiospores or sporangia are noted.

Yeast Identification Scheme



Yeast Morphology-Flow Chart

<table>
<tr><td colspan="3">Brown to black colony</td><td colspan="2">Direct Mount</td><td></td><td>Yeast only</td><td>Phaeococcomyces</td></tr>
<tr><td colspan="3" rowspan="2"></td><td colspan="2" rowspan="2"></td><td rowspan="2"></td><td>Yeast and hyphae</td><td>Aureobasidium
Exophiala
Wangiella</td></tr>
<tr><td rowspan="2">Pink to red colony</td><td rowspan="2">Satellite colonies</td><td>Present</td><td colspan="3">Forcibly discharged conidia present</td><td>Sporobolomyces</td></tr>
<tr><td colspan="4">Absent</td><td>Purify</td><td>Rhodotorula</td></tr>
<tr><td colspan="3" rowspan="2">White colony</td><td rowspan="2">Germ Tube Test</td><td colspan="2">Positive</td><td>Candida albicans</td></tr>
<tr><td>Negative</td><td>Purify</td><td>Dalmau plate
YBC-Vitek</td></tr>
<tr><td colspan="3" rowspan="2">Moist mycelial colony</td><td rowspan="2">Direct Mount</td><td colspan="2">Arthroconidia</td><td>Geotrichum
Trichosporon</td></tr>
<tr><td colspan="2">Other types of conidia</td><td>Hypohomycete</td></tr>
</table>

Morphology on Corn Meal Agar with Tween 80

Terminal Chlamydoconidia	*C. albicans*, *C. dubliniensis*
Abundant Pseudohyphae, pine forest arrangement, blastoconidia formed at or in between septa.	*C. tropicalis*
Elongated yeasts, Abundant Pseudohyphae (matchstick like appearance)	*C. krusei*
Giant Hyphae, blastospores at nodes	*C. parapsilosis*
Scant Pseudohyphae with chains of blastoconidia	*C. guilliermondii*
Yeasts only	*C. glabrata, C. famata, Pichia anomala, P. augusta*, *Cryptococcus neoformans*
Short, distinctly curved pseudohyphae with occasional blastoconidia at septa	C. lusitaniae
Arthroconidia with Blastoconidia	*Trichosporon* spp.
Arthroconidia without Blastoconidia	*Geotrichum* spp.

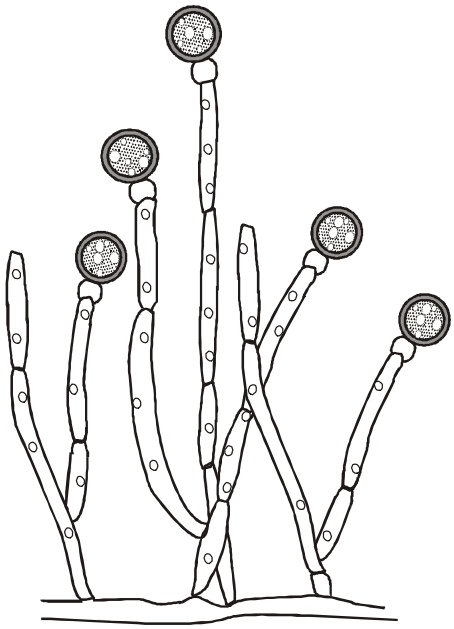


Fig. 7 : *Candida albicans*

C/C (SDA) White to cream coloured, smooth, soft.

MM (CMA) Hyphae and pseudohyphae formed with clusters of blastospores (4-5 □m) at internodes. Terminal chlamydospores (8-12 □m) formed in most species.

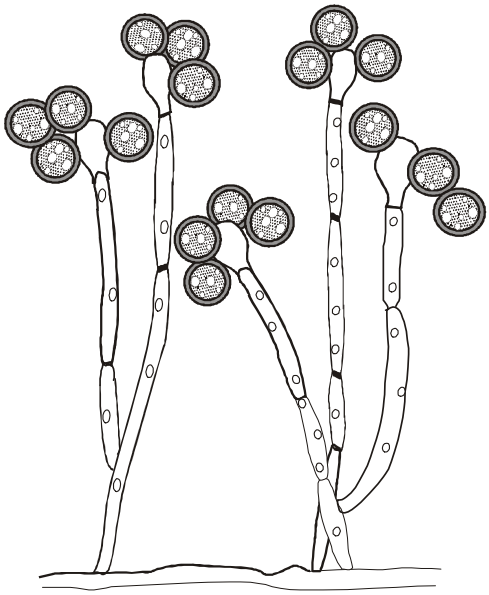


Fig. 8 : *Candida dubliniensis*

C/C (SDA) White to cream coloured, smooth, soft.

MM (CMA) Hyphae and pseudohyphae formed with clusters of blastospores (4-5 □m) at internodes. Terminal chlamydospores (8-12 □m) formed in clusters.

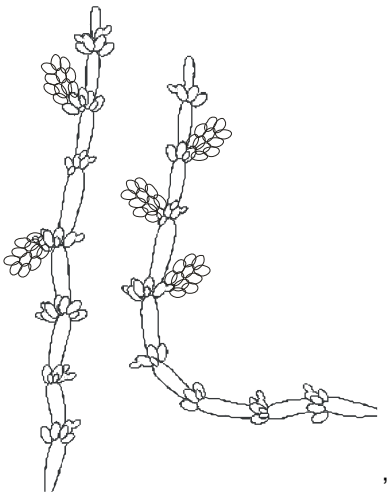


Fig. 9 : *Candida guilliermondii*

C/C (SDA) White to cream coloured, butyrous.

MM (CMA) Budding cells spherical to sub spherical to broadly ellipsoidal. Pseudomycelium may be present, hyphae not produced.

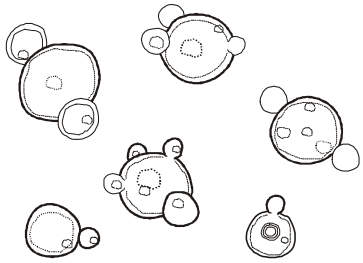


Fig. 10 : *Candida famata*

C/C (SDA) White to cream coloured, butyrous.

MM (CMA) Budding cells broadly ellipsoidal. 3.5 to 2-3.5 □m Pseudomycelium absent.

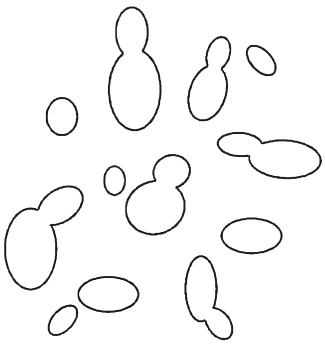


Fig. 11 : *Candida glabrata*

C/C (SDA) White to cream coloured, soft, glossy and smooth.

MM (CMA) Pseudomycelium absent. Some strains may form a few branched chains of ovoidal cells.

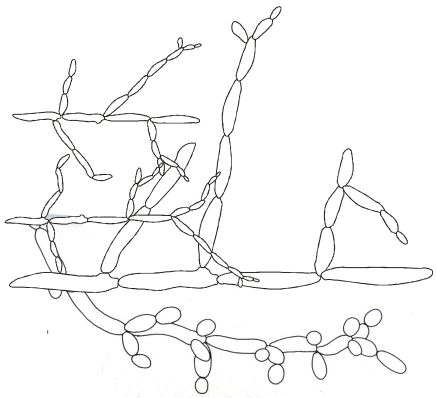


Fig. 13 : *C. parapsilosis*

C/C (SDA) Cream coloured to yellowish, glistening and soft, mostly smooth or partly or entirely winkled.

MM (CMA) Fine and coarse psedomycelium (giant forms). Blastospores (3.7 □m) single or in short chains at septa or terminal ends.

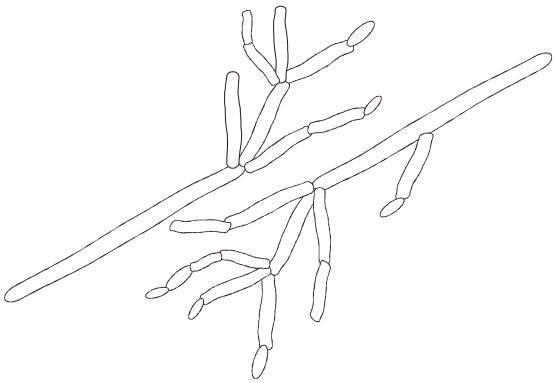


Fig. 14 : *Candida krusei*

C/C (SDA) White to cream coloured, butyrous.

MM (CMA) Budding cells broadly ellipsoidal to cylindrical (4-5 x 2.5 □m). Pseudomycelium often present, robust.

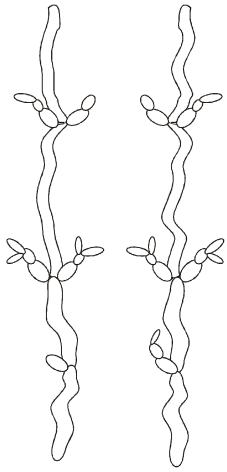


Fig. 15 : *Candida lusitaniae*

C/C (SDA) White to cream coloured, glistening, soft and smooth.

MM (CMA) Budding cells ellipsoidal. Hyphae and germ tubes absent; well developed Pseudomycelium often present, robust.

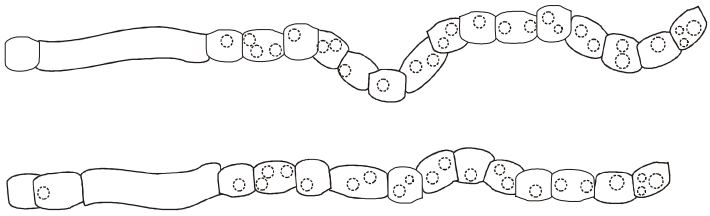


Fig. 12 : *Trichosporon species*

C/C (SDA) Colonies restricted, white, farinose, irregularly folded at the center, with a flat marginal zone.
MM (CMA) Budding cells and lateral conidia absent. Arthroconidia cylindrical.

5. Ascospore Production & Detection 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. Ascospores are produced under limited nutrients in the media.

The commonly used media are:

Malt extract agar (5% malt extract and 2% agar)

Acetate agar (0.5% sodium acetate trihydrate and 2% agar pH 6.5 - 7.0)

V-8 juice agar (commercially available)

Procedure: -

- Inoculate the yeast onto ascospores producing agar plates.
- Incubate aerobically at 25 ^{o}C.
- Examine the culture in 3-5 days and weekly thereafter for 3 weeks.
- Prepare wet mount of the yeast in distilled water.
- Examine the wet mount under oil-immersion objective.
- Observe for ascospores form, surface topography, size, color, brims and number of ascospores per ascus.
- If the ascospores are not seen in a wet mount, perform modified acid-fast stain (ascospores are acid fast).
- *Saccharomyces cerevisiae* should be included as positive control for production on media and staining procedure.

Ascospore morphology on Malt Extract Agar, (2-5 days growth)

1-4 hat shaped Ascospores in Asci	*Pichia anomala*, *Pichia norwegenesis*, *C. utilis* and *C. cifferi*
1-4 spherical or short ellipsoidal Ascospores	*Saccharomyces cerevisiae*
1-2 / ascus, spherical with warts	*C. famata*
4/ ascus, ellipsoidal with slimy sheath	*Blastoschizomyces capitatus*
1-2/ ascus, spherical	*C. krusei*
1-4/ ascus, spherical to hat shaped, protuberances on one or two edges	*C. lipolytica*
1-4/ ascus, clavate	*C. lusitaniae*

6. Ballistospore Formation:

Forcibly discharged conidia or ballistoconidia formation is an important criterion to distinguish *Sporobolomyces* from *Rhodotorula*. Presence of satellite conidia on the agar plate is the first indication for the formation of ballistoconidia.

Procedure: -

- Prepare the plates by pouring thin layer of malt extract agar or yeast extract agar on the top and bottom of the petri plate.
- Inoculate the bottom part of the plate with the yeast. Cover the inoculated part with the top part containing agar.
- Incubate the plate at 25 ^{o}C for 2-3days so that the inoculated surface is on the top.

Formation of the ballistospores can be seen by new growth in the form of mirror image of the original colony on the uninoculated bottom surface.

Sugar Fermentation

Procedure:

- Prepare liquid fermentation medium containing peptone (1%), sodium chloride (0.5%), Andrade's indicator (0.005%). Sterilize by autoclaving at 120°C for 15 min at 15 pounds pressure. Add filter-sterilized sugar at the concentration of 2% to the medium. Pour into the sterile test tubes (approx. 5ml) and place sterile Durham's tube into each tube.
- Plug the tubes with colour coded cotton plugs.
- Inoculum preparation is done by suspending heavy inoculum of yeast grown on sugar free medium.
- Inoculate each carbohydrate broth with approximately 0.1 ml of inoculum.
- Incubate the tubes at 25°C up to 1 week. Examine the tubes every 48-72hrs interval for the production of acid (pink color) and gas (in Durham's). Production of gas in the tube is taken as fermentation positive while only acid production may simply indicate that carbohydrate is assimilated.

10. Sugar assimilation test (Auxanographic technique)

The Disc Impregnation - Pour plate Auxanographic method of Wickerham and colleagues has withstood the test of time and are easier to perform than the liquid auxanographic technique. (which were claimed to be more sensitive and specific, but were later not found so).

- Preparation of yeast nitrogen base.

Yeast nitrogen base is prepared using the ingredients as follows:-

Potassium dihydrogen orthophosphate (KH_2PO_4)	-	1.0g
Magnesium sulfate ($MgSO_4$)	-	0.5g
Ammonium sulfate (NH4SO4)	-	5.0g
Noble agar	-	25.0g
Distilled water	-	1 L

Autoclave at 115^0C for 15 min.

OR

- Prepare YNB and agar separately as follows:

1. YNB (Difco) - 6.7gm
   Distilled water - 100ml

Sterilize by filtration and store at 4°C.

2. Agar - 20.0g
   Distilled water - 980ml

Dispense in 18-ml quantities in 18 X 150mm screw-capped tubes. Autoclave at 121°C and store at 4 °C.

- Prepare a yeast suspension from a 24-48 hrs old culture in 2ml of YNB by adding heavy inoculum. Add this suspension to the 18ml of molten agar (cooled to 45 °C) and mix well. Pour the entire volume into a 90mm petri plate.
- Allow the petri plate to set at room temperature until the agar surface hardens.
- Place the various carbohydrate-impregnated discs onto the surface of the agar plate.
- Sugar discs can be obtained commercially or can be prepared as follows:

Punch 6mm-diameter disc from Whatman no. 1 filter paper. Sterilize the disc by placing them in hot air oven for 1h. Add a drop of 10% filter sterilize sugar solution to each disc. Dry the disc at 37 °C and store at 4 °C in airtight container.

- Incubate the plates at 37^0 C for 3-4 days.
- The presence of growth around the disc is considered as positive for that particular carbohydrate. Growth around glucose disc is recorded first which serves as positive control (viability of yeast).

9. Nitrate Assimilation Test (Auxanographic technique): -

Preparation of Yeast Carbon base:

Potassium dihydrogen orthophosphate (KH_2PO_4)	-	1g
Magnesium sulfate ($MgSO_4$)	-	0.5g
Glucose	-	20g
Noble agar	-	25g
Distilled water	-	1L

Mix the reagents by boiling and autoclave at 121^0 C for 15min.

OR

Prepare yeast carbon base and agar separately:

a).	Yeast carbon base (Difco)	-	11.7g
	Distilled water	-	100ml

Sterilize by filtration and store at 4 °C until use.

b).	Agar	-	20g
	Distilled water	-	980ml

Autoclave at 121^0 C for 15min. Dispense in 18ml quantities in 18 X 150mm screw-capped tubes.

- Make a yeast suspension in molten YCB agar and pour into 90mm diameter petriplate and allow cooling.

OR

- Prepare the suspension from a 24-48hrs culture in 2ml of YCB equal to Mc Farland No. 1 standard. Add this suspension to 18ml of molten agar cooled at 45 °C. Mix well and pour into 90mm petriplates.
- Allow the plate to cool to room temperature.
- Divide the plate into two halves and label them.
- Place the potassium nitrate disc (KNO_3) and peptone disc on the surface of the agar on corresponding labeled halves.
- Potassium nitrate discs are prepared as follows:

  Potassium nitrate - 30g

  Distilled water - 1 L

  Mix the reagents and autoclave at 15psi for 15 min. Take 6mm disc (punched from Whatman No. 1 filter paper) and saturate with the potassium nitrate solution and dry the discs in sterile petri plate and store at 4°C until use.

- Peptone disc is prepared similarly as KNO_3 disc.

  Peptone - 30g

  Distilled water - 1 L

Incubate the plate at 37°C for 7 days. Check for the growth around the disc. The test is considered valid if the growth is present around the peptone. The growth around the KNO_3 impregnated disc is considered positive.

10. Urea Hydrolysis:

The Christensen's urea agar is recommended and is prepared according to the manufacturer instructions. Using a loop inoculate a small amount of the yeast colony on the agar surface.

- Inoculate appropriate control (*C. albicans* - negative control, *C. neoformans* - positive control).
- Incubate the slant at 25^0 C for 2-5 days. Read the test and the control tubes.
- A deep pink color indicates a positive test.

11. Rapid Urea Hydrolysis:

The rapid urea hydrolysis test is done in microtitre plates and used to screen for *C. neoformans.*

- Reconstitute each vial of Difco urea® broth with 3ml of sterile water on the day to be used.
- Dispense 3-4 drops into each well of the microtitre plate.
- Transfer a heavy inoculum of freshly isolated colony of yeast into urea broth into a well.
- Seal the plate with a tape and incubate for 4 hrs at 37^{0}C.
- Pink to red color is positive test.
- Similarly inoculate the controls, *C. neoformans* as positive control and *C. albicans* or uninoculated well as negative control.

12. Temperature Studies:

Another important step in the identification of the yeast is by determining the ability to grow at an elevated temperature. It can be used to distinguish *C. neoformans* from other species of *Cryptococcus* and *C. dubliniensis* from *C. albicans*.

Procedure: -

- Inoculate two tubes of malt extract agar with the isolate.
- Incubate one tube at 37 ^{o}C and other at 25 ^{o}C.
- Examine the tube everyday up to 4-7 days for the presence of growth.
- Growth must be present in both the tubes before concluding that the yeast has the ability to grow at 37^{0}C.

13. Canavanine Glycine Bromothymol Blue Agar (CGB) Test

This test is used to differentiate between *C. neoformans* from *C.* gattii. The latter hydrolyses glycine to form ammonia to alter the pH of the medium towards alkalinity and thus change its color 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 inoculum of *C. neoformans* or *C gattii* with a straight wire loop.
3. Put a positive control (*C gattii*) and negative control (*C. neoformans*) simultaneously.
4. Incubate at 25^{0} C for 1 – 5 days.
5. At the end of 5 days positive result is shown by color change from greenish yellow (pH 5.8) to cobalt blue (pH 7.0).

14. Phenoloxidase test:

Cryptococcus neoformans when grown on the medium containing bird seed agar produces dark brown or black colonies containing melanin.

Procedure:

- Prepare birdseed agar (BSA) (*Guizotia abyssinica* / Niger seeds) or Caffeic acid agar in the test tube (See Annexure for media preparation).
- Streak the culture of yeast/ *C. neoformans* on the media.
- Incubate the media at 25 - 30 ^{o}C and observe every day up to one week.
- *C. neoformans* produces dark brown or black colonies with in 3-5days of inoculation.

15. Commercial Yeast Identification System:

Principle:

Commercially available identification systems are described in the chart below. Most commonly used systems are: API 20C Yeast Identification Systems (API Analytab Products, Plainview, NY) and Biomerieux Vitek System (Hazelwood, MO) .The API 20C system requires less preparation of reagents. The Vitek system is an automated system.

Group I (for single species)	
Albicans – Sure (*C. albicans*)	Germ tube (*C. albicans*)
Albicans ID (*C. albicans*)	Rapidec albicans (*C. albicans*)
Albistrip (*C. albicans*)	*C. albicans* screen (Murex CA 50) (*C. albicans*)
Bacticard Candida(*C. albicans*)	Bichrolate krusei (*C. krusei*)
Fluroplate (*C. albicans*)	Caffeic acid disk (*C. neoformans*)
Group II (for several species)	
API 20CAUX system	BBL Minitek Yeast Set
CandiSelect	Candida Check
Chrom Agar	Fungiscreen H
Group III (for multiple genera)	
AMS – Yeast biochemical Card	Microbial Identification System (MID)
Microscan Rapid Yeast ID	Quantum II
RapID Yeast Plus System	

Both the systems are based on the modification of the auxanographic assimilation techniques. When the organism is able to assimilate particular carbohydrate (in a capsule of reconstituted substrates of API systems/ in the wells containing the substrate of Vitek). Change in color is observed. Both the systems require morphological studies and germ tube test in order to obtain more complete profile of yeast identification.

Specimen:

A pure culture of 24-48hrs old yeast cells growing on non-selective agar (e.g. -SDA) is required.

Reagents and material required but not supplied with the kit.

a. For API20C yeast identification system:
    1. Sterile wooden applicator stick.
    2. Sterile Pasteur pipettes.
    3. Squeeze bottle.
    4. Incubator (30^{o}C).
    5. Waterbath
    6. Sabouraud's Dextrose Agar plates.
b. For Vitek yeast identification system:
    1. Sterile wooden applicator stick
    2. Sterile tubes containing 1.8ml
    3. Colorimeter(Vitek colorimeter)
    4. Incubator (30^{o}C)
    5. Sabouraud's Dextrose Agar plates.

Procedure: -

a. API 20C yeast identification system:

- Melt the basal broth medium in the ampoules by keeping in the autoclave or by boiling in water bath
- Allow to cool to 48-50 ^{o}C.
- Prepare an incubation tray. Use a squeeze bottle to dispense 20ml of water into the tray and then place the sample into the incubation tray.
- Open the ampoule as per the manufacturer's instructions and inoculate the molten medium using applicator stick. Adjust the density to < 1+ on the wickerham card.
- Inoculate 0.2ml yeast suspension into 20 capsules by using Pasteur pipette and place the lid on the tray.
- Incubate the tray at 28- 30^{o}C for 72 hrs. read the results after 24, 48, 72 hrs.

b. Vitek Yeast Identification system:

- Prepare yeast suspension in tubes containing 1.8ml of saline. Adjust the density to McFraland No.2 standard using Vitek colorimeter.
- After labeling the yeast cards, place the card in the filling stand with a transfer tube that is in yeast suspension.
- Inoculate the card using the filling nodule.
- Seal the card with sealer module and incubate at 30^{o}C for 24hrs or 58 hrs depending on the readings provided in the instrument.

Results:

a. API 20C yeast identification system:

1. It does not include rhamnose and urea.
2. Germ tube test and morphological studies should be included.
3. Sometimes it gives one to three different yeast identification for an individual isolate.
4. It takes 3 days to obtain final results.

b. Vitek Yeast Identification system:

1. Yeasts with abundant capsules and isolates with extensive mycelial growth are sometimes difficult to suspend.
2. Morphological studies and other tests may be required when some strains react similarly in the test system.

Results obtained usually after 24hrs and few isolates may require additional incubation.

Mould Identification

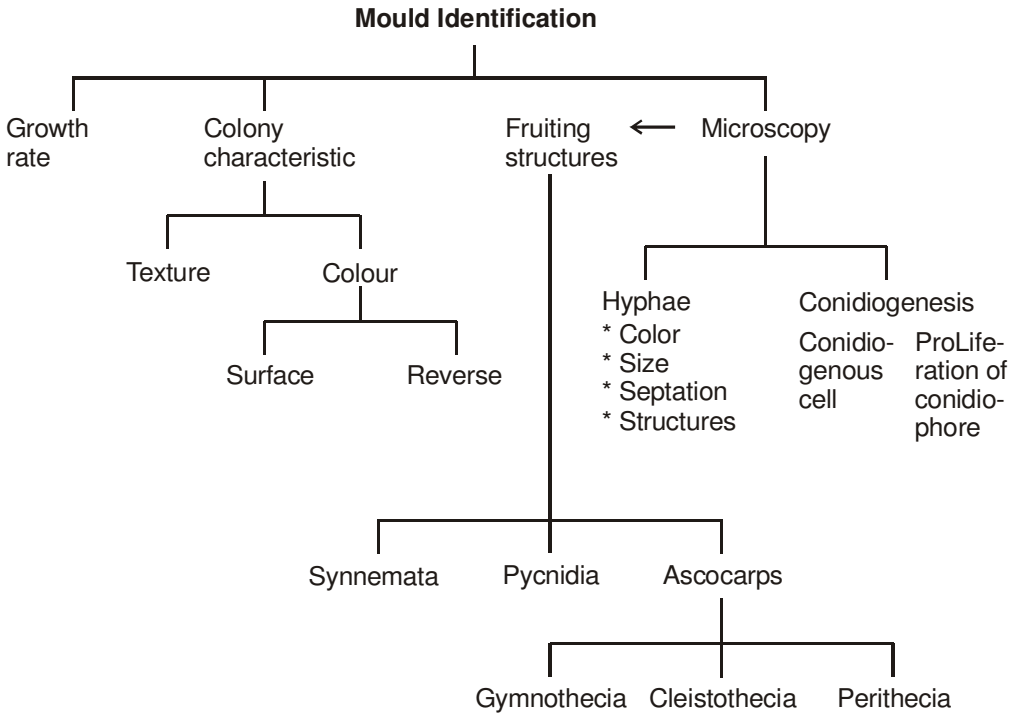


Fig. 1 : Mould Identification Scheme

Techniques used for identification

Colony Characteristic

Through extremely valuable are not given adequate importance.

- Media routinely used for studying the morphology are Sabouraud dextrose agar, malt extract agar, potato dextrose agar or Czapek-dox agar. Important data like growth rate, color, texture, diffusible pigment, exudates, aerial and submerged hyphae, and colony topography can be obtained by gross examination.
- Structures like ascocarps, pycnidia, sclerotia, sporodochia and synnemata can be studied with the help of dissecting microscope.

Tease Mount

It is the quickest way to mount fungi for microscopic examination. Though being the most common technique used in mycology laboratory; identification is often difficult because of the dislodgement of conidia and spores from the conidiogenous cell.

Procedure

a) Standard Tease Mount

1. Place a small drop of lactophenol cotton blue (mounting medium) on a clean microscopic slide.
2. Remove aseptically a small portion of growth midway between the colony center and edge. Place the removed colony on a drop of lactophenol cotton blue on a slide.
3. Tease the fungus using a pair of dissecting needles so as to have a thin spread out.
4. Gently place a cover slip at the edge of the drop of mounting fluid.

5. Avoid trapping air bubbles. Excess of lactophenol can by wiped out using a blotting paper.
6. For preserving the mount, seal the edges of coverslip with nail polish/varnish.

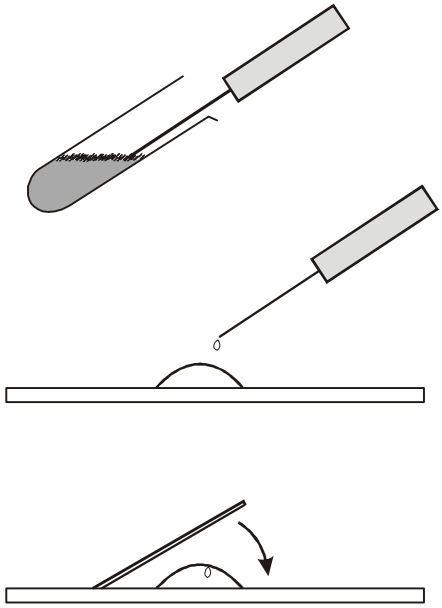


Fig. 2 : Standard Tease Mount

b) Tape Technique

1. Place a drop of mounting fluid on the slide.
2. Take 2 cm long tape from a tape roll. Touch one end of the tape to a forceps/ stick and then lightly touch the colony with the stick/forceps.
3. Lay the tape with the surface containing the fungus face down into the mounting medium on the slide. Detach the tape from the stick.
4. Examine the mount directly.

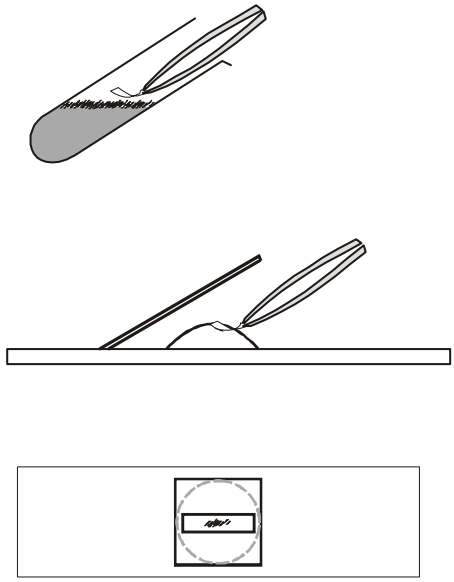


Fig. 3 : Tape Technique

c) Mounting Zygomycetes and fruiting structures

Sporangia, ascocarps and other fruiting bodies trap air bubbles making preparations unsatisfactory for microscopic study. Thus following techniques are recommended.

1) Take a drop of 95% ethanol, ethylacetate or water with 0.05% Tween 80 on a slide . All these are wetting agents, preventing formation of air bubbles around fruiting structures.
2) Transfer fungus from the culture to the drop of fluid. Add a drop of the mounting fluid and examine as in the standard technique.

III Confirmation of dimorphic fungi

Dimorphic fungi exist in different morphological forms at different temperatures and under separate nutritional conditions. They grow as moulds at 25^0C on routine media while in the tissue or on special media at 37^0C they grow either as yeasts or spherules.

Blastomyces dermatidis, Histoplasma capsulatum, Paracoccidioides brasiliensis, Penicillium marneffei and *Sporothrix schenckii* grow as yeast in tissue and at 37^0C. *Coccidioides immitis* produce spherules and endospores at 37^0C in tissue and when grown on special medium.

Temperature, medium composition and CO_2 concentration can all affect the mould to tissue form conversion. For *P. brasiliensis* and *H. capsulatum* nutrition seems to be extremely important while in *S. schenckii* and *C. immitis* yeast and spherule form respectively is enhanced by atmospheric CO_2.

Mould to tissue form conversion of dimorphic fungi

	Fungus	**Media and conditions**	**Similar looking fungi at 25^0C**
1)	*Blastomyces dermatitidis*	Kelley's agar, 37^0; KT medium	*Chrysosporium spp.*
2)	*Coccidioides immitis*	Modified convers medium, 40^0C, 5 to 10% CO_2	*Malbranchea* spp., *uncinocarpus* spp., *Auxarthron spp., Archniotus spp.*
3)	*H.capsulatum*	BHIA with glutamine, 37^0 C Pine's Agar Medium	*Sepedonium spp.*
4)	*P. brasiliensis*	BHIA, 37^0C	*Chrysosporium spp.*
5)	*S. schenckii*	BHIA, 37^0 C, 5-10% CO_2	*Acrodontium spp.*
6)	*P.marneffei*	5% sheep BA, BHIA, 37^0C	*Penicillium spp. other than P. marneffei*

- Carry out all procedures within biological safety cabinet.
- Do not set up slide cultures for dimorphic fungi.
- For quality control a known culture can be inoculated simultaneously to check for conversion conditions.

IV Cylcoheximide resistance

The following fungi are resistant to cycloheximide at 30^0C i.e. all of them grow in the presence of cycloheximide.

B. dermatitidis	*S. schenckii*
C. immitis	*H. capsulatum*
P. brasiliensis	Most of the dermatophytes

While the following fungi are inhibited by cycloheximide

Absidia	*Rhizopus*
Scedosporium	Some strains of *Candida* & *Aspergillus*
Mucor	*Cryptococcus*

V Slide culture technique

Whenever it is difficult to identify moulds with tease mounts, slide cultures can be put up. Nutritionally deficient media like corn meal agar, potato dextrose agar etc. is good for enhancing sporulation.

1) Prepare the 'Setup"

- In a 100 mm glass petri dish, place a filter paper, V-shaped glass rod, a microscopic slide and a coverslip.
- Autoclave the whole setup at 121^0C for 15 min
- Store the setups in a petri dish canister for a maximum period of 2 weeks.

2) Procedure

- Aseptically cut 1 cm square agar blocks from CMA or PDA.
- Transfer the agar block on to the slide in the setup.
- Transfer very small amount of the colony to the four sides of the agar block.
- With a sterile forceps place the coverslip 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.
- Place the slide culture in a petridish canister and incubate in the dark.
- Slide culture is ready to be taken down when mature conidia or spores are observed

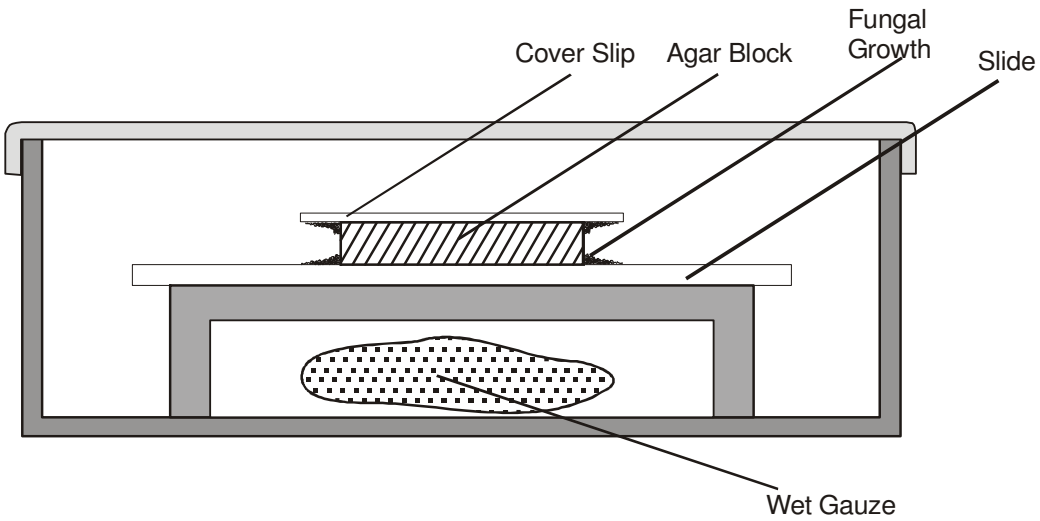


Fig. 4: Slide Culture Technique

3) "Taking – down" the slide culture

- Take a small drop of mounting medium (LCB) on a microscopic slide.
- With the forceps, carefully remove the coverslip from the agar block. Do not push or pull the coverslip.

- Pass the coverslip through the blue portion of flame very quickly. This will heat fix the fungus and its spores (overheating can result in collapse of the hyphae).
- Carefully place the coverslip on the mounting medium so as to avoid trapping any air bubbles.
- Wipe off the excess of mounting medium. Seal the edges of coverslip with nail polish.
- Second mount can also be prepared from the microscope slide of the slide culture setup, by removing the agar block. Put a drop of lactophenol and a coverslip. The coverslip can be sealed with nail polish.
- A drop of 95% alcohol or ethyl acetate may be used to soak the colony before using lactophenol blue to avoid air bubble.
- The block can be dropped aseptically on a suitable media for the growth. This would help to maintain the fungus in the laboratory.

VI Temperature Tolerance

Thermotolerance is a useful characteristic that can be used as an aid in the identification of several medically important moulds.

Thermotolerance of some Medically Important Fungi:

Fungus	**Upper Growth Limits (^{0}C)**
Aspergillus fumigatus	48 – 50
Cladophialophora bantiana	42 – 43
Cladophialophora carrionii	35 – 36
Fonsecaea pedrosoi	38
Rhizomucor pusillus	50 – 55
Trichophyton mentagrophytes	37
Exophiala dermatitidis	40

VII Techniques used for identification of few fungi

	Suspected mould	**Technique utilized / Recommended**
1)	*A. fumigatus*	Temperature tolerance 48^{0}C
2)	*B. dermatitidis*	Exoantigen, mould to yeast conversion, Gen probe technique
3)	*Basidiobolous sp.*	Forcibly ejected sporangiola
4)	*Cladophialophora carrionii*	Temperature tolerance, 35^{0}C
5)	*Conidiobolus spp*	Forcibly ejected conidia
6)	*H. capsulatum*	Exoantigen, mould to yeast conversion Gen probe technique
7)	*P. brasiliensis*	Mould – yeast conversion
8)	*Rhizomucour pusillus*	Temperature tolerance, 55^{0}C
9)	*Sepedonium sp.*	Morphology and negative exoantigen for *Histoplasma*
10)	*Sporobolomyces*	forcibly ejected conidia
11)	*Exophiala dermatitidis*	Temperature tolerance 40^{0}C
12)	*Cladophialophora*	Temperature tolerance 42^{0}C

Specific and Rapid Identification of Medically Important Fungi by Exoantigen detection

Exoantigens are defined as antigens or soluble immunogenic macromolecules produced by fungi early in their development. The exoantigen test depends on interaction between antigens produced by fungi in culture and homologous antibodies generated specifically to precipitate them. The precipitate(s) formed are checked for fusion with reference precipitate(s) by immunodiffusion test. Thus the specific identification may be accomplished within 2-5 days while conventional identification has been known to take weeks or months. The rapid identification benefits the patient by permitting administration of therapy soon after receipt of a mature isolate.

Exoantigen technique obviates the need for time consuming temperature dependent conversion, and fungi can immunologically be identified in their typical, atypical or nonsporulating state.

Exoantigen analysis is extremely useful for determining taxonomic relatedness among morphologically similar and dissimilar fungi. The technique also reduces the potential for exposure to biohazardous fungi by eliminating the need for the invitro conversion or cultural manipulation used in animal inoculation.

Commercial Reagents

Commercial exoantigen reagents are available for identifying *B .dermatitidis, C. immitis,* and *H.capsulatum.* These may be obtained from Immuno-Mycologics, Inc. (Normal, Oklahoma) and the Nolan-Scott Biological Labs, Inc. (Tucker, Georgia). If properly titered, the commercial fungal reagents used in ID tests for detecting antibody in sera can be used in exoantigen tests. The serodiagnostic reagents must be checked for appropriate antibodies and must be rebalanced for the exoantigen test for *B. dermatitidis* and *H. capsulatum.* Coccidioidomycosis serodiagnostic reagents must also be carefully checked, since they are frequently devoid of the HS antibody homologous to the earliest specific exoantigen elaborated by *C. immitis*.

Criteria for Immunoidentification

The only antigen considered positive is antigen that forms a distinct line of identity with the reference antigen-antibody precipitate. Lines of non-identity or lines of partial identity are considered negative.

Although false-positive reactions have not been encountered, false-negative reactions may occur. They may be attributable to technical error, absence of specific homologous antibody in the reference antiserum, or inadequate antigen concentration due to insufficient growth or to improper growth conditions. Tests should be repeated when mycological or clinical judgment suggests that a negative culture should be positive. The cultures that are negative by the exoantigen test be further examined by conventional morphologic tests to make certain that they are not known pathogens.

Unknown antigens in duplicate are used in adjacent lateral wells rather than to test different antigens in adjacent wells. This is necessary because some isolates, such as *C.immitis,* produce antigen so abundant that precipitates can extend in front of an adjacent well. Such an extended line in front of a well with a negative extract could be misinterpreted as a positive result.

The exoantigen test procedure has been evaluated extensively with Centers for Disease Control reference reagents and commercial reagents. It has been found to be extremely sensitive and specific for identifying systemic fungal pathogens. Positive results are recognized as the equivalent of cultural isolation and identification by conventional tests.

Safety Considerations

To assure that laboratory workers do not become infected, we recommend that all fungal cultures be handled in a biological safety hood and that broth cultures or extracts be treated with a preservative.

The preservative chosen should be one that renders the culture nonviable without destroying its exoantigens. Although 0.2% formaldehyde can effectively kill the mycelial forms of *B.dermatitidis, C. immitis,* and *H.capsulatum,* it unfortunately also inactivates the *B.dermatitidis* A and *C.immitis* HL antigens. Solutions of 0.025 thiomersal have consistently killed these fungi in broth culture but not in slant extracts. 0.02% merthiolate can be used to treat broth cultures and slant extracts for 24 hr. On rare occasions viable elements can survive in the thiomersal-treated extract. Thiomersal does not deleteriously affect the exoantigens. For those workers who prefer to use 0.02% thiomersal and yet be assured that they are working with sterile extracts, sterilization of the extracts by passage through a 0.45-□m membrane filter is recommended..

Applicability to identification of Fungal Pathogens

I Identification of Dimorphic Fungi

Blastomyces dermatitidis

A specific precipitin, designated – A, is found in patients with blastomycosis. The specific A antigen is found among isolates of *B. dermatitidis* from all parts of the world, with the exception of some African isolates of *B. dermatitidis.* All isolates of *B. dermatitidis* also share an antigen designated K. Thus, it is apparent that at least two serotypes of the fungus exist, one that possesses the A antigen and one that does not. The A-deficient serotype appears to be prevalent only in Africa. The predominant K antigen is also found in *Chrysosporium parvum*.

At times some isolates of *B. dermatitidis* fail to produce sufficient A antigen in slant extraction but invariably produce it in shake broth cultures.

b. *Coccidioides immitis*

Unconcentrated slant extracts concentrated 25 times are excellent sources of diagnostic exontigens. These exoantigens may be either heat stable (HS) (remaining stable after boiling for 10 min.) or heat labile (HL or F) (losing activity after heating at 60^0C). HS is the earliest antigen to appear. HL is detected in later growth stages, and the F antigen the last. Commercial reagents should contain a combination of the three antigens.

c. *Histoplasma capsulatum*

The mycelial form of genus *Histoplasma* are characterized by the production of tuberculate macroconidia. A number of saprophytic fungi in the genera *Arthroderma, Chrysosporium, Corynascus, Renispora* and *Sepedonium* resemble *Histoplasma* varieties. But the soluble H and M antigens are elaborated only by *H. capsulatum var. capsulatum*, *H. capsulatum var duboisii, H. capsulatum var farciminosm.*

The exoantigen technique does not allow differentiation of the three varieties of *H. capsulatum*.

d. *Paracoccidioides brasiliensis*

Protein antigen E (Equivalent Antigen 1) and antigens 2 and 3 are all specific for *P.brasiliensis*.

e. *Sporothrix schenckii*

Antisera prepared in rabbits against yeast form cells of *S. schenckii* produce 3 precipitin bands upon reaction with antigens from the mycelial form of this fungus.

II Identification of opportunistic filamentous fungi

i) *Aspergillus Group* Identification of aspergilli is dependent on morphologic studies. However, group specific antisera have been developed for specific identification of Aspergillus species of medical importance. Exoantigens have proven reliable for identifying the sterile albino-type isolate of *A. fumigatus.*

ii) *Basidiobolus and Conidiobolus*

Two heat stable exoantigens N and Y are common to *B. haptosporus and B.ranarum. B.meristosporus, B.microsporus, C. coronatus* and *C. incongruous* share only the N antigen. Other heterologus zygomycetes produce neither of the antigen. The sharing of the N antigen by the *Basidiobolus* and *Conidiobolus* species implies a taxonomic relationship.

iii) *Dermatophytes*

Theoretically, exoantigen tests should provide ideal means of rapidly identifying dermatophytes. However, it has not been possible to develop exoantigen tests for dermatophytes.

iv) *Penicillium marneffei*

It produces two distinct exoantigens which permit its differentiation from other species of *Penicillum.*

III) Dematiaceous Fungi

i) *Cladosporium sp., Cladophialophora sp.*

Specific antigens among isolates of *C. bantiana* and *C. carrionii* permit rapid specific identification and separation from the common saprophytic species i.e. *C. cladosporioides* and *C. herbarum*.

ii) *Exophiala jeanselmei*

E. jeanselmei has 3 serotypes. Isolates that cause mycetomas have been identified as serotype 1, whereas those that cause phaeohyphomycosis belong to serotype 1, 2, & 3.

Summary

Exoantigen technique is accepted for accurate and rapid identification of fungal pathogens. This acceptance is emphasized by the fact that positive exoantigen results obtained with the dimorphic fungi *B.dermatitidis, C.immitis, H.capsulatum* and *P.brasiliensis* and no longer considered presumptive but considered definitive for species identification.

Since the test is simple and reagents for many of the pathogens are commercially available, the test can be performed in most laboratories. Identification of numerous fungi could be facilitated by application of exoantigen techniques. Some fungi cannot be classified on the basis of morphologic and biochemical qualities alone. Supplementary data obtained with exoantigen analysis could undoubtedly aid in resolving such problems.

Zygomycota

Zygomycetes are a group of fungi whose members generally produce coenocytic (nonseptate) or rarely septate hyphae which branch irregularly and have an irregular (10-25□m) diameter. This class is characterized by its sexual reproduction resulting in formation of zygospores. Asexual reproduction is typified by a sac like structure called sporangium which contains spores. Two orders of interest to medical mycology are *Entomophthorales* which have forcibly discharged spores and the *Mucorales* in which the spores arise by cleavage of the sporangial plasma and are passively liberated.

General Features

1. Cultures have rapid growth with woolly texture. Colour may be white to grey to black.
2. Some *Zygomycetes* have vegetative hyphae in form of runners which are termed as stolons. The stolon when touches the substrate is termed as node. Internode is the arch between two nodes.
3. Rhizoids develop at the node while sporangiophores might arise at the node or the internode. The relationship of stolon to the origin of rhizoid and sporangiophore is a useful distinguishing feature between several general.

4. Asexual structure: sporangium may have its sporangiospores in a row termed merosporangium or a reduced sporangia with few sporangiospores called sporangiola.

5. Columella is the sterile dome like area at the apex of sporangiophore. Apophysis is the swelling in the sporangiophore which merges with the columella. Collarette are the remnants of sporangial wall remaining attached to the junction of columella and sporangiophore after rupture of sporangium.

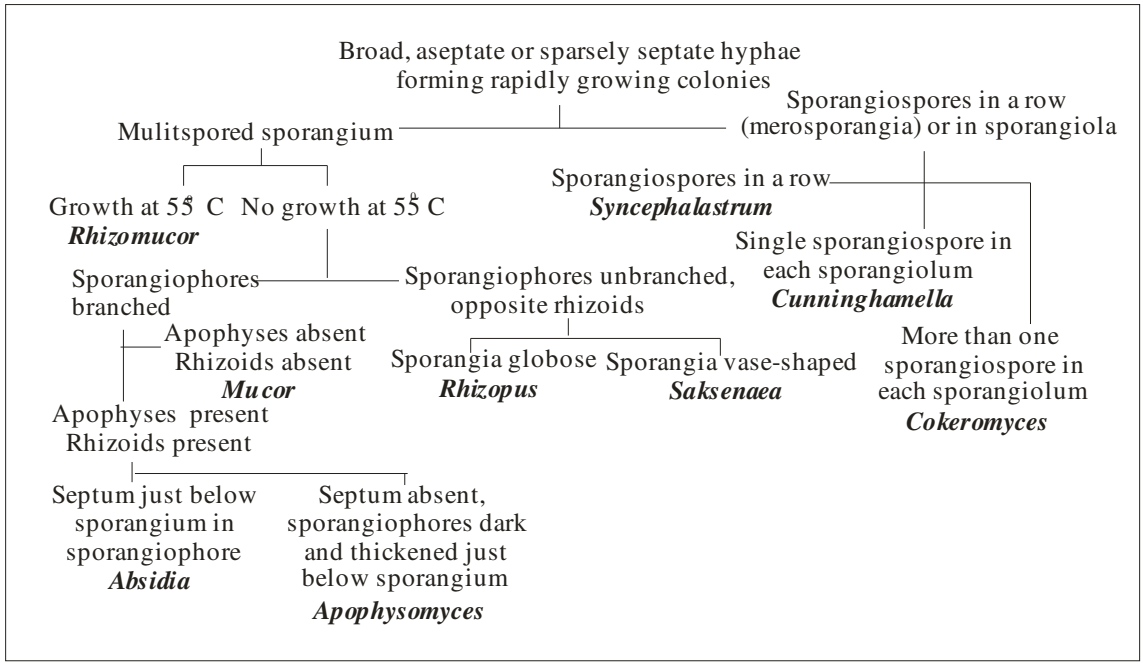


Fig. 5: Key to identify medically important *Zygomycetes* (Compiled by Dr. Arvind A. Padhye)

Identification of Dermatophytes

Dermatophytes are the keratinophilic fungi capable of invading keratinous tissue. All dermatophytes are classified into three Genera namely *Epidermophyton, Microsporum* and *Trichophyton*. Differentiation into three Genera is based on the colony characteristics on Sabouraud dextrose agar and microscopic morphology. The accurate identification of some dermatophytes requires additional tests such as growth on special media for enhancement of pigment production or sporulation, physiological tests to determine special nutritional requirements/capability to perforate hair/ to hydrolyze urea/ to tolerate high temperature.

Colony characteristics:- examination of the colony characteristics on the Sabouraud dextrose agar is one of the important criteria for the identification of dermatophytes. The colony should be observed for the pigmentation on the surface and reverse, texture on the surface (cottony, wooly, velvety, powdery, ceribriform, granular or glabrous) and topography (elevation, margin and folding).

Microscopic characteristics:- on microscopic examination, the appearance and arrangement of the macroconidia and/microconidia aid in the identification of most of the dermatophytes. The characteristics of the macroconidia especially the size, shape, number of septations, characters of their walls and the way they are borne on the hyphae are important to describe the Genus of the dermatophyte (**Table**). Various other non specific structures that are helpful in identification in conjunction to characteristic macroconidia are chlamydospores, spirals, nodular organs, pectinate organs, arthroconidia and racquet mycelium

Wood's Lamp

The woods lamp is a source of long-wave ultra violet light and can be used to detect the fluorescence in hair which is a feature of infection by some dermatophytes. It is especially useful for the detection of inconspicuous scalp lesions. Apart from its use in diagnosis, Wood's lamp can be used to select infected hairs for laboratory investigation.

The ''Wood's filter'' is opaque to all light except for a band between 320 and 400 nm with a peak at 365 nm. Fluorescence of tissue occurs when light of shorter wavelengths, initially emitted by Wood's light, is absorbed and radiation of longer wavelengths, usually visible light, is emitted. The output of Wood's lamp is generally low. Different dermatophytes growth on the hair absorbs this waveband, which, upon absorption, fluoresces at longer visible wavelengths resulting in the enhanced emission.

Precautions

The lamp should be allowed to warm up for about 1 min. The examination room should be very dark. Black occlusive shades or a windowless room are preferred. It is also essential that the examiner becomes dark-adapted in order to see the contrasts clearly.

Urea hydrolysis (see section)

Urease test

A number of dermatophytes have the ability to hydrolyze urea and, therefore, a test that demonstrates the production of urease can be utilized in their differentiation.

Procedure

- Prepare urea agar medium using Difco Urea Agar Base with the addition of 0.4% glucose and dispense in tubes.
- After inoculation, incubate at 26^0C for 7 days

- Examine for the change in colour from straw to red which indicates the production of urease

Results

T. interdigitale -positive; *T. rubrum* –negative
T. mentagrophytes -positive; *T. erinace*i – negative
T. megninii - positive;

In vitro hair perforation test

In vitro hair invasion by dermatophytes species varies from that of *in vivo* invasion. The fungus will not produce the type of hair invasion that is seen *in vivo*. As a result of the growth of the fungus in the artificial culture media along with the hair some dermatophytes produce perforations and these form wedge- shaped tunnels or holes in the hair. This test is utilized to differentiate especially between *T. mentagrophytes* and *T. rubrum* which are difficult to differentiate based on morphological characteristics.

- Collect a short length of human hair (1cm long) preferably from a child or pure blonde, place in a petri dish and autoclave at 121^0C for 10min.
- To it add 25 ml of sterile distilled water and 2-3 drops of filter sterilized 10% yeast extract.
- Inoculate the plate with the small fragment of test fungi.
- Incubate the plates at room temperature (25^0C).
- Remove the hair segments from the plates at regular intervals and mount on the slide containing lactophenol cotton blue and examine the hairs under microscope.
- The plates should be examined for up to 4 weeks before considering the test as negative

Results

- Positive (wedge shaped perforations)- *T. mentagrophytes*
  -All *Microsporum* species
- Negative – *T. rubrum*

Table 1: Characteristics of Macroconidia

Genus	Size (µ)	Septations	Wall thickness	Wall surface	Occurrence	Attachment
Trichophyton	8-86/4-14	1-12	Thin-thick	Smooth	Usually rare	Single/clusters
Microsporum	6-160/6-25	1-15	Thick	Rough	Usually numerous (rare in *M. audouinii*)	Single
Epidermophyton	20-40/7-12	1-6	Thick	smooth	Numerous	Single/clusters of 2/3

Table 2: Characteristics of Microconidia

Genus	Shape	Occurrence	Attachment
Trichophyton	Globose	Numerous (exception- *T. schoenleinii)*	Single/clusters
Microsporum	Pyriform/clavate	Rare/numerous	Single
Epidermophyton	Pyriform/clavate	Absent	Single

Table 3: Characters of clinical materials obtained from dermatophytosis

Finding on Wood's lamp examination of scalp	Examination of KOH mount		Dermatophyte implicated
	Skin scrapping	Hair	
Bright yellow to green fluorescence	Hyphae and chains of arthroconidia	Small spores arthroconidia 2-5µm in dia, easily dislodged from hair	*M. audouinii* *M. canis* *M. ferrugenium*
No fluorescence/dull fluorescence	Hyphae with arthroconidia in mass and chains	Large spores (5-8 µm) arranged in chains or masses	*M. gypseum* *M. nanum* *M. vanbreusighemii*
No fluorescence	Hyphae and chains of arthroconidia	Chains of spores on surface of hair	*T. mentagrophytes* *T. veruccosum*
	Hyphae and chains of arthroconidia	Chains of arthroconidia inside the shortened hair stubs	*T. soudanese* *T. tonsurans* *T. violaceum*
	Cup-like clusters with chains of arthroconidia	Hyphae with air bubbles or tunnels and fat globlet and inside hair	*T. schoenleinii*

Table 4: Cultural and other characteristics of dermatophytes

Dermatophyte spp	Colony morphology on SGA	Microscopic morphology	Other tests	Comments
Epidermophyton floccosum	Slow growing, flat, fluffy,soon becomes velvety or powdery; turn tan to olive-brown/ green; reverse yellowish tan	Microconidia absent, numerous macroconidia broad, blunt to clavate with smooth thin wall and 2-6 celled. Chlamydospores are usually plenty	Urease positive. Hair perforation negative	Do not invade hair
M. audouinii	Slow growing, flat, velvety with whitish tan to brown surface, reverse salmon to pale brown	Macroconidia and microconidia usually rare. Macroconidia if present are large with beak and constriction in the	Urease negative. Hair perforation negative	Poor growth on Rice grain media

		middle and smooth or echinulate surface		
M. canis	Rapid growing, initially white with pale to yellow pigment; later tan, cottony with central knob. Reverse yellow to orange brown	Macroconidia numerous, large, spindle shaped, thick echinulate wall and 8-15 celled. Microconidia few, sessile and drop shaped	Urease positive. Hair perforation positive	Teleomorph- *Arthroderma otae*. Grows confluently on Rice grain media with yellow pigment
M. gypseum	Rapid growing, powdery to granular with cinnamon to rosy buff. Reverse pale yellow to brownish	Macroconidia numerous, ellipsoid, thin, echinulate wall and 3-9 septa. Microconidia are rare, drop shaped and sessile.	Urease positive. Hair perforation test positive	Good growth on Rice grain media
M. ferrugineum	Slow growing, waxy with many folds and yellowish to deep rust color surface. Reverse yellow to orange	Macroconidia and microconidia absent. Hyphae well formed with arthroconidia	Urease negative. Hair perforation test negative	Geographically restricted
M. nanum	Rapid growing, flat, powdery to fluffy with sandy surface. Reverse brownish red to dark red	Macroconidia numerous, ellipsoidal, thin walled, rough surface, 1-celled. Microconidia pear shaped and sessile.	Urease test positive. Hair perforation test positive	Poor growth at 37^0C. Teleomorph- *Arthroderma obtusum*
M. cookie	Rapidly growing, powdery granular to velvety, yellowish to greenish brown surface. Reverse wine red	Macroconidia few, broader thick-walled and rough. Microconidia abundant and drop shaped	Urease test positive. Hair perforation test positive	Teleomorph- *A. cajetani*
M. vanbreuseghemii	Rapid growing, flat, powdery with yellowish tan to pink surface.	Macroconidia numerous. Cylindrofusiform, rough, thick-walled with 7-10 celled		Teleomorph- *A. grubyi*

	Reverse no pigmentation or yellowish			
M. persicolor	Rapid growing, flat, fluffy to powdery, yellowish to pinkish buff surface. Reverse peach to rose	Macroconidia often absent, spindle shaped, smooth thin walled and around 6 celled. Microconidia subglobose to club shaped and seen in clusters.	Urease test positive. Hair perforation test positive	Teleomorph- *A. persicolor*. Spiral hyphae, resembling *T. mentagrophytes* is produced
Trichophyton mentagrophytes complex. Zoophilic [*T. mentagrophytes sensu strict*(former *T. mentagrophytes* var quinckeanum)], animal adapted form of *T. interdigitale* and *Trichophyton* anamorph of *A. benhamiae*	Rapid growing, flat, powdery to granular, with cream to buff or tan surface. Reverse pale to red brown	Macroconidia rare, club shaped, smooth, microconidia abundant, spherical, arranged in clusters	Urease test positive. Hair perforation positive	Teleomorph- *A. vanbreuseghemii,* *A benhamiae*
T. *mentagrophyte* complex (anthropophilic) [human adapted form of *T. interdigitale*]	Rapid growing, powdery to fluffy, cream to buff white surface; reverse pale to red brown	Macroconidia rare, club shaped, smooth, microconidia abundant, spherical or drop shaped and seen in clusters. Spiral hyphae present	Urease test positive. Hair perforation positive	Macroconidia can be induced on SGA + 3-5% NaCl. Teleomorph- *A. vanbreuseghemii* *A benhamiae*
T. mentagraphytes (nodular variant and morphotype of *T. interdigitale*) [earlier *T. krajdenii*]	Slow growing, cottony with white to cream surface and yellow margin. Reverse deep yellow	Macroconidia rare, microconidia abundant, drop shaped and sometime round coiled yellow nodular bodies and yellow granular. Spiral hyphae rarely seen	Urease test positive. Hair perforation test positive	
T. *rubrum* (cosmopolitan	Slow growing, flat/heaped,	Macroconidia rare; pencil shaped.	Urease test positive. Hair	

variant)	velvety or powdery which becomes highly folded, surface white to cream to deep rose. Reverse wine red; rarely yellowish orange	Microconidia drop shaped abundant, scanty or not formed.	perforation test- negative	
T. rubrum (Afro-Asiatic variant)	Slow growing, powdery to velvety, cream to deep red surface. Reverse wine red	Macroconidia abundant, club shaped and rarely rat tail extension. Microconidia drop shaped to round with many chlamydospores	Urease test positive. Hair perforation - negative	
T. violaceum	Slow growing, heaped, glabrous, cream to lavender deep purple surface	Macroconidia rare. Microconidia usually not found on SGA. Production of microconidia enhanced on thiamine medium	Urease test positive/weak. Hair perforation - negative	
T. tonsurans	Slow growing, powdery surface which turn to velvety. Surface becomes tan or bright yellow or rose. Reverse yellowish to mahogany red.	Macroconidia rare, small pencil or club shaped, thin and smooth wall. Microconidia abundant tear shaped produced on undifferentiated hyphae or simple condiophores. Some microconidia enlarge and appear like balloons.	Urease test positive. Hair perforation test- mostly negative	
T. veruccosum	Very slow growing, folded, white or yellow powdery. Reverse no pigment	Macroconidia rare, string bean shaped, thin and smooth walled 3-5 celled. Microconidia abundant on media supplemented with	Urease test negative. Hair perforation test- negative	Thiamine essential for sporulation

		thiamine and inositol. They are small and borne on hyphae. On SGA chlamydospores are seen		
T. schoenleinii	Slow growing, heaped up, town and leathery, white to tan, glabrous or white to powdery surface	Macroconidia absent. Microconidia very rare. Hyphae appear knobby and clubbed at ends. (Favic Chandeliers). Chlamydospores abundant.	Urease test variable. Hair perforation test- negative	

Growth on Dermatophyte test medium

Dermatophyte Test Medium is a selective medium used for the isolation of pathogenic fungi from cutaneous specimens. This medium is intended to be used as a primary isolation medium. Dermatophytes produce typical morphology and a pink to red color in the medium surrounding the colony within 10—14 days of incubation. Occasionally, a bacterial contaminant may produce a color change in the medium within this period but can be distinguished by colonial morphology.

PRINCIPLE

Dermatophyte Test Medium contains papaic digest of soybean meal as an amino acid source and other nitrogenous substances necessary for fungal growth. Dextrose provides an energy source and phenol red, a colorimetric indicator, is used to visualize the pH shift in the medium

Results

NCCLS Control Organisms (ATCC Strains)

Trichophyton mentagrophytes (ATCC 9533)	White cotton-like growth, pink to red
Aspergillus niger (ATCC 16404)	Inhibition, partial to complete
Candida albicans (ATCC 10231)	White/off-white growth, no color change
Escherichia coli	Inhibition, partial to complete

This medium is intended to be used as a primary isolation medium. Dermatophytes produce typical morphology and a pink to red color in the medium surrounding the colony within 10—14 days of incubation. Occasionally, a bacterial contaminant may produce a color change in the medium within this period but can be distinguished by colonial morphology. Disregard any color changes in the medium after 14 days of incubation. This may be caused by contaminating fungi.Identification of dermatophytes requires a pure culture. Morphological, biochemical and/or serilogical tests should be performed.

Procedure

Inoculate the specimen as soon as possible after it is received in the laboratory. Place the specimen onto the center of the agar with sterile forceps. Press carefully to ensure firm contact with the agar surface. Incubate the inoculated media at 22-25℃ for up to 14 days. (Do not incubate cultures at 33-37℃.)

Examine the culture daily for a change in the color of the medium and evidence of fungal growth. Disregard any color changes in the medium after 14 days of incubation. This may be caused by contaminating fungi.

Precautions

The ability to detect microorganisms by culture techniques can be affected by the following factors: improper specimen collection, storage and inoculation, initiation of antiinfective therapy prior to specimen collection, improper culture incubation temperatures and atmospheres, improper length of culture incubation, and improper storage and handling of culture media

Hair bait technique

The hair-bait technique introduced by Vanbreuseghem (1952) for the isolation of keratinophilic fungi, including dermatophytes.

Procedure

- Fill half of a petri dish (10mm) with soil that is moistened with sterile distilled water. The amount of water varies from sample to sample, depending on the moisture content of the sample.
- Bait this with strands of autoclaved horse hair.
- After one or two weeks of incubation at room temperature, Examine the growth on the hair and cultured on Sabouraud's agar medium containing cycloheximide and chlortetracycline.
- Detailed morphology of each strain can be studied and the fungi identified.

Paraffin bait Technique

This technique is used for the isolation of the Nocardia species from the sputum specimen and soil

Procedure

- Inoculate about one ml of the decontaminated sputum sampleis/washings of siol into a sterile carbon-free broth of the following composition: sodium nitrate, 2 g; monobasic potassium phosphate, 0.008 g; manganese chloride, 0.002 g; distilled water, 1000 ml; pH, 7.2.
- Paraffin-coated glass rods are prepared and sterilized and one rod each aseptically introduced into the carbon-free broth inoculated with the sputum/soil suspension.
- The baited suspensions should be incubated at 37℃ for up to 15 days.

Interpretation

Growth usually appears on the paraffin bait in seven to ten days as brown, yellow, pink, or white tuft-like growths. Suspend the growth from each tuft and streaked onto glucose nutrient agar plates. Suspected colonies should be identified to species.

Serodiagnosis of Fungal Infections

As conventional diagnostic tests for fungal diseases are of low sensitivity, there continues to be a need for more rapid, non – culture methods for diagnosis. Tests for detection of antibodies, rapid detection of specific fungal antigens have the potential to yield rapid diagnostic information that can guide the early and appropriate use of antifungal therapy. Some progress has been made in these areas. Though the tests for detection of antibodies to casual agents of the mycoses were first used in the early years of twentieth century. These tests are all the more important in systemic opportunistic mycoses. Despite decades of effort, establishment of test procedures of acceptable performance for individual mycoses has not always been easy, and in some instances has not yet been attained. However serological tests may provide a rapid means of diagnosing fungal infections, as well as the means to monitor the progression of the infection and the patient's response, to therapy by comparing serial determinations of antibody or antigen titers. Some important facts in the development or standardization of serological tests in fungal diseases are to be remembered.

- The complicating factors in serology include the morphological and biochemical complexity of fungal cell, the wide-spread existence of cross-reacting antigens among pathogenic fungi, the absence of international standards.
- Some reagents in use today have been developed empirically. A, few such as the immunodiffusion test for histoplasmosis, have nevertheless being highly successful and still have a role in clinical mycology laboratory.
- Antigens in current use range from crude culture filtrates containing on array of metabolic products, to highly purified materials of known composition.
- Intact fungal cells have been proved satisfactory as a basis for immunofluorescence procedures, and are still used in a complement fixation test for histoplasmosis.
- Materials predominantly derived from cell walls, from internal components or from extracellular products have also been widely used.
- With the understanding of pathogenesis of individual fungal disease, some specific cell wall, cytoplasmic and secreted fungal components are purified to develop specific test. Examples of these methods include galactomannan of *Aspergillus fumigatus* or extracellular acidic carboxyl proteinase of *Candida albicans*. Other macromolecules derived from *C albicans* and suitable for development of specific tests include the enzyme enolase or a 47 KDa heat stock protein.
- Test for antibodies are generally less helpful for patients who are severely immunocompromised and having infection due to opportunistic fungi like *Candida, Aspergillus* or *zygomycetes*.
- In candidiasis, the interpretation of antibody titer is difficult due to host-parasite relationship that exists for this disease, ranging from harmless commensalism to true invasion.
- Sequential determinations of antibody titers in some instances provide objective measurements for following the course of illness or for monitoring treatment. Significance can only rarely be ascribed to results from a single sample.
- More success has been achieved with tests for detection of free antigen in body fluid like in cryptococcosis. The latex agglutination test for cryptococcosis, introduced four decades ago, remain one of the most valuable of all serological tests for the mycoses.
- Good success has also been achieved for the determination of antigen test in histoplasmosis especially after the development of enzyme linked immunosorbent test replacing the earlier radio-immunoassay.

- Antigenemia in patients with invasive aspergillosis is ofen sporadic rather than sustained. If multiple samples are not examined, occurrence of antigenemia may be missed.
- In assessing performance of a test, the relationship between the time the serum was taken for analysis and the time when active tissue invasion was taking place is also of importance. This is rarely taken into account, but antibody or antigen profiles rarely remain constant. Thus, whenever possible to do so, it is better to have tests made on a succession of sera rather than rely on results obtained with a single sample.

Table -1: Serological tests for Systemic mycosis

	Histoplasmosis	**Blastomycosis**	**Coccidioidomycosis**	**Sporotrichosis**	**Cryptococcosis**	**Candidiasis**	**Aspergillosis**	**Zygomycosis**
Test for antibody								
Precipitation (double diffusion)	++	++	++			±	±	+
CIE	++	++				±	±	
Latex agglutination (	±			+	+			
whole cell agglutination					+			
Complement fixation	++	±	++					
Immunofluorescence					+		±	
Immunoblot/ ELISA					+			
Test for Antigenemia								
Latex agglutination		++			+++	+	+	
ELISA	++							
RIA	++	?						

Despite all those difficulties in Serodiagnosis of fungal diseases, perhaps the most reliable and widely used test for detection of antibodies is for histoplasmosis and coccidioidomycosis. Both the complement fixation and immunodiffusion (ID) tests has been found to be useful for diagnosis of these infections. Complement fixation titers of > 1:32 may be diagnostically significant, whereas lower titer may represent early infection, a cross-reaction, or residual antibody from previous infection. ID test is less sensitive than complement fixation test, but may be useful for determination of cross-reaction. As both tests detect different antibodies, both should be performed for maximum sensitivity. The antibody test especially ID test is also useful to determine allergic broncho-pulmonary aspergillosis (ABPA)and aspergilloma. Precipitions can be found in > 90% patients with aspergilloma and ~70% of patients with ABPA. It can also be used as a prognostic marker.

Though significant advances have been made in detection of fungal antigen or metabolite by-product in serum or other body fluid, most of the tests are available only in research laboratories. Exception to this is the latex agglutination or ELISA tests for detection of capsular polysaccharide antigen of *C. neoformans*. The commercially available tests for cryptococcal antigen detection, detects > 95% of cryptococcal meningitis and ~70% of disseminated cryptococcal infections. Another useful antigen test available is the test for detection of *Histoplasma* antigen. This antigen test has been shown to be rapid (<24 h), sensitive (55-95%), specific (> 98%) and reproducible. The detection of antigen in urine or serum is the common procedure, though it may be detected in spinal fluid or alveolar lavage fluid.

Mannan, a polysaccharide component of *Candida* cell wall, has the disadvantage of short serum half-life and remains as immune complex. Although mannan can be detected by several methods, complicated techniques are required to dissociate mannan-antibody complex like heat dissociation or enzyme digestion of antibody. Similarly galacto-mannan is detected in aspergillosis. This particular test is evaluated in several laboratories in recent years with varying degree of success. Few cross-reactions are also observed especially with *Penicillium* species. False positive reactions are observed after vancomycin therapy. Various immuno assay formats have been designed to detect galactomannan antigen either free or in immune complexes in serum, broncho alveolar lavage fluid or urine. In two of the largest studies on patient's samples radio immunoassay for antigen detection showed 74% sensitivity and 90% specificity. The value of galactomannan detection by the Pastorex Aspergilus Latex agglutination test (Bio – Rad laboratories, France) has been evaluated by number of groups and the test showed sensitivity of up to 95% with serum samples from invasive aspergillosis. The sandwich ELISA test that employs rat monoclonal antibody EB-A2 known as Platelia Aspergillus (Bio – Rad laboratories, France) is one of the most sensitive method currently available. Lower limit of detection of galactomannan for the sandwich ELISA was 0.5 - 1.0 ng/ml of serum. Furthermore, the sandwich ELISA became positive earlier than latex particle agglutination test. Another advantage of the ELISA is the possibility that antigen titers in serum can be monitored during treatment. A decrease in the concentration of galactomannan in serum is indicative of response to treatment.

Immunodiffusion test for *Aspergillus*

Principle: - The test is based upon double diffusion as originally described by Oudin and Ouchterlony. Antibodies placed into one well on the gel matrix (e.g. Agar, agarose, polyacrylamide, gelatin etc) and homologous antigen placed into another well some distance from the antisera, will diffuse and react with each other. Visible lines of precipitation are formed in the gel matrix where the antigens and antibodies have combined in relative concentration (equivalence zone).

Materials required:

a. Glass petridishes 5cm & 20 cm diameter.
b. Agar gel cutter (template) 4-well pattern.
c. Pasteur pipettes with teats
d. Antigens : *A. fumigatus* culture filtrate
   A. flavus culture filtrate
   A. niger culture filtrate
e. Serum : Patient serum
   Positive antiserum (known positive)
   Negative antiserum (known negative)
f. BOD incubator.
g. 1% purified agar (Noble agar or equivalent).

h. Citrate buffer (pH 7.2)

i. 1% amido black solution.

Preparation of the reagents:

1. Citrate buffer (pH 7.2)

Sodium citrate	8.5g
Trisodium citrate	4.5g
Sodium azide	1.0g
Distilled water	1 L
Adjust pH to 7.2	

2. Amido Black solution.

Amido Black	= 0.1g
Acetic acid (12%)	= 45ml
Sodium acetate (1.6%)	= 45ml
Glycerol	= 10ml

Preparation of the agar plates:-

1. Dissolve 1% noble agar (Difco) or ultra pure agar in 100ml of citrate buffer.
2. Place the flask on an asbestos pad over a burner and heat it with agitation until the agar is completely dissolved. Allow it to cool to 60-70^{0}C.
3. Pour 6ml of molten agar in each petri plate (5cm diameter) and allow it to solidify at room temperature (25 -30^{0}C).
4. Place the plates in the refrigerator for at least 2h or until use.

Performance of the test:

1. Remove the agar plates from the storage. Punch out the pattern with the help of the agar gel cutter so that minimum distance between the wells is 6mm.
2. Label each well on the underside of each plate using a glass-marking pen.
3. A total of four petriplates are required to test one patient's sera. Fill the central well of the four petriplates with the patient's sera (90µl). Leave the plates at room temperature for 30 min.
4. Fill the peripheral wells with different *Aspergillus* antigens (A*. fumigatus, A. flavus, & A. niger*). Fill the wells in a clockwise manner from the notched end.
5. Add positive and negative control serum into two peripheral wells separately.
6. Cover the petriplates and place them into bigger petriplate (20-cm diameter) containing formol saline (10% formalin and 0.9% sodium chloride).
7. Cover the bigger petriplate (serving as moist chamber) and keep it in BOD incubator at 25^{o}C.
8. Examine the plates everyday for a week for the appearance of precipitating bands.

Staining:

1. After the development of the precipitating bands transfer the whole gel carefully into a big petridish containing normal saline.
2. Wash the gel by changing normal saline atleast 3-4 times every 3-10hrs to remove non-specific proteins. Finally wash with distilled water for one hour.
3. Stain the precipitating band by placing the gel in amidoblack solution for 3-5 minutes or till desired result is achieved.
4. Wash the excess stain gently in saline followed by differentiation in 2% acetic acid solution.
5. Wash the gel again with water and place in on a glass plate. Allow it to dry at 37^{o}C for permanent mount.

Reading of the test:

Unstained plate can be read by using any bright indirect light source to aid in seeing the precipitating bands. A dark background is usually preferred. For example the plates may be held next to the shade of a high intensity lamp whose light beam is directed straight downwards.

Light boxes, providing indirect light against a dark background, prepared specifically for reading immuno diffusion plates are also commercially available.

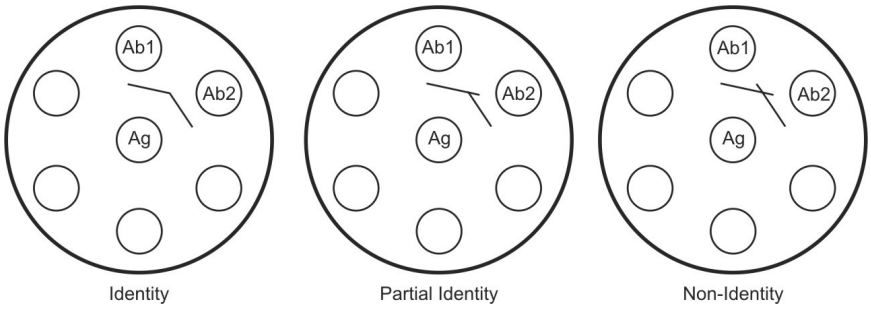


Fig. 1 : Immuno Diffusion Test – Oudin and Ouchterlony Method

Interpretations of results:

If two identical antigens (Ag1) are placed in adjacent wells of an Ouchterlony agar gel plate and an appropriate antiserum (Ab) in the central well, the precipitating band formed between each antigen and antibody (Patient serum) will meet to fuse giving a band of identity. If different antigens (Ag1 & Ag2) are placed into adjacent peripheral wells and the antiserum containing antibodies directed against both the antigens, the precipitating band formed will not fuse but will cross because different antigens are involved thus giving precipitation bands of non-identity.

If one antigen solution contains molecules of several antigen specifications on the same molecule (Ag1) and a second antigen solution containing molecules with only some of the antigenic specifications of the first antigen are placed in adjacent wells against antiserum (Ab) which contain antibodies against all specificity's present in the first solution (Ag), band of partial identity or spur formation will occur

Preparation of *Aspergillus fumigatus, A. flavus* and *A. niger* antigens used in immunodiffusion Test

For the detection of the antibodies in fungal diseases various antigens such as whole cell antigens, purified cell wall antigens cytoplasmic material in the form of carbohydrates and protein fraction of the organism have been tried. For immunodiffusion test, culture filtrate antigens are used and are prepared as described below.

Isolates:- Various strains of *Aspergillus* (*A. flavus*-6, *A. fumigatus*-3, *A. niger*-2) prevalent in locality and causing invasive fungal infections are chosen for the antigen preparation.

- Streak one or two Sabouraud dextrose agar slant with the organism.
- Incubate the slant at 25^0C for 2 days.
- After obtaining typical growth on incubation, inoculate the fungi on to synthetic medium like glucose asparagine broth/ Sabouraud dextrose broth (approx. 200ml) in a 500 ml flask.
- Inoculate by cutting approximately 0.5cm squares in the surface of the slant on which the fungi is growing and allow the slant to float on the broth.
- Incubate the broth at 25 ^{o}C (without shaking) for 5 weeks.
- Add formalin (40% formaldehyde) to a final concentration of 0.5% in the broth.

- Check the culture for microbial contamination by making smears from broth cultures and stain by Gram's technique. If contamination is noted discard the culture.
- Filter the killed broth culture by using Whatman No. 40 filter paper.
- Chill the filtrate at 4 ^{o}C.
- Add two volumes of cold acetone (double the amount of culture filtrate obtained) drop by drop to the culture filtrate (add approx. 10ml acetone every few minutes). Maintain the culture filtrate in constant agitation with a magnetic stirrer during this period.
- After adding all acetone, allow the mixture to stir on the magnetic stirrer for additional 15-min. in cold.
- Transfer the precipitated filtrate to a large centrifuge vessel.
- Centrifuge at 3,500 X g for 30min in cold centrifuge (4^{0}C).
- Discard the supernatant and resuspend the sediment in two volumes of cold acetone.
- Centrifuge at 3500 X g for 30min in cold centrifuge (4^{0}C).
- Discard the supernatant, loosen the stopper of the centrifuge bottle and place it in a desiccator containing phosphorus pent oxide or calcium chloride.
- Allow the precipitate to dry overnight at 25^{0}C.
- Resuspend the pellet in sterile distilled water (approx. 1/10th the volumes of the original culture filtrate).
- Agitate the vessels for few minutes and centrifuge at 3500 X g for 30 min to remove undissolved precipitates.
- Recover the supernatant and discard the precipitate.
- Take a small quantity of the antigen and determine its carbohydrate content by phenol-sulfuric acid test*.
- Adjust the solubilized antigen by diluting with sterile distilled water to contain 1,000-1,500 µg carbohydrate per ml.
- Add Merthiolate to a final conc. of 1:10,000 and store the antigen at 4^{0}C.

Evaluation of the *Aspergillus* antigens: -

The antigen used in the double immunodiffusion is acceptable when it gives satisfactory results when tested simultaneously with the standard reference antigens.

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* Phenol sulfuric acid test: -

- Mix 1 ml of antigen with 1 ml of 5% phenol solution.
- Add 5ml of 96% sulfuric acid.
- Allow the mixture to stand at room temperature for 30 min.
- Record the OD against blank at 490nm.
- Construct the reference curve after taking OD from the known quantities of glucose (0-75µg/ml).

Counterimmunoelectrophoresis (CIEP)

Counterimmunoelectrophoresis is also known as cross over or immunoelectrophoresis. During this electrophoresis, the antigens migrate towards the anode because their isoelectrophoretic points are lower than the pH of the buffer and antibodies migrate towards the cathode by endosmosis.

Equipment and solutions required

1. 1% agarose in Tris Buffer
2. 5×7-cm glass slides
3. Biopsy punch
4. Needles for picking out plugs or suction device
5. Anti- *Candida* antibody (control)
6. Anti- *Aspergillus* antibody (control)
7. *Candida* antigen 2 and 20mg/ml (antigen used in ID)
8. *Aspergillus* antigen, culture filtrate and somatic antigen (antigen used in ID)
9. Pipettes and pipette tips
10. CIEP tank and power pack
11. Lint wicks
12. Running buffer
13. 5% Trisodium citrate
14. 2% saline
15. Distilled water
16. Filter paper strips
17. Drying oven at 80^0C
18. Staining racks
19. 0.5%amido black stain
20. Destain
21. Light box

Preparation of Agar slides

Chemicals needed:

Agarose

Barbital buffer, 0.075M, pH 8.6

1. Add 99ml barbital buffer to a 250ml beaker.
2. Add 1.0g agarose to the buffer.
3. Heat to near boiling on a hot plate until agarose is completely dissolved.
4. Place precoated slides (7x5cm2) on a flat, level surface.
5. Working rapidly, pipette out required amount of agarose with a glass pipette.
6. Place the tip of the pipette at the centre of the slide and allow the agarose to flow out onto the slide uniformly.
7. While the agar is still fluid, remove any bubbles by playing the flame over the agar surface.
8. Cover the slides with half of a petri dish and allow to dry for approximately 20min.
9. With a template and an agar punch, cut wells (4mmdiameter) and remove agarose plugs with a Pasteur pipette.

Procedure

1) Label the wells using a solution of 5% Alcian blue applied with a fine paint brush as an 'A' or a 'C' for *Aspergillus* and *Candida.*

2) Fill control wells (15µl) at the top:

Candida Control - Positive *Candida* control sera in left hand side of pair (C+)

Candida antigen (20mg/ml) in right hand side of pair

Aspergillus control- Positive *Aspergillus* control sera in left hand of pair (A+)

*Aspergillu*s antigen (somatic) in right hand side of pair.

3. Fill in sample wells (15µl) using sera (T1-T5) in the left-hand side of each pair of wells, and antigen in the right hand side of each pair of wells. For *Aspergillus*, use culture filtrate antigen in the right hand column and somatic antigen in the left-hand column. For *Candida* use 2 mg/ml and 20 mg /ml as a reference.

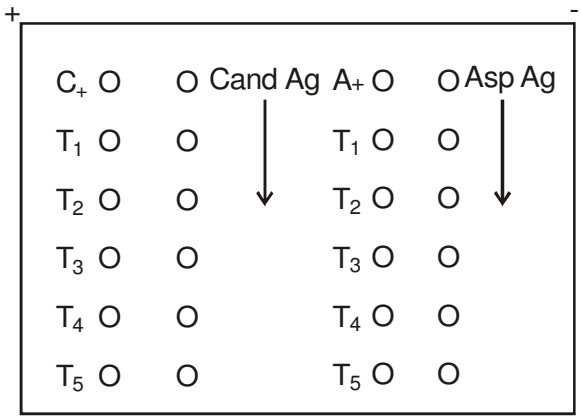

Fig. 2 : CIEP Well Pattern

4) Place the slide into the CIE tank, soak two lint wicks in running buffer. Fold "furry side up", squeeze excess buffer from folded edge and place along the edge of the gel, upto but not over the wells. (Note, to prevent running buffer exhaustion ,swap polarity of tank after each run. Wicks can be used twice turning over after the the first run then discarded).
5) Check buffer level in tank, close lid and plug into power pack. Run at 2mA/cm for 1hr 30min (for 5×7cm slides, 10mA per slide in the tank)
6) After electrophoresis, wash in 5% trisodium citrate (plus 0.1% azide to prevent bacterial growth) for 30min and overnight in a final saline wash.
7) Wash in distilled water for 30min (to prevent stain from binding non-specifically)
8) Remove, place on paper towel or and cover slides with piece of filter paper of same size making sure it is wetted uniformly.
9) Dry in oven at 37°C for about overnight till completely dry. Remove slides from oven. Gently wet the filter paper covering the slide in case difficult to remove it from the agarose surface.
10) Redry slide for a further 5min or so.
11) Place slides in staining rack and stain in 0.5% amido black stain (in 35% methanol,19% acetic acid) for 5min
12) Destain in 2 changes of destain dilution (35% methanol, 10% acetic acid) till background is clear (about 5min each) and remove and blot dry.
13) Observe in light box.

Reading

Positive - Titre ≥1/16

Complement Fixation Test

Complement fixation (CF) tests are being used principally for the serodiagnosis of histoplasmosis and coccidioidomycosis.

Materials required:

1. U- bottomed microtitre plates
2. Complement
3. Haemolysin
4. Antigens
5. Sera
6. 2.8% sheep red blood cell suspension
7. Hemolysin

Procedure

1) Dilute test sera 1/5 (total 400µl required)
2) Dilute control sera 1/6 (total 600µl required).
3) Incubate test and control sera at 56°C for 30min.
4) Label a U- bottomed microtitre plate as follows:

   Test:- 3 row of 4 wells to titrate sample from 1/5, 1/10, 1/20, and 1/40 for the mycelial and yeast antigens and a serum control.

   Positive controls:- 2 rows of 6 wells titrated from 1/5, 1/10, 1/20, 1/40, 1/80, 1/160 with mycelial antigen and serum control.

   Antigen controls: 2 wells, 1 for each antigen (yeast and mycelial)

   Complement controls: 4 wells (plus 2 used to prepare dilutions)

	1/5	1/10	1/20	1/40	1/80	1/160		
THM	O	O	O	O				
THY	O	O	O	O				
TSC	O	O	O	O				
CHM	O	O	O	O	O	O		
CSC	O	O	O	O	O	O		
CHY	O	O	O	O	O	O		
CSC	O	O	O	O	O	O		
			Complement controls				Comp.	Dil
AgC	O	O	O	O	O	O	O	O
HM	HY	3	HY	½	0			

THM Test Histoplasma Mycelium Antigen
THY Test Antigen Histoplasma Yeast Antigen
TSC Test Serum Control
AgC Antigen Controls
CHM Control Histoplasma Mycelium
CHY Control Histoplasma Yeast Antigen
CSC Control Serum Control

Fig. 3 : CFT Well Pattern

5) Add diluents as follows:
   a) Add 25µl to wells from 1/10 upward and each antigen control well.
   b) Add 50µl to first 3 complement control wells and first complement dilution well.
   c) Add 75µl to fourth complement control well.

d) Add 125µl to second complement dilution well.

6) Cover plates till sera are ready.
7) Mix inactivated sera, and add 25µl aliquots to first and second wells of test and positive control rows.
8) Make solutions across the rows starting from the second well onwards, changing tips between each row and discarding the final 25µl.
9) Just before adding complement, reconstitute complements (freeze-dried guinea pig complement in Richardson's preservative) in chilled water. Adjust strength of complement by dilution. Keep at 4°C until needed.
10) Add 25µl antigen to test and positive control rows and antigen controls; add 25µl diluent to all serum control rows.
11) Mix plates on shaker briefly.
12) Immediately add 25µl complement solution to all wells except complement control wells. Put 25µl complement in first control well. Add 25µl to both complement dilution wells, then transfer 25µl from first dilution well to third complement control
13) All wells should contain 75µl final volume.
14) Shake for 20 seconds uncovered. Put lids on, place inside plastic bag and leave in refrigerator (4°C) overnight.
15) Spin down 2ml sheep red blood cells (sterile sheep RBCs in Alsevers preservative) at 3,000 rpm.
16) Discard supernatant and wash three times in buffered diluent, inverting the tube to mix. Unsuitable cells tend to stick to the bottom and should be left there. Ensure the supernatant is clear and there is no or little haemolysis.
17) Shake last pellet gently to resuspend and transfer 200µl of RBC's to 5ml diluent with a pipette with the end cut off the minimize damage to the RBCs.
18) Add 25µl haemolysin (sheep haemolysin raised in rabbits) to 5ml diluent.
19) Mix the haemolysin and RBC suspensions well by pouring back and forth 4-5 times.
20) Place the RBC/haemolysin mixture and the microtitre plates with the serum/antigen/complement mixtures at 37^0C for 30min.
21) Mix RBCs gently and add 25µl to each well of microtitre plate.
22) Cover plate with lid and shake at 37^0C for 30min. Place plate in plastic bag at 4^0C for at least 2hrs.

Interpretation:

Read plate recording each well as 1 complete haemolysis, 2 slight inhibition of haemolysis, 3 partial haemolysis, 4 complete inhibition of haemolysis with button of intact cells at bottom of well and clear supernatant.

Cryptococcal Antigen Latex Agglutination Test

Principle

Cryptococcal Latex Agglutination is an antigen detection test that employs latex particles sensitized with anti cryptococcal antibodies. When these latex particles come in contact with the polysaccharide antigen of *C. neoformans* in the specimen, the two form a complex causing visible agglutination.

Materials required:

1. Reagent latex

2. Control latex
3. Sample diluent
4. Positive control
5. Negative control
6. Slide
7. Disposable stirrers

Specimens used:

CSF -should be clear, if blood tinged; centrifuge and use supernatant

Serum - heat inactivate at 56°C for 30min.

1) Mark 4 wells of the cardboard slide: test ,+C and -C (positive and negative controls)
2) Place 50µl of the test (neat) and 1/100 dilution in the appropriate wells.
3) Place 50µl of *Cryptococcus* antigen control onto the +C well and negative control into the -C well.
4) Resuspend the latex reagent before using by shaking gently (not vortexing) and place 50µl on each well.
5) Mix each well with a clean applicator stick (plastic pipette tips can be used, but not wooden sticks).
6) Shake at 100rpm for 10min at room temperature.
7) Read by looking for agglutination by comparing with the controls. Sera with extremely high titers of Cryptococcal antigen may be negative with neat but positive at 1/100 dilution (prozone effect).

Results:

Reactive result is indicated by distinct large clumps against a clear or slightly milky background.

Nonreactive result is indicated by a smooth, milky suspension with absence of agglutination

Pronase treatment

This is performed when both the control and the test give agglutination.

Pronase is a commercially available enzyme (Protease, SIGMA)

1. 150mg of the enzyme powder is dissolved in 20ml of glycine buffer. This can be aliquoted and stored in ampoules up to 3 months.
2. Add 300µl of the test sample to 200µl of pronase solution in a test tube and allow it to stand for 5min.with intermittent shaking.
3. Place in water bath at 56°C for 15min.
4. Transfer the tube to a boiling water bath and allow to remain in it for 5min.
5. Centrifuge at 1000rpm for 15min and repeat the test using supernatant.

Tube agglutination test of *Cryptococcus neoformans or Candida* spp.

Materials required

1. Physiological normal saline
2. Test tubes 10×75mm, 12×75mm
3. Serological pipettes
4. Test tube rack
5. Water bath
6. Cryptococcal whole cell antigen

Preparation of antigen:

1. Streak a Sabouraud's dextrose slants with a standard strain of *Cryptococcus neoformans* and incubate for 25 °C for 48hrs.
2. Prepare the inoculum for broth culture by suspending the organisms from the slant in a few ml of Sabouraud's dextrose broth.
3. Inoculate the suspension of organisms from step 2 into a Erlenmeyer flask containing 500ml of sterile glucose aspargine broth and incubate at 25° C on an incubator shaker (180rpm) for 72- 92hrs.
4. Add formalin to the broth culture to a final concentration of 0.4% (10ml of 40% formaldehyde for 100ml of broth culture).
5. Allow the broth culture to stand at 4° C for 24hrs.
6. Prepare smear of the culture and stain it with Gram stain to check for bacterial contamination. If contamination is noted, discard the culture and begin again at Step 1
7. Transfer the broth culture to 2 centrifuge tubes and centrifuge at 650g for 15min.
8. Remove the supernatant fluid and resuspend the sediment cells in enough formalized saline to fill the tube. Repeat the procedure for at least two times.
9. Prepare a 25% suspension of the cells by adding 3 volumes of formalized saline per 1 volume of packed cells.
10. Store this, as stock antigen suspension at 4° C. It is stable upto 3 months.

Preparation of working antigen

1. Add 1ml of stock antigen suspension to a 15/20ml-centrifuge tube.
2. Add approximately 9ml of formalized saline to the antigen and mix.
3. Centrifuge at 650g for 15min, remove the supernatant fluid and resuspend the sediment cells in approximately 9ml formalized saline.
4. Repeat step 3 two times for a total of three washing cycles. Finally resuspend the cells to final volume of 25ml with formalized saline.
5. Adjust the volume

Performance of test

1. Transfer 0.2 ml of patient serum and positive and negative control serum using auto pipette. Heat inactivate these sera at 56°C for 30min in a water bath.
2. Label and set up a row of nine 10×75mm test tube to make 1:4,1:8,1:16,1:32,1:64,1:128,1:256,1:512,1:1024 dilutions of each serum to be tested (including positive and negative control sera).
3. Using 1ml pipette, add 0.3ml of inactivated serum to tube 1.
4. Add 0.3ml of normal saline starting from tube 1 to tube 9.
5. Mix and transfer 0.3ml from tube 1 to tube 2. Continue mixing and transferring 0.3ml through tube 9. Discard 0.3ml from the ninth tube.
6. Repeat step C to E with each serum to be tittered.
7. Add 0.3ml of the working *C. neoformans* antigen to each dilution of positive, negative and patient' sera.
8. Shake the rack thoroughly to mix the tube contents.

9. Place the rack of tubes in a 37°C water bath and incubate for 2hrs. Shake the rack at the end of the first hour and again at the end of the incubation period.
10. Transfer the rack to a 4°C refrigerator and let the tubes remain at that temperature for overnight.
11. After overnight incubation, remove the rack from refrigerator, taking care not to disturb the sediment antigen.

Reading of results

Examine the tubes with any good light source against a dark background.

1. First read the negative control serum dilution series. There must be no agglutination of antigen in any tube of this dilution series. Button formation is usually noticed.
2. Read the positive control dilution series. The titer of this serum is the dilution factor of the highest dilution of showing any degree of agglutination. The positive reference antiserum must reach its anticipated titer within a two-fold dilution for the test to be considered valid.
3. If all controls in the tests are satisfactory, read the tubes of the patient's serum dilution series. Titers of these sera are determined in the same way as in the positive reference antiserum.

Interpretation:

Titer equal to or more than 8 is considered significant. Simultaneously attempt should be made for culture of specimen or *Cryptococcus* antigen detection test.

Intradermal test

Skin tests are used for different purposes:

1. Etiological diagnosis (helps only in non-endemic area)
2. Epidemiological study Immunological status of the subject e.g. atopic and non- atopic groups.

Requirement

i. Tuberculin syringe
ii. Needle 26 gauge
iii. Antiseptic solution
iv. Antigen
   - *Aspergillus fumigatus* (100 PNU/ml, Hollister -Steir, USA)
   - *Candida albicans* (1000 PNU/ml, Hollister -Steir, USA)
   - *Histoplasma capsulatum* (Histoplasmin(yeast derived) Histolyn Cyl ,Berkly Biologicals, CA)
   - *Sporothrix schenckii* (Culture filtrate antigen from yeast phase *of Sporothrix schenckii*
v. Shaving blade
vi. Filter paper
vii. Steroid and antihistaminic, injection for anaphylactic reaction if any)

Procedure

1. The antigenic product to be administered should be first inspected visually for any particulate matter or discoloration prior to administration.
2. Intradermal administration
   - Cleanse the rubber stopper of the vial with antiseptic before withdrawing extract.
   - Clean the anterior surface of lower arm (preferably) with antiseptic. Introduce the needle between the superficial layers of the skin (away from the blood vessel) and inject the extract.

- Simultaneously inoculate negative control solution. The negative control should be the same diluting fluid as for the antigenic extract or buffer saline (usually sterile Phosphate Buffered Saline (PBS) pH 7.2 is used as negative control). Diluting fluid is injected similarly as the antigenic extract.

3) Precaution

- Patient should avoid antihistamines for 24hrs
- Drugs like ephedrine, adrenaline, aminophylline and isoprenaline should not be administered within 8hrs.

4) Reading and interpretation

Read test after 15min and following up to one hour, and then at 6-8hrs. For delayed type reaction, it is read after 24, 48 and 72hrs.

Type I Reaction develops within minutes, maximal after 10-20 min and resolves within 1 to 1 1/2 hrs. The urticarial weal and flare are occasionally accompanied by itching.

Type III Reaction read after 6 hrs. Any amount of subcutaneous edema is considered positive

Type IV Delayed type of hypersensitivity read after 48-72hrs.
Induration more than 5 mm is considered positive.

Pulmonary Aspergillus Lung Disease

Asthma	-	Type I
Alveolitis	-	Type III reaction, occasionally type I also
ABPA	-	Type I, III, IV
Aspergilloma	-	Type IV

In case of Histoplasmosis, an induration of ≥5mm is taken as skin-positive

In case of Sporotrichosis, an induration of > 10mm is taken as skin-positive.

Preparation and standardization of the antigens used in Skin testing

The following general procedure is used for the preparation of the antigens:

1. Preparation of the fungal growth
2. Grinding
3. Defatting
4. Extraction
5. Clarification
6. Dialysis
7. Sterilization
8. Sterility testing
9. Standardization

1. Preparation of the fungal growth:

- Streak one or two Sabouraud dextrose agar slant with the organism obtained from stock culture.
- Incubate the slants at 25^{o}C for 2-3 days (incubation varies with different fungi).
- After obtaining typical growth on incubation, the organism is used to inoculate synthetic medium like glucose aspargine broth.
- Glucose asparagine broth is prepared by using the following formula:
    - L- asparagine - 7.0g
    - Ammonium chloride (NH_4Cl) - 7.0g
    - Dipotassium phosphate (K_2HPO_4) C.P. - 1.31g

- Sodium citrate C.P. - 0.9g
- Magnesium sulfate anhydrous ($MgSO_4$) - 1.5g
- Ferric citrate U.S.P. VIII (scales) - 0.3g
- Dextrose (AR) - 10.0g
- Glycine C.P. - 25g
- Distilled water - 1 L

Dissolve aspargine in about 300ml of hot distilled water at 50°C. Dissolve each of the organic salts in 25ml of distilled water, ferric citrate being dissolved in hot water. Add each salt in order, starting with K_2HPO_4 to the hot asparagine solution and mix well each time the salt is added.

- Inoculate by cutting approximately 0.5cm squares in the surface of the slant on which the fungi is growing and allow the slant to float on the broth.
- Incubate the broth at 30°C/25°C (without shaking) for 3-4 weeks.
- Kill the culture by adding Merthiolate to a final concentration of 1:10,000 in a broth.
- Check the culture for microbial contamination by making smears from broth cultures and stain with Gram stain. If contamination is noted discard the culture.
- Filter the killed broth culture by using filter paper.
- Separate the mycelial mat and transfer in 95% alcohol or acetone and chill at 4°C for 24hrs.
- Squeeze the mycelial mat between the two layers of the filter paper and dry in a desiccator over anhydrous calcium chloride.

2. Grinding:

- For efficient extraction of antigenic material from the dried fungal pellicle, finely pulverize in pestle and mortar and then pass through a sieve of 150meshes/ sq. cm to get a fine powder.
- Use a portion of the powder to raise antisera in rabbit to test the potency of the prepared antigen.

3. Defatting:

- This step is done to allow efficient extraction of proteinaceous material and to obtain clear extract.
- Add 3-4 volumes of ether or acetone to the extract. Shake the mixture well and decant the oil layer. Repeat the process with fresh ether or acetone until no color is visible in the supernatant.

4. Extraction:

- Extract the active allergenic/ antigenic substances from the defatted powder using highly alkaline buffer (pH 8.0) of the following composition.
    - Sodium chloride 5.0g
    - Potassium phosphate (monobasic) 0.31g
    - Sodium phosphate (dibasic, anhydrous) 7.0g
    - Phenol crystals 4.0g
    - Double distilled water 1L.
- Add the extracted powder to 500ml of buffer in the Erlenmeyer flask.
- Allow the mixture to stand at room temperature for 72 hrs.
- Shake the mixture for a period of 30min using magnetic stirrer in a refrigerator for at least 8 -10 times during the 72h extraction period.

5. Clarifications, Dialysis and Sterilization:

- After extraction separate the soluble ingredients by filtration using whatman No. 1 filter paper.
- Dialyze the filtrate against distilled water to remove the irritating substances and coloring materials.
- Sterilize the extract using Millipore filters or membrane filters.
- Collect the filtrate aseptically into different sterile containers, label and store at $4^{o}C$.

6. Sterility testing:

Inoculate the filtrate into blood agar, brain heart infusion broth and thioglycollate broth and incubate at $37^{o}C$ for 7 days.

7. Standardization:

Accurate methods for determination of allergenic potency have not been yet established. Commonly used method for giving appropriate expression of the potency of allergenic components includes:

1. Weight by volume method (1:50) 1 g of the substance is extracted with 50ml of extraction fluid (buffered saline pH 8.0).
2. Total nitrogen content by micro Kjeldahl method.

Total protein content by biuret method.

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Molecular Methods in Diagnosis of Fungal Infections

The increasing incidence of systemic fungal infections and the failure to diagnose those infections antemortem by conventional procedures has focused more attention on the rapid and accurate diagnosis of invasive fungal infections using various molecular biology techniques. Nucleic acid hybridization and amplification methods are fundamental to molecular diagnosis. Polymerase chain reaction (PCR) offers the potential for the rapid and early detection of fungal DNA. PCR is the enzymatic exponential amplification of a specific target region using short primers leading to detectable amount of amplified DNA from one or a few original sequences. The parameters influencing the clinical usefulness of a PCR assay includes: -

I. Method employed for isolation of fungal DNA from clinical specimen.
II. The sensitivity and specificity of the assay to rapidly detect and identify DNA in a variety of specimen.
III. Need for repeated sampling of clinical specimen for PCR in a particular fungal disease.
IV. Relevance of the test result as PCR is likely to predict infection before other diagnostic tests.

The five major components of a PCR assay are: -

I. Sample preparation
II. Selection of target region
III. Post PCR detection methods to identify the amplified product.
IV. Standardization of reproducible PCR conditions and format.
V. Precautions to minimize false positive and negative results.

Specimen preparations can have a significant impact on the sensitivity and reproducibility of a molecular diagnostic test. Sample preparation method should release intracellular DNA from the fungal cell wall. The requirement for fungal DNA extraction varies with the nature of the specimen. The optimum specimen for PCR may also differ with the disease entity. Serum is the most convenient sample used; but when leukocytes are known to phagocytose fungal hyphae, whole blood, plasma or buffy coat layer may result in higher DNA yield.

The different DNA extraction protocol described involves: -

- Disruption of fungal cell wall.
- Release of DNA by lysis of cell membrane.
- Lysis of white and red blood cells.
- Purification of DNA.

DNA extraction:

In earlier protocols zymolase or lyticase enzymes were used to disrupt the fungal cell wall. This results in formation of fungal spheroplasts. Zymolase has efficiently been used in *Candida* and *Cryptococcus* spp., but found to be ineffective in disrupting cell wall of moulds. Lyticase has greater activity against the cell wall of moulds. Mechanical disruption with heat-alkali treatment, vortexing in presence of glass bead, repeated freezing and thawing or grinding in liquid nitrogen has also been effectively used. In whole blood, lysis protocol usually consists of combined heat-alkali treatment procedures and cell membrane lysis.

The availability of commercial DNA extraction kit has facilitated DNA purification. The QIA amp DNA mini kit (Quiagen) has been successfully applied to clinical samples to purify fungal DNA. In addition to above mentioned methods, CTAB, a strong cationic detergent that releases the DNA by solubilizing cell membrane has been used as another fungal DNA extraction method. After the cell wall and membrane lysis, standard phenol: chloroform extraction procedures are used to purify the DNA.

Target Selection:

Targets that can be used in molecular diagnostic tests for fungal infection include single and multicopy nuclear, mitochondrial genes and RNA. Multicopy gene targets have high sensitivity because of the large number of target molecules detected.

Multicopy gene targets:

Ribosomal DNA gene cluster:- rDNA gene is a tandem array of at least 50-100 copies in the haploid genome of all fungi. It comprises of small subunit SSU rDNA (18S), 5.8S gene and large subunit LSU (28S) gene. Separating 18S and 5.8S is ITSI and 5.8S and 28S is ITS2 called internal transcribed spacer regions. Between each transcript is an intergenic spacer region (IGS). Some basidiomycetes may contain fourth gene called 5SrRNA. Within a single repeat there are regions that have different rates of evolutionary changes and hence may be used to examine evolutionary histories of group of taxa. The coding regions are highly conserved, ITS regions are moderately variables called divergent domain while IGS region contains the hyper variable sequences in rDNA. So this allows the design of universal primers based on conserved regions as well as species-specific probes from variable regions that can be used to identify the species. Universal primers ITS1 and ITS2 have been designed from conserved sequence of the coding region. Various species specific probes have been designed like 27A and Ca3 probes from the *C. albicans* and CARE-1 from *Cryptococcus neoformans.*

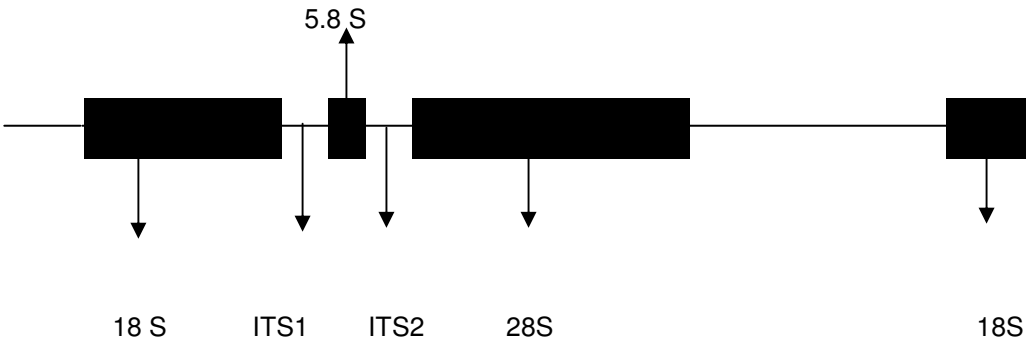

Fig1 : Ribosomal gene cluster

Some other multicopy genes that have been targeted include Candidal – secreted aspartic proteinase gene (SAPs), which comprises at least 9 related members in the *C. albicans* genome and *Aspergillus* mitochondrial (mt) gene.

Single copy gene targets:

In case of yeasts especially in *Candida* spp. various genes including actin, chitin synthase, heat shock proteins and lanosterol 14 alpha-demethylase can be targeted. But there is less data available on single copy gene targeted PCR to detect moulds. In *P. brasiliensis* 43 Kda glycoprotein has been targeted. In *Aspergillus* species – copper zinc superoxide dismutase is a potential target.

PCR based detection methods:-

Nested PCR: Consists of a second round of PCR using a separate primer set internal to the first round which increases amplification of a specific region in the first PCR amplified target. This method increases the sensitivity and is more discriminative.

Multiplex PCR: Allows detection of several different fungal isolates in the same PCR reaction. It uses highly specific sets of primers which are designed to anneal at the same temperature and the PCR products are designed to be of different sizes to allow discrimination.

PCR-RFLP: Various groups of fungal pathogens in clinical samples can be detected by amplifying targeted site by PCR followed by restriction enzyme digestion. This combined PCR – restriction fragment length polymorphism (RFLP) analysis is applicable to a wide variety of species and is relatively easy to perform.

Direct sequencing of amplified product: The nucleotide sequence of an amplified target sequence can be determined and the information is used to identify the fungal pathogen by comparison of the sequence obtained with the sequences deposited in the database collection (GenBank and Fungal Genome Initiative).

Real-Time PCR: Holds promise for the rapid diagnosis of fungal infections. This technology, unlike traditional PCR, has a typical run time of less than 1 h and both amplification and detection occur in a closed system eliminating cross contamination. In addition, if hybridization probe technology is used as the method of detection, melting temperature analysis may be used to differentiate microorganisms. The Taq Man probe used in Real Time PCR consists of a reporter dye with a fluorescence derivative at the 5′ end, a 3′ quencher dye, and a 3′ blocking phosphate group. The fluorescence emission of the reporter dye is suppressed in the intact probe by faster type energy transfer. During PCR, probe is cleaved by the exonuclease activity of Taq polymerase only when it is hybridized to a complementary target. When probe specific PCR has been generated, an increase in reporter dye fluorescence resulting from the cleavage between the reporter and quencher dye occur. The amount of reporter dye released is proportional to the amount of DNA amplified by PCR. Real-Time PCR is becoming a standard procedure in molecular diagnosis of fungal infections.

Hybridization based detection methods

These techniques use probes with sequence homology to target DNA and can be used to detect fungal specific sequences in DNA or RNA samples. Most of these techniques employ post amplification hybridization.

Fluorescent Insitu Hybridization (FISH): In this technique fluorescent probes are used for insitu analysis of organisms, even those that are unculturable.

Microtiter hybridization assay: This technique consists of generation of biotinylated amplified product followed by its capture onto a streptavidin coated microtiter plate wells and hybridization with a labelled probe. The reaction product is detected in an enzyme linked immuno assay (EIA) to provide a colorimetric or fluorescence readout.

Reverse Hybridization Line Probe Assay: In this technique species specific oligonucleotide probes deposited on a strip are allowed to hybridize to biotin labeled amplified target sequence. After hybridization and stringent washing, detection is possible by addition of a streptavidin conjugate and a substrate which results in a purple precipitate at the probe lane.

Hybridization on DNA Chips: DNA chips comprise arrays of a large number of probes that are fixed onto a support at relatively high densities. PCR products labeled with fluorescent dye can be hybridized to all

spotted probes simultaneously in a single hybridization reaction and the resulting hybridization pattern is read by fluorescence detection. Oligonucleotide arrays can be custom made to identify presence of specific fungal species using ITS or ribosomal RNA sequence information.

The detection and identification of fungal pathogens by DNA based methods can yield results sooner than cultivation. Sensitivity of these methods is higher than cultures and serologic tests for diagnosis of invasive fungal infections. The DNA detection results have correlated with clinical improvement and effect of treatment. However, molecular diagnostic methods too have some limitations as these methods may not distinguish individuals who are colonized from these who are infected. False positive results ranging from 8% to 38% have been reported in diagnosis of candidiasis and aspergillosis. False negative results have also been reported in 21% of patients with *Candida* infections.

Preparation of solutions:

Lysis buffer for mycelial DNA extraction

EDTA	50 mM
Tris HCl (pH 8)	100 mM
SDS	3%

Yeast DNA extraction solution:

SDS	1%
EDTA	2.5 mM
Sodium acetate	25 mM
Protinase K	267 µg/ ml

TE buffer

Tris HCl (pH 8.0)	100 mM
EDTA (pH 8.0)	10 mM

Phenol: chloroform: isoamyl alcohol mix

Tris equilibrated phenol (molecular biology grade)	25.0 ml
Chloroform	24.0 ml
Isoamyl alcohol	1.0 ml

3M Sodium acetate

Sodium acetate (un hydrous)	246.09 g
Distilled water	1000ml

70% ethanol

Absolute ethanol	70.0 ml
Distilled water	30.0ml

RNase solution

RNase	10 mg
Distilled water	1.0 ml

Proteinase K solution

Proteinase K	20 mg
TE buffer	1.0 ml

TAE buffer 50X

Tris base	242.0 g
Glacial acetic acid	57.1 ml

0.5M EDTA (pH 8.0)	100.0 ml

TBE buffer 5X

Tris base	54.0 g
Boric acid`	27.5 g
0.5M EDTA (pH 8.0)	20.0 ml

Gel loading buffer (6X) for DNA

Bromophenol blue	0.25%
Xylene cyanol FF	0.25%
Glycerol	30.0%

Ethidium bromide solution for DNA staining

Stock solution(10 mg%)

Ethidium bromide powder	10.0 mg
Distilled water	1.0 ml

Working concentration in 1x gel running buffer (0.5 µg/ml)

Preparation of primer solution:

Total amount of oligonucleotide primer = 33 × OD× 1000/MW = Z nm.

To make a primer stock solution of 100pmol/µl add 10×Z µl of TE buffer. This stock solution is diluted 4 times to make a working primer solution of 25 pmol/µl. Primer solutions are stored in aliquots at -20^0 C. Primers are thawed on ice just before use in PCR reactions.

DNA isolation Protocols:

Isolation of genomic DNA from yeast:

- Place a loopful of culture in 5 ml of yeast extract-phosphate-dextrose (YPD) broth in a 200 ml flask. Incubate overnight at 30^0 C.
- Pellet down the growth in 1.5 ml of microfuge tube at 7500 rpm for 2 min.
- Wash the pellet twice with sterile dist. water and centrifuge again for 4 min. Suspend the pellet in 200 µl of lysis buffer. Add 0.3 g of sterile glass beads.
- Mix well by vortexing and then add 200 µl of equal amount of phenol: chloroform to the cell suspension.
- Vortex this mixture vigourously for one min followed by cooling on ice for 30 sec. Repeat this process of vortexing and cooling five more times. Add 200µl of TE buffer and again vortex briefly.
- Centrifuge the mixture at 13000 rpm at room temperature for 5 min. Take the aqueous phase in a clear sterile microfuge tube and add equal volume of chloroform: isoamyl alcohol (25:24:1). Centrifuge at 10000 rpm for 7 min.
- Collect the aqueous phase and add 1/10th volume of 3 M sodium acetate and equal volume of chilled isopropanol. Mix and incubate at -20^0 C for 2 hours for DNA precipitation.
- Collect the DNA precipitate at 10000 rpm for 5 min and decant the supernatant.
- Add 0.5 ml 70% ethanol, vortex briefly to wash the pellet. Centrifuge at 10000rpm for 5 min, aspirate out the supernatant carefully and allow the pellet to dry at 37^0 C for one hour.

- Resuspend the pellet in appropriate volume of TE buffer. The DNA is allowed to dissolve for atleast 6h at room temperature with intermittent mixing. One µl of RNase is added and incubated at 37^0 C for 30 min. The DNA is electrophoresed on a 0.8% agarose gel to check the quality and quantity.

Isolation of Genomic DNA from mycelial fungi:

- Grow the mycelial isolate on Sabouraud's dextrose agar (SDA) slope for three to four days at 25^0 C. Collect the conidia and wash with sterile normal saline.
- Inoculate into Sabouraud's dextrose broth and incubate at 37°C in a rotary shaker (150rpm) for about three days.
- Recover mycelia by filtration through a 50 ml syringe which is packed with sterile glass wool. The fungal mat formed in the syringe is washed with acetone.
- Transfer the mat to a Whatman No1 filter paper and let it dry at 37 °C.
- Thoroughly dried mycelial mat (0.3-0.5 g) is transferred to a clean mortar and a small volume of liquid nitrogen is added to it. Grind the frozen mat quickly to make fine powder using a pestle and adding more liquid nitrogen.
- To this powder immediately add 2-3 ml of lysis buffer (100 mM Tris HCl, pH 8.0, 50 mM EDTA, 3% SDS), preheated to 65°C and mix well to make a slurry. Transfer 0.7 ml of this suspension to a 1.5 ml microfuge tube.
- Add 1.4 µl of proteinase K (20 mg/ml) and incubate the tube at 55^0C for 1 hour with occasional mixing.
- To this add 700 µl of buffered phenol: chloroform (1:1), vortex briefly and centrifuge at room temperature for 5 min at 10000 rpm.
- Transfer the top aqueous phase to another sterile micro centrifuge tube without disturbing the interface and add equal volume of chloroform: isoamyl alcohol (24:1) to the supernatant. Repeat centrifugation and collect the upper aqueous phase into a fresh tube.
- Add $1/10^{th}$ volume of sodium acetate and two volumes of ice cold ethanol to allow the DNA to precipitate.Gently invert the tubes to mix the contents and to see the threads of DNA precipitation.
- Centrifuge at 10000rpm for 2-5 min at room temp. Without disturbing the pellet decant the supernatant and wash the pellet twice with 500 µl of ice- cold 70% ethanol.
- Allow the pellet to dry at 37^0C till the ethanol evaporates completely.
- Resuspend the pellet in appropriate amount of TE buffer. The DNA is allowed to dissolve for atleast 6 h at room temperature with intermittent mixing.
- One µl of RNase is added and incubated at 37^0 C for 30 min. The DNA is electrophoresed on a 0.8% agarose gel to check the quality and quantity.

Isolation of genomic DNA from serum for detection of yeast:

- Take 200µl of serum in a clean microfuge tube and add equal volume of yeast DNA extraction solution.
- Add 5µl of proteinase K to the above solution and incubate the mixture at 60^0C in a water bath for a period of 2 hours.
- Centrifuge the above mixture at 10000g for 10min.

- Collect the supernatant in other sterile microfuge tube and add equal volume of phenol: chloroform: isoamyl alcohol (25:24:1).
- Vortex it very briefly and spin down the solution at 10000g for 10min.
- To the aqueous phase add equal volume of chilled isopropyl alcohol. Allow the DNA to precipitate for for 2 hours at -20^{0}C.
- Centrifuge to collect the DNA pellet at 10000g for 10min. The supernatant is removed and the DNA pellet is washed in 70% alcohol.
- The DNA pellet is allowed to air-dry, dissolved in 30µl of TE buffer and the DNA is quantitated in a spectrophotometer.

Isolation of DNA from blood to detect mycelial fungal DNA by PCR:

- Take 500 µl of blood in a 2 ml microfuge tube and 1.5 ml of RBC lysis buffer (25 mM Tris HCl pH 8.0), mix and incubate for 10 min at room temperature.
- Centrifuge at 10000 rpm for 30 sec and remove the supernatant.
- Resuspend the pellet in 150 µl of leukocyte resuspension buffer (0.9% NaCl, 15 mM EDTA, 15 mM Tris HCl pH 8.0, 21 mM β-mercapto ethanol, 125 units lyticase/ml) and incubate at 37^0 C for 1 h.
- Then add 6.5 µl of 10% SDS followed by 7.5µl proteinase K solution and incubate the tube at 55^0 C for 1 hour with occasional mixing.
- Add 100 µl of aspergillus extraction buffer (400 mM Tris HCl pH 8.0, 1 M NaCl, 20 mM EDTA, 2% SDS) and incubate the tube for 30 min at 65^{0}C.
- Add equal volume of phenol: chloroform: isoamyl alcohol (25:24:1), vortex briefly and centrifuge at room temperature for 5 min at 10000 rpm.
- Transfer the top aqueous phase to another sterile micro centrifuge tube without disturbing the interface and add equal volume of chloroform: isoamyl alcohol (24:1) to the supernatant. Repeat centrifugation and collect the upper aqueous phase into a fresh tube.
- Add equal volume of isopropyl alcohol and keep in -20^0 C to precipitate the DNA. Centrifuge to collect the DNA pellet at 10000rpm for 10min and decant the supernatant. Wash the DNA pellet in 500 µl of ice- cold 70% ethanol.
- Resuspend the pellet in appropriate amount of TE buffer. The DNA is allowed to dissolve for atleast 6 h at room temperature with intermittent mixing.
- One µl of RNase is added and incubated at 37^0 C for 30 min. The quality of DNA is checked by electrophoresis on a 0.8% agarose gel.
- All DNA samples are stored at -20^0 C until use.

PCR Protocols:

Optimization of a particular PCR can be time consuming and complicated because of various parameters that are involved. These parameters include the followings:

- Quality and concentration of DNA template.
- Design and concentration of primers.
- Concentration of Mg^{2+} ions.
- Concentration of four dNTP's.

- PCR buffer system.
- Selection and concentration of DNA polymerase.
- PCR thermal cycling conditions.
- Addition and concentration of PCR additives and co-solvents.

Optimization of PCR may be affected by each of these parameters individually as well as the combined interdependent effect of these parameters.

Materials:

- Template DNA
- Forward and reverse primers
- $MgCl_2$
- dNTP's (dATP, dCTP, dGTP, dTTP)
- 10 X PCR buffer having 500mM KCl, 100mM Tris Cl, pH 8.0
- DNA Polymerase (Taq, Pfu, Tsg polymerase)
- PCR additives (glycerol, DMSO, formamide) - Optional

Setting up PCR:

The common volume of PCR used per reaction mixture is usually 10, 25 or 50µl. All the reaction components can be mixed together in a 0.5 ml PCR tube in any sequence except for DNA polymerase, which should be added at the end. It is recommended to mix all the component right before PCR cycling.

For each PCR, master mix is prepared by adding following components:

For 50µl reaction mixture

PCR buffer with $MgCl_2$:	5 µl
Primers forward and revers (100 µMl)	2 µl each
dNTP's (2.5 mM)	4 µl
DNA polymerase (3U/µl)	0.5 µl
Template DNA (200-500 ng)	2-10µl
DD H_20	upto 50 µl

PCR Cycling:

A common PCR program starts with an initial denaturation step at 95^0 C for 2 –5 min followed by 30 cycles of denaturation at 94^0 C for 1 min, primer annealing at 45-65^0 C (decided by primer Tm) for 1 min and primer extension at 72^0 C for 1 min and a final Extension step at 72^0 C for 5-10 min.

Verifying PCR Amplification:

About 2 to 5µl of PCR product is run on a 1-2% agarose gel to check for the presence of fungal specific DNA in the sample. The gel is visualized by staining with ethidium bromide. A successful PCR amplification should display a specific band with the expected size without nonspecific bands and smear.

Some important Facts:

- The quality and concentration of DNA templates can directly affect outcome of PCR.
- Proper concentration (usually 0.1-1 µM) and appropriate primers are very critical for successful PCR. The optimal primer size is usually between 18-20 bases. Both forward and reverse primers should have melting temperature within limits of 2-5^oCof each other. Avoid complementary

sequences between primers to reduce primer dimer formation. Primers with Tm higher than 50^{o}C will generally give more specific results.

- Mg concentration is critical to the success rate of PCR amplification. It may affect DNA polymerase activity. Excess Mg results in accumulation of non-specific products. Common Mg concentration used in PCR is between 0.5- 2mM.

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Antifungal Susceptibility Testing

Reference method for Broth Dilution Antifungal Susceptibility Testing of Yeasts: Approved Standard – Second Edition (M 27-A2, CLSI, USA)

Introduction

The method described here is intended for testing yeasts that cause invasive infections. These yeasts encompass *Candida* species, and *Cryptococcus neoformans*. The method has not been used in studies of the yeast form of dimorphic fungi, such as *Blastomyces dermatitidis* or *Histoplasma capsulatum* var. *capsulatum*.

M27-A2 is a "reference" standard. It is being developed through a consensus process to facilitate the agreement among laboratories in measuring the susceptibility of yeasts to antifungal agents. An important use of a reference method is to provide a standard basis from which other methods can be developed, which also will result in interlaboratory agreement within specified ranges. For example, broth microdilution methods, described here, have been configured to produce results paralleling those obtained by the reference method.

Antifungal Agents

Source

Antifungal standards or reference powders can be obtained commercially, directly from the drug manufacturer or from reputed company as pure salt. Pharmacy stock or other clinical preparations/ formulations **should not** be used. **Acceptable powders bear a label that states the drug's generic name, its assay potency [usually expressed in micrograms (µg) or International Units per mg of powder], and its expiration date**. The powders are to be stored as recommended by the manufacturers, or at -20^{o}C or below in a desiccator (preferably in a vacuum). When the desiccator is removed from the freezer, it is to be allowed to come to room temperature before it is opened (to avoid condensation of water).

Weighing Antifungal Powders

All antifungal agents are assayed for standard units of activity. The assay units can differ widely from the actual weight of the powder and often differ within a drug production lot. Thus, a laboratory must standardize its antifungal solutions based on assays of the lots of antifungal powders that are being used.

Either of the following formulae may be used to determine the amount of powder or diluent needed for a standard solution:

$$\text{Weight (mg)} = \frac{\text{Volume (mL)} \times \text{Concentration (µg/ML)}}{\text{Assay Potency ((µg/mg)}} \quad (1)$$

.or

$$\text{Vol. (mL)} = \frac{\text{Weight (mg)} \times \text{Assay Potency (µg/mg)}}{\text{Concentration (µg/mL)}} \quad (2)$$

The antifungal powder should be weighed on an analytical balance that has been calibrated with Standards. Usually, it is advisable to accurately weigh a portion of the antifungal agent in excess of that required and to calculate the volume of diluents needed to obtain the concentration desired.

Example: To prepare 100 ml of a stock solution containing 1280 µg/mL of antifungal agent with antifungal powder that has a potency of 750 µg/mg, use the first formula to establish the weight of powder needed:

$$\text{Weight (mg)} = \frac{\text{100 mL (Target Vol.)} \times \text{1280 µg/ML (Desired Conc.)}}{\text{750 µg/mg (Potency)}} = 170.7 \text{ mg} \quad (3)$$

Because it is advisable to weigh a portion of the powder in excess of that required, powder was deposited on the balance until 182.6 mg was reached. With that amount of powder weighed, formula (2) above is used to determine the amount of diluent to be measured:

$$\text{Volume (mL)} = \frac{\text{182.6 mg (Powder Weight)} \times \text{750 µg/mg (Potency)}}{\text{1280 µg/mL (Desired Concentration)}} = 107.0 \text{ mL}$$

Therefore, the 182.6 mg of the antifungal powder is to be dissolved in 107.0 mL of diluent.

Preparing Stock Solutions

Antifungal stock solutions are to be prepared at concentrations of at least 1280 µg/mL or ten times the highest concentration to be tested, whichever is greater. There are some antifungal agents, however, of limited solubility that can require lower concentrations. In all cases, information provided by the drug manufacturer should be considered as part of determining solubility.

Use of Solvents other than water

Some drugs must be dissolved in solvents other than water (see Table 1). Information on the solubility of an antifungal compound should be included with the drug. Such drugs should be dissolved at concentrations at least 100 times higher than the highest desired test concentration. Commonly used agents include: dimethyl sulfoxides (DMSO), ethyl alcohol, polyethylene glycol, and carboxy methyl cellulose. When such solvents are used a series of dilutions at 100 times the final concentration should be prepared from the antifungal stock solution in the same solvent. Each intermediate solution should then be further diluted to final strength in the test medium. This procedure avoids dilution artifacts that result from precipitation of compounds with low solubility in aqueous media.

For example to prepare for a broth macrodilution test series containing a water-insoluble drug that can be dissolved in DMSO for which the highest desired test concentration is 6 µg/mL first weigh 4.8 mg (assuming 100% potency) of the antifungal powder and dissolve it in 3.0 mL DMSO. This will provide a stock solution at 1,600 µg/mL. Next, prepare further dilutions of this stock solution in DMSO. (See Tables 1 and 2). The solutions in DMSO will be diluted 1:50 in test medium and a further two fold when inoculated, reducing the final solvent concentration to 1% DMSO at this concentration (without drug) should be used in the test as a dilution control.

The example above assumes 100% potency of the antifungal powder. If the potency is different, the calculations as mentioned above should be applied.

Filtration

Normally, stock solutions do not support contaminating microorganisms and they can be assumed to be sterile. If additional assurance of sterility is desired, they are to be filtered through a membrane filter. Paper, asbestos, or sintered glass filters, which may adsorb appreciable amounts of certain antifungal agents, are not to be used. Whenever filtration is used, it is important that the absence of adsorption by appropriate assay procedures is documented.

Storage

Small volumes of the sterile stock solutions are dispensed into sterile polypropylene or polyethylene vials, carefully sealed, and stored (preferably at -60°C or below but never at a temperature greater than -20°C). Vials are to be removed as needed and used the same day. Any unused drug is to be discarded at the end of the day. Stock solutions of most antifungal agents can be stored at -60°C or below for six months or more without significant loss of activity. In all cases, any directions provided by the drug manufacturer are to be considered as a part of these general recommendations and should supersede any other directions that differ. Any significant deterioration of an antifungal agent may be ascertained by comparing with the quality control strains.

Number of concentrations Tested

The concentrations to be tested should encompass the breakpoint concentrations and the expected results for the quality control strains. Based on previous studies, the following drug concentration ranges should be used: amphotericin B, 0.0313 to 16 μg/mL; flucytosine, 0.125 to 64 μg/mL.; ketoconazole, 0.0313 to 16 μg/mL.; itraconazole and new triazoles 0.0313 to 16 μg/mL; and fluconazole 0.125 to 64 μg/mL.

Test Procedures

Broth Medium

A completely synthetic medium is RPMI 1640 (with glutamine, without bicarbonate, and with phenol red as pH indicator) was found at satisfactory for testing the filamentous fungi and has been used to develop the proposed standard.

Buffers

Media should be buffered to a pH of 7.0 ± 0.1 at 25°C. A buffer should be selected that does not antagonize antifungal agents. Tris buffer is unsatisfactory because it antagonizes the activity of flucytosine. Zwitterion buffers are preferable to buffers that readily traverse the cell membrane, such as phosphate buffers, because, theoretically, the latter can produce unexpected interactions with antifungal agents. One buffer that has been found to be satisfactory for antifungal testing is MOPS (3-(N-morpholino) prophanesulfonic acid] (final concentration 0.165 mol/L for pH 7.0). The pH of each batch of medium is to be checked with a pH meter when the medium is prepared; the pH should be between 6.9 and 7.1 at room temperature (25°C). MIC performance characteristics of each batch of broth are evaluated using a standard set of quality control organisms

Preparing Diluted Antifungal Agents

The steps for preparation and storage of diluted antifungal agents are as follows:

1) Use sterile, 12 x 75 mm plastic test tubes to perform the tests
2) Use a growth control tube containing RPMI 1640 broth without antifungal agents (but with nonaqueous solvent where necessary) for each organism tested.
3) Close the tubes with loose screw-caps, or plastic or metal caps.

Water Soluble antifungal agents

When two fold dilutions of a water-soluble antifungal are to be used, they may be prepared volumetrically in broth (Table – 1). The procedure for antifungals that are not soluble in water is different from that for water-soluble agents and is described below. When running a small number of tests, consulting the schedule in Table 2 is recommended.

The total volume of each antifungal dilution to be prepared depends on the number of tests to be performed. Because 0.1 mL of each antifungal drug dilution will be used for each test, 1.0 mL will be adequate for about nine tests, allowing for pipetting. A single pipette is used for measuring all diluents and then for adding the stock antifungal solution to the first tube. A separate pipette is used for each remaining dilution in that set. Because there will be a 1:10 dilution of the drugs when combined with the inoculum, the working antifungal solutions are ten times more concentrated than the final concentrations.

Many persons find working with 1:10 dilutions (as shown in Table 1 easy and convenient. However, some automated pipettes deliver only 1.0 or 01 mL volumes; therefore, a ratio of 1:11 would be preferable. It is unimportant whether the final test volume is 1.0 mL or 1.1 mL. If 1:11 dilutions are made, the dilution scheme should be altered so that the same final concentrations or drug are obtained.

Water Insoluble antifungal agents

For antifungal agents that cannot be prepared as stock solutions in water, such as ketoconazole, amphotericin B, or itraconazole, a dilution series of the agent should be prepared first at 100 x final strength in an appropriate solvent. Each of these nonaqueous solutions should now be diluted tenfold in RPMI 1640 broth.

For example, if a dilution series with final concentrations in the range 16 μg/mL to 0.0313 μg/mL is desired, a concentration series from 1,600 to 3.13 μg/mL should have been prepared first in DMSO. To prepare 1 mL volumes of diluted antifungal agent (sufficient for 10 tests), first pipette 0.9 mL volumes of RPMI 1640 broth into each of 11 sterile test tubes. Now, using a single pipette, add 0.1 mL of DMSO alone to one 0.9 mL lot of broth (control medium), then 0.1 mL of the lowest (3.13 μg/mL) drug concentration in DMSO, then 0.1 mL of the 6.25 μg/mL concentration and continue in sequence up the concentration series, each time adding 0.1 mL volumes to 0.9 mL broth. These volumes can be adjusted according to the total number of tests required. Because there will be a 1:10 dilution of the drugs when combined with the inoculum, the working antifungal solutions are tenfold more concentrated than the final concentrations.

Inoculum Preparation

The steps for preparation of inoculum are as follows:

1) All organisms should be sub-cultured from sterile vials onto Sabouraud dextrose agar or peptone dextrose agar and passaged at least twice to ensure purity and viability. The incubation temperature throughout must be 35^{o}C.
2) The inoculum should be prepared by picking five colonies of ~ 1 mm in diameter from 24 hour old culture of *Candida* species or 48 hours old cultures of *C. neoformans*. The colonies should be suspended in 5 mL of sterile 0.145 mol/L saline (8.5 g/L NaCl; 0.85% saline).
3) The resulting suspension should be vortexed for 15 seconds and the cell density adjusted with a spectro-photometer by adding sufficient sterile saline to increase the transmittance to that produced by a 0.5 McFarland standard (see Appendix) at 530 nm wavelength. This procedure will yield a yeast stock suspension of 1 x 10^6 to 5 x 10^6 cells per mL. A working suspension is made by a 1:100 dilution followed by a 1:20 dilution of the stock suspension with RPMI 1640 broth medium which results in 5.0 x 10^2 to 2.5 x 10^3 cells per mL.

Inoculating Broth

Before adjusting the inoculum, 0.1 mL of the various antifungal concentrations are placed in 12 x 75 mm tubes. The growth control receives 0.1 mL of drug diluent without antifungal agent. Within 15 minutes after the inoculum has been standardized (up to two hours if inoculum is kept at 4°C), 0.9 mL of the adjusted inoculum is added to each tube in the dilution series and mixed. This results in 1:10 dilution of each antifungal concentration and 10% dilution of the inoculum.

Incubation

With the exception of *C. neoformans*, tubes are incubated (without agitation) at 35°C for 46 to 50 hours in ambient air. When testing *C. neoformans*, tubes should be incubated for a total of 70 to 74 hours before determining results.

Reading Results

The MIC is the lowest concentration of an antifungal that substantially inhibits growth of the organism as detected visually. The amount of growth in the tubes containing the agent is compared with the amount of growth in the growth control tubes (no antifungal agent) used in each set of tests as follows:

Amphotericin B: For amphotericin B end points are typically easily defined and the MIC is read as the lowest drug concentration that prevents any discernible growth. Trailing end points with amphotericin B are not usually encountered.

Flucytosine and azoles antifungal: For flucytosine and especially for azoles, such as fluconazole and ketoconazole, end points are typically less sharp and may be a significant source of variability. A less stringent end point (slight turbidity is allowed above the MIC) has improved interlaboratory agreement and also discriminates between putatively susceptible and resistant isolates. When turbidity persists, it is often identical for all drug concentrations above the MIC. The amount of allowable turbidity can be estimated by diluting 0.2 mL of drug-free control growth with 0.8 mL of media, producing an 80% inhibition standard. Even dispersion of clumps that can become evident after incubation can make end-point determination more reproducible. Reference strains of defined susceptibility can also be used in the training of new personnel.

Interpretation of Results

Interpretive breakpoints have been established at present only for some organism-drug combinations. The clinical relevance of testing other organism-drug combinations remains uncertain, but the relevant information can be summarized as follows:

Amphotericin B: Experience to date indicates that amphotericin B MICs for *Candida* spp. isolates are tightly clustered between 0.25 and 1.0 µg/mL. When isolates that appear resistant to amphotericin B in animal models are tested by M27, MIC values greater than 1 µg/mL may be obtained. Unfortunately, the M27 methodology does not consistently permit detection of such isolates and all that can at present be concluded is that if an M27 amphotericin B MIC of > 1 µg/mL is obtained for a *Candida* spp. isolates, then that isolate is likely resistant to amphotericin B. Current work suggests that testing with Antibiotic Medium-3 supplemented with 2% glucose permits more reliable detection of resistant isolates. However, the reproducibility of this method is still under study and laboratories that choose to do this testing must carefully compare their results with those obtained for isolates with known responses to amphotericin B.

Flucytosine: Based largely on historical data and partially on the drug's pharmacokinetics, interpretive breakpoints for *Candida* spp. and flucytosine have been established.

Fluconazole : Based on a large data package presented by flucoanzole's manufacturer, interpretive breakpoints for *Candida* spp. and fluconazole have been established . These data are principally drawn from studies of oropharyngeal candidiasis and of invasive infections due to *Candida* spp. in non-

neutropenic patients and their clinical relevance in other settings is uncertain (in recent meta- analysis, the breakpoint is confirmed). In addition, these interpretive breakpoints are not applicable to *C. krusei*, and thus identification to the species level is required in addition to MIC determination. The utility of testing isolates of *C. neoformans* is currently under intense study, and recent data do suggest a correlation between elevated MIC and clinical failure.

Ketoconazole : Experience to data using the procedures described in this standard indicates that yeast MICs vary between 0.0313 and 16 µg/mL. However, data are not yet available to indicate a correlation between MIC and outcome of treatment with ketoconazole.

Itraconazole: Based on a large data package presented by itraconazole's manufacturer, interpretive breakpoints for *Candida* spp. and itraconazole have been established. These data are entirely from studies of oropharyngeal candidiasis, and their clinical relevance in other settings is uncertain. In addition, the importance of proper preparation of drug dilutions for this insoluble compound can not be over emphasized. Use of the incorrect solvents or deviation from the dilution scheme suggested in Table 2 can be lead to substantial errors due to dilution artifacts.

New Triazoles: Experience to date with posaconazole-, ravuconazole-, and voriconazole-using procedures described in this standard indicates that yeast MICs vary between 0.03 and 16 µg/mL with the majority of isolates inhibited by <1 µg/mL of all three agents. However, data are not yet available to indicate a correlation between MIC and outcome of treatment with these agents.

Broth Microdilution Modifications

A substantial body of data has now been presented that documents excellent concordance between results obtained by the broth macrodilution methodology described above and a broth microdilution adaptation. The ease of performance of broth microdilution tests is very attractive, and most clinical laboratories will probably choose to implement this method rather than the broth macrodilution method. The steps and testing conditions that are relevant to the broth microdilution test are discussed in detail.

The 10-fold drug dilutions described for the broth macrodilution procedure should be diluted 1:5 with RPMI to achieve the two times strength needed for the broth microdilution test. The stock inoculum suspensions are prepared and adjusted, as described, under the broth macrodilution test. The stock yeast suspension is mixed for 15 seconds with a vortex diluted 1:50 and further diluted 1:20 with medium to obtain the two times test inoculum (1×10^3 to 5×10^3 CFU/mL). The (two fold) inoculum is diluted 1:1 when the wells are inoculated and the desired final inoculum size is achieved (0.5×10^3 to 2.5×10^3 CFU/mL).

The broth microdilution test is performed by using sterile, disposable, multiwell microdilution plates (96 U-shaped wells). The 2x drug concentrations are dispensed into the wells of rows 1 to 10 of the microdilution plates in 100 mL volumes with a multichannel pipette. Row 1 contains the highest (either 64 or 16 µg/mL) drug concentration and row 10 contains the lowest drug concentration (either 0.12 or 0.03 µg/mL). These trays may be sealed in plastic bags and stored frozen at -70°C for up to 6 months without deterioration of drug potency. Each well of a microdilution tray is inoculated on the day of the test with 100 µL of the corresponding 2x diluted inoculum suspension, which brings the drug dilutions and inoculum densities to the final concentrations mentioned above. The growth control wells contain 100 µL of sterile drug-free medium and are inoculated with 100 µL of the corresponding diluted (2X) inoculum suspensions. The QC organisms are tested in the same manner and are included each time an isolate is tested. Row 11 of the microdilution plate can be used to perform the sterility control (drug-free medium only).

The microdilution plates are incubated at 35°C and observed for the presence or absence of visible growth. The microdilution wells are scored with the aid of a reading mirror, the growth in each well is compared with that of the growth control (drug-free) well.

A numerical score, which ranges from 0 to 4, is given to each well using the following scale: 0, optically clear; 1, slightly hazy; 2, prominent decrease in turbidity; 3, slight deduction in turbidity; and 4, no reduction of turbidity.

For isolates in which clumping hinders applying these definitions, dispersion of the yeast suspension by pipetting, vortexing or other techniques can help. The MIC for amphotericin B is defined as the lowest concentration in which a score of 0 (optically clear) is observed and for 5-FC and the azoles, as the lowest concentration in which a score of 2 (prominent decrease in turbidity) is observed. Prominent decrease in turbidity corresponds to approximately 50% inhibition in growth as determined spectrophotometrically. The microdilution MICs read at 48 hours (72 hours for most *C. neoformans*) provide the best agreement with the reference broth macrodilution method.

Impact of Time Reading: 24 Hours versus 48 Hours

The M27-A2 methodology for *Candida* recommends an end point reading at 48 hours. For most isolates, the difference between readings at 24 hours versus 48 hours is minimal and will not alter the interpretative category (i.e., does not change whether the isolate would be categorized as “susceptible” or “resistant”). However, recent work has begun to include 24-hour readings, because (a) MICs can often be read at 24 hours; and (b) readings taken at 24 hours may be more clinically relevant for some isolates. Isolates for which the earlier reading is important show a dramatic rise in MIC between 24 hours and 48 hours due to significant trailing growth (partial inhibition of growth over an extended range of antifungal concentrations). Estimated as occurring in about 5% of isolates this trailing growth can be so great as to make an isolate that appears susceptible after 24 hours appear completely resistant at 48 hours. Two independent in vivo investigations of this phenomenon that employed murine models of disseminated candidiasis, have shown that isolates with this behavior should be categorized as “susceptible” rather than “resistant.” This concept has been corroborated by a demonstration that trailing growth can be eliminated by lowering the pH of the test medium to 5 or less and by a clinical demonstration that oropharyngeal candidiasis due to such isolates respond to a low dose of fluconazole used to treat typical susceptible isolates. In light of these observations, both 24-hour and 48-hour microdilution MIC ranges are provided for the two QC strains and eight systemic antifungal agents (Table 3).

Other Modifications

In addition to ongoing efforts to simplify the procedures described in this standard, some more fundamental modifications of the method have been developed in response to specific problems and are described in Table 5. These modifications are not part of the current methodology, but interested laboratories may wish to explore their clinical relevance.

Quality Control

Growth Control: Each broth macrodilution series should include a growth control of basal medium without antifungal agent to assess viability of the test organisms. With the broth tests, the growth control also serves as a turbidity control for reading end points.

Purity Control: A sample of each inoculum is streaked on a suitable agar plate and incubated overnight to detect mixed cultures and to provide freshly isolated colonies in the event retesting proves necessary

End Point Interpretation Control: End point interpretation is monitored periodically to minimize variation in the interpretation of MIC end points among observers. All laboratory personnel who perform these tests should read a selected set of dilution tests independently. The results are recorded and compared to the results obtained by an experienced reader.

Quality Control Strains: Ideal reference strains for quality control of dilution tests have MICs that fall near the middle of the concentration range tested for all antifungal agents; e.g. an ideal control strain would be

inhibited at the fourth dilution of a seven dilution series, but strains with MICs at either the third or fifth dilution would also be acceptable.

Table 4 lists expected ranges for strains found to be acceptable as quality control strains. Also shown are additional strains that can be useful for conducting reference studies.

Table 1: Scheme for Preparing Dilutions of Water Soluble Antifungal Agents to be used in Broth Dilution Susceptibility Tests

Drug - Starting Concentration (µg/ml)	Stock 5120	2	4 160	8	16	32 20	64	128	256 2.5	511	Remarks
Tube #	TUBE 1	2X TUBE 2	4X TUBE 3	2X TUBE 4	4X TUBE 5	8X TUBE 6	2X TUBE 7	4X TUBE 8	8X TUBE 9	2X TUBE 10	
Source	From Stock 1.0	From Tube 1	From Tube 1	From Tube 3	From Tube 3	From Tube 3	From Tube 6	From Tube 6	From Tube 6	From Tube 9	Step 1
Add DRUG Amount (ml) +		1.0 +	1.0 +	1.0 +	0.5 +	0.5 +	1.0 +	0.5 +	0.5 +	1.0 +	Row 1
Add Solvent RPMI (ml)	7.0	1.0	3.0	1.0	1.5	3.5	1.0	1.5	3.5	1.0	
Intermediate Drug Concentration (µg/ml)	640	320	160	80	40	20	10	5	2.5	1.25	
Add Drug from Tube Row 1 Above (ml) +	1.0 +	1.0 +	1.0 +	1.0 +	1.0 +	1.0 +	1.0 +	1.0 +	1.0 +	1.0 +	Step 2 Row 2 5X (1:4)
RPMI 1640 (ml)	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	
Final Concentration at 1:5 (µg/ml)	128	64	32	16	8	4	2	1	0.5	.25	2X
From Row 2 add Drug to Micro titer plate (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	Step 3 (1:1)
Add Inoculum to Plate (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	Step 4 (T.Vol 0.2ml)
FINAL DRUG CONC in Well at 1: 100 (µg/ml)	64	32	16	8	4	2	1	0.5	0.25	0.125	

Table 2: Scheme for Preparing Dilution Series of Water-Insoluble Antifungal Agents to be used in Broth Dilution Susceptibility Tests

Drug - Starting Concentration (µg/ml)	1,600	2	4	8 200	16	32	64 25	128	256	511	Remarks
Tube #	TUBE 1 (Stock) (100 X)	2X TUBE 2	4X TUBE 3	8X TUBE 4	2X TUBE 5	4X TUBE 6	8X TUBE 7	2X TUBE 8	4X TUBE 9	8X TUBE 10	
Source	From Tube 1	From Tube 1	From Tube 1	From Tube 1	From Tube 4	From Tube 4	From Tube 4	From Tube 7	From Tube 7	From Tube 7	Step 1
Add DRUG Amount (ml) +	-	0.5 +	0.5 +	0.5 +	0.5 +	0.5 +	0.5 +	0.5 +	0.5 +	0.5 +	Row 1
Add Solvent DMSO (ml)	-	0.5	1.5	3.5	0.5	1.5	3.5	0.5	1.5	3.5	
Intermediate Drug Concentration (µg/ml)	1,600	800	400	200	100	50	25	12.5	6.25	3.13	
Add Drug from Tube Row 1 Above (ml) +	0.1 +	0.1 +	0.1 +	0.1 +	0.1 +	0.1 +	0.1 +	0.1 +	0.1 +	0.1 +	Step 2 Row 2 (1:50)
RPMI 1640 (ml)	4.9	4.9	4.9	4.9	4.9	4.9	4.9	4.9	4.9	4.9	
Final Concentration at 1:50 (µg/ml)	32	16	8.0	4.0	2.0	1.0	0.5	0.25	0.125	0.0625	(2X)
From Row 2 add Drug to Micro titer plate (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	Step 3 (1:1)
Add Inoculum to Plate (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	Step 4 (T.Vol 0.2ml)
FINAL DRUG CONC in Well at 1: 100 (µg/ml)	16	8	4	2	1	0.5	0.25	0.125	0.0625	0.0313	

*DMSO = **Dimethylsuloxide**

Table 3. Recommended MIC limits for Two Quality Control and Four Reference Strains for Broth Macrodilution Procedures:

MIC(µg/ml) Ranges for microdilution tests							
Organism	**Antifungal Agent**	**Range**	**24h Mode**	**% within Range**	**Range**	**48h Mode**	**% within Range**
Candida parapsilosis ATCC® 22019	Amphotericin B	0.25-2.0	0.5	97	0.5-4.0	2.0	92
	Fluconazole	0.06-0.25	0.12	99	0.12-0.5	0.25	98
	5FC	0.5-4.0	2.0	98	1.0-4.0	2.0	99
	Itraconazole	0.12-0.5	0.25	96	0.12-0.5	0.25	98
	Ketoconazole	0.03-0.25	0.06/0.12	98	0.06-0.5	0.12	98
	Voriconazole	0.016-0.12	0.06	100	0.03-0.25	0.06	100
	Ravuconazole	0.016-0.12	0.06	96	0.03-0.25	0.06	98
	Posaconazole	0.06-0.25	0.12	97	0.06-0.25	0.12	99
Candida krusei ATCC® 6258	Amphotericin B	0.5-2.0	1.0	100	1.0-4.0	2.0	100
	5FC	4.0-16	8.0	98	8.0-32	16	99
	Fluconazole	8.0-64	16	100	16-128	32	100
	Itraconazole	0.12-1.0	0.5	96	0.25-1.0	0.5	100
	Ketoconazole	0.12-1.0	0.5	96	0.25-1.0	0.5	99
	Voriconazole	0.06-0.5	0.25	98	0.25-1.0	0.5	100
	Ravuconazole	0.06-0.5	0.25	93	0.25-2.0	0.5	100
	Posaconazole	0.06-0.5	0.25	100	0.12-1.0	0.5	99

Table 4: Recommended MIC limits for Two Quality Control and Four Reference Strains for Broth Macrodilution Procedures:

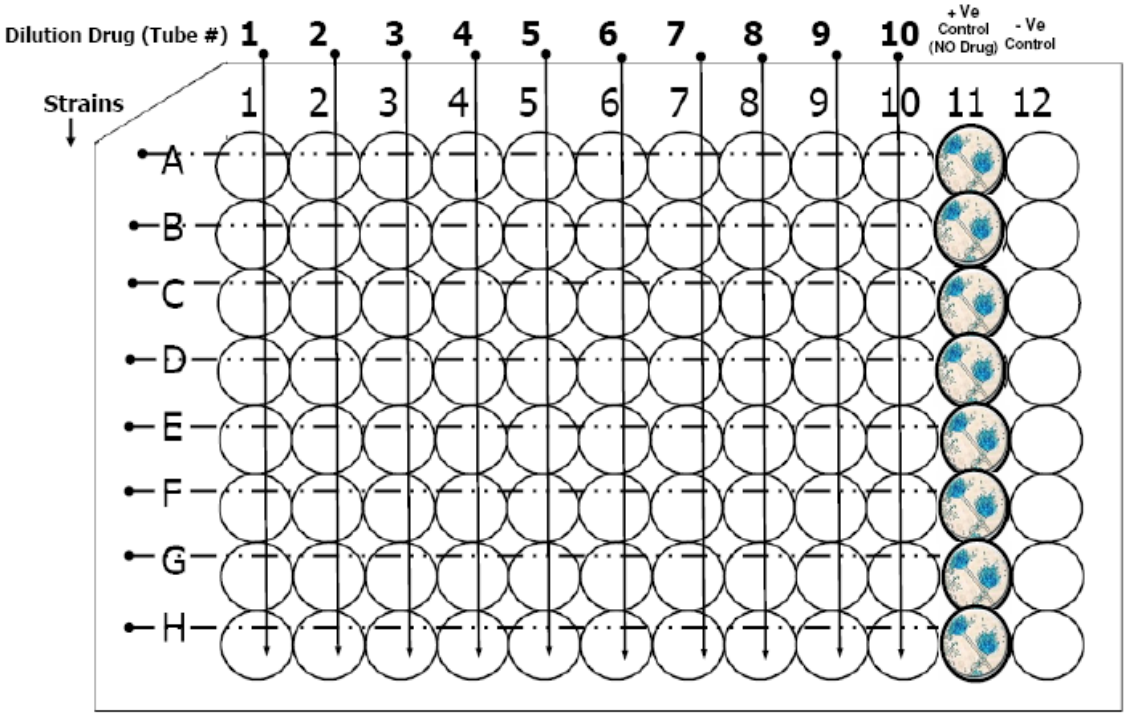
Organsim	Purpose	Antifungal Agent	MIC Range (µg/mL)	% of MICs within Range
Candida parapsilosis ATCC® 22019	QC	AmphotericinB Fluconazole Itraconazole Ketoconazole 5FC	0.25-1.0 2.0-8.0 0.06-0.25 0.06-0.25 0.12-0.5	99.1 99.1 99.0 99.0 98.6
Candida krusei ATCC® 6258	QC	AmphotericinB Fluconazole Itraconazole Ketoconazole 5FC	0.25-2.0 16-64 0.12-0.5 0.12-0.5 4.0-16	99.5 99.1 94.0 100.0 96.8
Candida albicans ATCC® 90028	Reference Fluconazole 5FC	Amphotericin B Fluconazole 5FC	0.25-1.0 0.25-1.0 1.0-4.0	99.5 95.9 91.9
Candida parapsilosis ATCC® 90018	Reference	Amphotericin B Fluconazole 5FC	0.5-2.0 0.25-1.0 ≤0.12-0.25	96.4 98.2 9.5
Candida tropicalis ATCC® 750	Reference	Amphotericin B Fluconazole 5FC	0.5-2.0 1.0-4.0 ≤0.12-0.25	93.7 95.5 99.5

Note: ATCC is a registered trademark of the American Type Culture Collection

Table 5. Modifications for Special Circumstances

Drug	Organism	Modification
Amphotericin B	*Candida* spp	Use of antibiotic Medium 3 may enhance detection of resistance, but this medium is not standardized and substantial lot-to-lot variability is possible
All drugs	*C. neoformans*	Use of Yeast Nitrogen Base may ehance the growth of C. neoformans and improve the clinical relevance of antifungal MICs
All drugs	All organisms	Supplementation of the test medium so that it contains glucose at a final concentration of 20 g/L may simplify endpoint determination

Suggested Plating Scheme for Micro Broth



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Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard (M -38A)

Introduction

CLSI document M38A is a “reference” standard developed through a consensus process to facilitate the agreement among laboratories in measuring the susceptibility of moulds to antifungal agents. An important use of a reference method is to provide a standard basis from which other methods can be developed.

The advantages which a standardized method provides are ease of performance, economy, or more rapid results. Any method that produces concordant results with this reference method, it would be considered to be in conformity with CLSI document M 38-A.

This method (M 38- A) is intended for testing filamentous fungi or moulds that cause invasive infections. These moulds encompass *Aspergillus* species, *Fusarium* species, *Rhizopus* species, *Pseudallescheria boydii*, and mycelial form of *Sporothrix schenckii.* Although other opportunistic monilaceous and dematiaceous moulds have been evaluated, Caution should be used when interpreting the MIC results from other mould/drug combinations. This method has not been used for antifungal susceptibility testing of the yeast form of dimorphic fungi, such as *Blastomyces dermatitidis* or *Coccidioides immitis, Histoplasma capsulatum* variety *capsulatum or Penicillium marneffei or S. schenckii*.

Antifungal Agents

For details of the source of antifungal, weighing antifungals, preparing stock solutions, use of solvents other than water (appendix), storage of stock solutions and number of concentrations tested are similar as mentioned above in M 27-A2.

Test Procedure

The preparation of broth medium and buffers in this protocol is the same as mentioned above in M 27-A2.

Preparing Diluted Antifungal Agent

The steps for preparation and storage of diluted antifungal agents are as follows:

1) Use sterile, plastic test tubes to prepare drug dilutions and sterile, disposable, multiwell microdilution plates (96 U shaped wells) to perform the tests

2) Use a growth control well containing RPMI 1640 broth without antifungal agents (but with nonaqueous solvent where necessary) for each organism tested.

When two fold dilutions of a water-soluble antifungal are to be used, they may be prepared volumetrically in broth (Table – 6). The procedure for antifungals that are not soluble in water is different from that for water-soluble agents and is described below. When running a small number of tests, consulting the schedule in Table 7 is recommended.

The total volume of each antifungal dilution to be prepared depends on the number of tests to be performed. Because 0.1 mL of each antifungal drug dilution will be used for each test, 1.0 mL will be adequate for about eight tests (one microdilution tray), allowing for pipetting. A single pipette is used for measuring all diluents and then for adding the stock antifungal solution to the first tube. A separate pipette is used for each remaining dilution in that set. Because there will be a 1:2 dilution of the drugs when combined with the inoculum, the working antifungal solutions are two times more concentrated than the final concentrations.

For antifungal agents that cannot be prepared as stock solutions in water, such as ketoconazole, amphotericin B, or itraconazole, a dilution series of the agent should be prepared first at 100 x final strength in an appropriate solvent. Each of these nonaqueous solutions should now be diluted 1:50 in RPMI-1640 medium`.

For example, if a dilution series with final concentrations in the range 16 μg/mL to 0.0313 μg/mL is desired, a concentration series from 1,600 to 3.13 μg/mL should have been prepared first in DMSO. To prepare 5 mL volumes of diluted antifungal agent (sufficient for 45 tests), first pipet 4.9 mL volumes of RPMI 1640 broth into each of 10 sterile test tubes. Now, using a single pipet, add 0.1 mL of DMSO alone to one 4.9 mL lot of broth (control medium), then 0.1 mL of the lowest (3.13 μg/mL) drug concentration in DMSO, then 0.1 mL of the 6.25 μg/mL concentration and continue in sequence up the concentration series, each time adding 0.1 mL volumes to 4.9 mL broth. These volumes can be adjusted according to the total number of tests required. Because there will be a 1:2 dilution of the drugs when combined with the inoculum, the working antifungal solutions are twofold more concentrated than the final concentrations.

Inoculum Preparation

When the risk of substantial spatter or areosolozation is present, the manipulation should be performed in a Class IIA or IIB biological safety cabinet.

Initial work demonstrated that reliable non-germinated conidial or sporangiospore suspensions could be prepared by a spectrophotometric procedure, and that concentration of viable conidial or sporangiospore test inocula in a range of approximately $0.4x10^4$ to $5x\ 10^4$ CFU/mL provided the most reproducible MIC data. To induce conidium and sporangiospore formation, most fungi (*Aspergillus*

species, *P. boydii, R. arrhizus* and *S. schenckii*) should be grown on potato dextrose agar for 7 days at 35^0C. *Fusarium* species should be incubated for 18 to 72 hours at 35^0 C and then until day 7 at 25^0 C to 28 ^{0} C. Cover seven-day- old colonies with approximately 1ml of sterile 0.85% saline, and prepare a suspension by gently probing the colonies with the tip of a transfer pipette. Addition of one drop (approximately 0.01ml) of Tween 20 will facilitate the preparation of *Aspergillus* inocula. The resulting mixture of conidia or sporangiospores and hyphal fragments is withdrawn and transferred to a sterile tube. After heavy particles are allowed to settle for 3 to 5 minutes, the upper homogenous suspension is transferred to a sterile tube, the cap tightened and mixed with a vortex mixer for 15 seconds. (Caution: remove the cap carefully as liquids adhering to the cap may produce aerosols upon opening.) The densities of the conidial or sporangiospore suspensions are read and adjusted to an optical density (OD) that all range from 0.09 to 0.11(80 to 82%transmittance) for *Aspergillus* species and *S. schenckii* and 0.15 to 0.17 (68 to 70 % transmittance) for *Fusarium* species, *P. boydii and R. arrhizus*. These suspensions will be diluted 1:50 in the standard medium. Inoculum suspensions of *P. boydii* may require a lower (50%) dilution factor. The 1: 50 inoculum dilutions will correspond to 2x the density needed of approximately 0.4 $x10^4$ to 5x 10^4 CFU/ml. The test inoculum will be made in sufficient volume to directly inoculate each well with 0.1 ml of the corresponding diluted inoculum suspension.

Inoculum quantification can be performed by plating 0.01ml of a 1:100 dilution of the adjusted inoculum on Sabouraud glucose agar to determine the viable number of CFU per milliliter. The plates will be incubated at 28 to 30^0 C and observed daily for the presence of fungal colonies. Colonies should be counted as soon as possible after growth becomes visible, especially for isolates of *R. arrhizus*. The incubation times will range from 24 hours or less (*R. arrhizus*) to 5 days (*P. boydii*)

Inoculating Broth

Inoculate each well on the day of the test with 0.1 ml of the 2x conidial or sporangiospore inoculum suspension. This step will dilute the drug concentrations, inoculum densities, and solvent if used to the final desired test concentrations. The growth control wells will contain 0.1ml of the drug diluents (2%) without antifungal agent. QC and reference organisms are also tested in the same manner and should be included each time an isolate is tested.

Incubation

All microdilution trays are to be incubated (without agitation) at 35^oC. Trays containing *Rhizopus* species should be examined after 21 to 26 hours of incubation before determining MIC results. Most other opportunistic filamentous fungi, including: *Fusarium* spp., *Aspergillus* spp., and *Sporothrix schenckii* are evaluated after 46 to 50 hours of incubation. *P. boydii* is examined after 70 to 74 hours.

Reading Results

The MIC is the lowest concentration of an antifungal that substantially inhibits growth of the organism as detected visually. For conventional microdilution procedure, the growth in each MIC well is compared with that of the growth control with the aid of a reading mirror. Each microdilution well is then given a numerical score as follows: 4-no reduction in growth; 3- slight reduction in growth or approximately 75% of the growth control (drug free medium); 2- prominent reduction in growth or approximately 50% of the growth control; 1- slight growth or approximately 25% of the growth control; and 0- optically clear or absence of growth.

Reference strains of defined susceptibility can be used in the training of new personnel.

Described below are the guidelines for reading the results for the various drugs.

Amphotericin B

For amphotericin B end points are typically easily defined and the MIC is read as the lowest drug concentration that prevents any discernible growth (score 0). Trailing end points with amphotericin B are not usually encountered and it is possible that such a pattern could reflect clinically relevant drug resistance.

Flucytosine, fluconazole, and ketoconazole

For flucytosine and especially for azoles, such as fluconazole and ketoconazole, end points are typically less defined that that described for amphotericin B which may contribute to a significant source of variability. A less stringent end point (slight turbidity is allowed above the MIC) has improved inter-laboratory agreement. For this class turbidity allowed corresponds to approximately 50% (half or more) reduction in growth, to the growth in the control well(drug – free medium). When this turbidity persists, it is often identical for all drug concentrations above the MIC.

Itraconazole

For itraconazole and the new triazoles, posaconazole, ravuconazole, and voriconazole end points are typically easily defined and the MIC is read as the lowest drug concentration that prevents any discernible growth (100% inhibition, or numerical score of 0). Trailing end points with these agents against *Aspergilllus* spp., and most other opportunistic pathogenic moulds are not usually encountered. It is possible that such a pattern could reflect clinically relevant drug resistance as it has been demonstrated for A. *fumigatus* strains that have been clinically resistant to itraconazole.

Interpretation of Results

Interpretive breakpoints have not been established at present. The clinical relevance of testing this group of fungal pathogens remains uncertain.

Amphotericin B

Experience to date using the procedure described in this standard indicates that amphotericin B MICs for most opportunistic filamentous fungal isolates are clustered between 0.5 and 2.0 µg/mL. However amphotericin B MICs for some species (*A. terreus*, *Acremonium strictum, S. apiospermum* and *S. prolificans*) can be above 2 µg/ml (MIC ranges of 2 to 16 µg/ml). Although little data are available regarding correlation between MIC and outcome of treatment with amphotericin B for the filamentous fungi, MIC above 2µg/ml have been associated with treatment failures and MIC below 2µg/ml with clinical cure.

Flucytosine

Filamentous fungi are usually not susceptible to flucytosine and most MICs are >64 µg/ml for these isolates. The exceptions are some isolates of *Aspergillus* species and phaeoid (dematiaceous) fungi.

Fluconazole

Filamentous fungi are usually not susceptible to fluconazole and most MICs are > 64µg/ml for these isolates, excepting some isolates of the dimorphic fungi and dermatophytes.

Ketoconazole

Experience to date using the procedures described in this standard indicates that mould MICs vary between 0.0313 ad 16 µg/mL. However, data are not yet available to indicate a correlation between MIC and outcome of treatment with ketoconazole.

Itraconazole and new Triazoles

The importance of proper preparation of drug dilutions for this insoluble compound cannot be over emphasized. Use of the incorrect solvents or deviation from the dilution scheme suggested in Table 7 can be lead to substantial errors due to dilution artifacts. As for ketoconazole, experience to date using the procedures described in this standard indicates that MICs for moulds vary between 0.0313 and 16 µg/ml. However, preliminary data indicate that high itraconazole MICs (>8µg/ml) are associated with clinical resistance to this agent when MICs are determined by the M38 A microdilution method after 48 hours of incubation. Data are not yet available to indicate a correlation between MIC and outcome of treatment with the new triazoles.

Broth Macrodilution Modifications

Data have been published that document good concordance between results obtained by the broth microdilution methodology described above and a broth macrodilution adaptation. The ease of performance of broth microdilution tests is very attractive, and most clinical laboratories will probably choose to implement this method rather than the broth macrodilution method. The steps and testing conditions that are relevant to the broth microdilution test are discussed in detail.

The 100-fold drug dilutions described for the broth microdilution procedure should be diluted 1:10 with RPMI to achieve the ten times strength needed for the broth microdilution test. The stock inoculum suspensions are prepared and adjusted, as described, under the broth microdilution test. The stock inoculum preparations are prepared and adjusted, as described, under broth microdilution test. The stock conidia or sporangiospore suspension is mixed for 15 seconds with a vortex diluted 1:100 with the medium to obtain test inoculum (0.4×10^4 to 5×10^4 CFU/mL).

The 10x drug concentrations are dispensed into 12 x75 sterile tubes in 0.1ml volumes. These tubes may be sealed in plastic bags and stored frozen at -70^0 C for up to six months without deterioration of drug potency. Each tube is inoculated on the day of the test with 0.9ml of the corresponding diluted inoculum suspension, which brings the drug dilutions and inoculum densities to the final concentration mentioned for the microdilution method. The growth control receives 0.1ml of 10 fold of the diluent without antifungal agent and is inoculated with 0.9ml of the corresponding diluted inoculum suspensions. The QC organisms are tested in the same manner and are included each time an isolate is tested.

Tubes are incubated at 35^0 C (without agitation) and observed for the presence or absence of visible growth. The tubes are scored and MICs determined as described for the broth microdilution procedure.

Quality Control

The goals of a quality control program are to monitor the following:

• The precision and accuracy of the susceptibility test procedure.

• The performance of reagents, testing conditions, and instructions used in the test.

• The performance of persons who conduct the tests and read the results.

The goals are best realized by, but not limited to, the use of quality control and reference strains selected for their genetic stability and for their usefulness in the particular method being controlled.

For detailed quality control parameters please refer to the original document, however the other quality control parameters include the following:

Growth Control: Each broth macrodilution series should include a growth control of basal medium without antifungal agent to assess viability of the test organisms. With the broth tests, the growth control also serves as a turbidity control for reading end points.

Purity Control: A sample of each inoculum is streaked on a suitable agar plate and incubated overnight to detect mixed cultures and to provide freshly isolated colonies in the event retesting proves necessary.

End Point Interpretation Control: End point interpretation is monitored periodically to minimize variation in the interpretation of MIC end points among observers. All laboratory personnel who perform these tests should read a selected set of dilution tests independently. The results are recorded and compared to the results obtained by an experienced reader. Specific reference strains with predetermined MICs are particularly helpful for this purpose, especially with itraconazole.

Quality Control Strains: Ideal reference strains for quality control of dilution tests have MICs that fall near the middle of the concentration range tested for all antifungal agents; e.g. an ideal control strain would be inhibited at the fourth dilution of a seven dilution series, but strains with MICs at either the third or fifth dilution would also be acceptable.

Table 8 lists expected ranges for strains found to be acceptable as quality control strains. Also shown are additional strains that can be useful for conducting reference studies.

Table 6. Scheme for Preparing Dilution Series of Water-soluble Antifungal Agents to be used in Broth Dilution Susceptibility Tests

Drug - Starting Concentration (µg/ml)	Stock 5120	2	4 160	8	16	32 20	64	128	256 2.5	511	Remarks
		2X	4X	2X	4X	8X	2X	4X	8X	2X	
Tube #	TUBE 1	TUBE 2	TUBE 3	TUBE 4	TUBE 5	TUBE 6	TUBE 7	TUBE 8	TUBE 9	TUBE 10	
Source	From Stock 1.0	From Tube 1	From Tube 1	From Tube 3	From Tube 3	From Tube 3	From Tube 6	From Tube 6	From Tube 6	From Tube 9	Step 1
Add DRUG Amount (ml) +		1.0 +	1.0 +	1.0 +	0.5 +	0.5 +	1.0 +	0.5 +	0.5 +	1.0 +	Row 1
Add Solvent RPMI (ml)	7.0	1.0	3.0	1.0	1.5	3.5	1.0	1.5	3.5	1.0	
Intermediate Drug Concentration (µg/ml)	640	320	160	80	40	20	10	5	2.5	1.25	
Add Drug from Tube Row 1 Above (ml) +	1.0 +	1.0 +	1.0 +	1.0 +	1.0 +	1.0 +	1.0 +	1.0 +	1.0 +	1.0 +	Step 2 Row 2 5X (1:4)
RPMI 1640 (ml)	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	
Final Concentration at 1:5 (µg/ml)	128	64	32	16	8	4	2	1	0.5	.25	2X
From Row 2 add Drug to Micro titer plate (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	Step 3 (1:1)
Add Inoculum to Plate (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	Step 4 (T.Vol 0.2ml)
FINAL DRUG CONC in Well at 1:100 (µg/ml)	64	32	16	8	4	2	1	0.5	0.25	0.125	

Table 7. Scheme for Preparing Dilutions of Water Insoluble Antifungal Agents to be Used in Broth Dilution Susceptibility Tests

Drug - Starting Concentration (µg/ml)	1,600	2	4	8 200	16	32	64 25	128	256	511	Remarks
Tube #	TUBE 1 (Stock) (100 X)	2X TUBE 2	4X TUBE 3	8X TUBE 4	2X TUBE 5	4X TUBE 6	8X TUBE 7	2X TUBE 8	4X TUBE 9	8X TUBE 10	
Source	From Tube 1	From Tube 1	From Tube 1	From Tube 1	From Tube 4	From Tube 4	From Tube 4	From Tube 7	From Tube 7	From Tube 7	Step 1
Add DRUG Amount (ml)	-	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	Row 1
+		+	+	+	+	+	+	+	+	+	
Add Solvent DMSO (ml)	-	0.5	1.5	3.5	0.5	1.5	3.5	0.5	1.5	3.5	
Intermediate Drug Concentration (µg/ml)	1,600	800	400	200	100	50	25	12.5	6.25	3.13	
Add Drug from Tube Row 1 Above (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	Step 2
+	+	+	+	+	+	+	+	+	+	+	Row 2
RPMI 1640 (ml)	4.9	4.9	4.9	4.9	4.9	4.9	4.9	4.9	4.9	4.9	(1:50)
Final Concentration at 1:50 (µg/ml)	32	16	8.0	4.0	2.0	1.0	0.5	0.25	0.125	0.0625	(2X)
From Row 2 add Drug to Micro titer plate (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	Step 3 (1:1)
Add Inoculum to Plate (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	Step 4 (T.Vol 0.2ml)
FINAL DRUG CONC in Well at 1: 100 (µg/ml)	16	8	4	2	1	0.5	0.25	0.125	0.0625	0.0313	

DMSO = Dimethyl sulfoxide

Table 8. Recommended MIC limits for Two Quality Control and Four Reference Strains for Broth Macrodilution Procedures:

Organism	Purpose	Antifungal Agent	MIC* Range(ug/mL)	% of MICs within Range
Candida parapsilosis ATCC® 22019	QC	Amphotericin B Fluconazole Itraconazole Ketoconazole Posaconazole Ravuconazole Voriconazole 5FC	0.5-4.0 1.0-4.0 0.12-0.5 0.06-0.5 0.06-0.25 0.03-0.25 0.03-0.25 0.12-0.5	99.1 99.1 99.0 99.0
Candida krusei ATCC® 6258	QC	Amphotericin B Fluconazole Itraconazole Ketoconazole Posaconazole Ravuconazole Voriconazole 5FC	1.0-4.0 16-128 0.25-1.0 0.25-1.0 0.12-1.0 0.25-1.0 0.12-1.0 8.0-32	99.5 99.1 94.0 100.0
Aspergillus flavus ATCC® 204304	Reference	Amphotericin B Itraconazole Posaconazole Ravuconazole Voriconazole	0.5-4 0.2-0.5 0.06-0.5 0.5-4 0.5-4	100.0 100.0 100.0 100.0 100.0
Aspergillus fumigatus *ATCC®*204305	Reference	Amphotericin B Itraconazole	0.5-2.0 0.12-1.0	100.0 100.0

* MIC ranges for *Candida* QC isolates are microdilution values after 48 hours of incubation.

Method for Antifungal Disk Diffusion Susceptibility Testing of Yeasts; Approved Guideline (M 44-A, CLSI, USA)

Introduction

Although CSLI broth dilution reference methods are available for antifungal susceptibility testing, there still remains a need for an alternative easy, quick and a cost-effective antifungal susceptibility method which could be made susceptibility of fungal organisms to various classes of antifungal agents that would make antifungal susceptibility testing more readily available to the clinical microbiology laboratory. The NCCLS Subcommittee on Antifungal Susceptibility Testing has developed a disk diffusion method for testing *Candida* species to fluconazole and voriconazole. Zone interpretive criteria (breakpoints) have been approved for fluconazole as well as quality control parameters for both fluconazole and voriconazole. One significant advantage of this method is that qualitative results can usually be determined after only 20 to 24 hours incubation as opposed to 48 hours with NCCLS document M27. There are currently more than ten systemically active antifungal agents and it is expected that this document will further encourage the development of disk diffusion testing for at least some of these agents.

The method described here is intended for testing *Candida* species. This method does not currently encompass any other genera and has not been used in studies of the yeast form of dimorphic fungi, such as *Blastomyces dermatitidis* or *Histoplasma capsulatum.* Moreover, testing of filamentous fungi (i.e., moulds) is not addressed in the current procedure. The method described herein must be followed exactly to obtain reproducible results.

Definitions

Antimicrobial Susceptibility Test Interpretive Category – 1) A classification based on an *in vitro* response of an organism to an antimicrobial agent at levels of that agent corresponding to blood or tissue levels attainable with usually prescribed doses of that agent; 2) Susceptible Antimicrobial Susceptibility Test Interpretive Category – A category that implies that an infection due to the isolate may be appropriately treated with the dosage of an antimicrobial agent recommended for that type of infection and infecting species, unless otherwise contraindicated; 3) Susceptible-Dose Dependent (S-DD)Antimicrobial Susceptibility Test Interpretive Category – A category that includes isolates with antimicrobial agent MICs that approach usually attainable blood and tissue levels and for which response rates may be lower than for susceptible isolates; 4) Resistant Antimicrobial Susceptibility Test Interpretive Category – Resistant isolates that are not inhibited by the usually achievable concentrations of the agent with normal dosage schedules or where clinical efficacy has not been reliable in treatment studies.

Reagents for the Disk Diffusion Test

Mueller-Hinton Agar + 2% Glucose and 0.5 µg/mL Methylene Blue Dye (GMB) Medium (see Appendix)

Of the many agar media available, supplemented Mueller-Hinton agar to be a good choice for routine susceptibility testing of yeasts.

pH of Mueller-Hinton Agar + 2% Glucose and 0.5 µg/mL Methylene Blue Dye Medium

The pH of each batch of prepared Mueller-Hinton agar should be checked. The agar medium should have a pH between 7.2 and 7.4 at room temperature after gelling. The pH can be checked by one of the following means:

- Macerate a sufficient amount of agar to submerge the tip of a pH electrode.
- Allow a small amount of agar to solidify around the tip of a pH electrode in a beaker or cup.
- Use a properly calibrated surface electrode.

Moisture on Agar Surface

If excess surface moisture is present, the agar plates should be dried in an incubator or laminar flow hood with the lids ajar until the excess moisture has evaporated (usually 10 to 30 minutes). The surface should be moist, but with no droplets on the agar surface or the petri dish cover.

Storage of Antimicrobial Disks

Cartridges containing commercially prepared paper disks specifically for susceptibility testing are generally packaged to ensure appropriate anhydrous conditions. Disks should be stored as follows:

- Refrigerate the containers at 8 °C or below, or freeze at -14 °C or below, in a nonfrost-free freezer until needed. The disks may retain greater stability if stored frozen until the day of use. Always refer to instructions in the product insert. The unopened disk containers should be removed from the refrigerator or freezer one to two hours before use so they may equilibrate to room temperature before opening. This procedure minimizes the amount of condensation that occurs when warm air contacts cold disks.
- Once a cartridge of disks has been removed from its sealed packaging, it should be placed in a tightly sealed, desiccated container.
- A disk-dispensing apparatus should be fitted with a tight cover and supplied with an adequate desiccant. The dispenser should be allowed to warm to room temperature before opening. The desiccant should be replaced when the indicator changes color.
- When not in use, the dispensing apparatus containing the disks should always be refrigerated.
- Only disks within their valid shelf life may be used. Disks should be discarded on the expiration date.

Turbidity Standard for Inoculum Preparation

To standardize the inoculum density for a susceptibility test, a $BaSO_4$ suspension with turbidity, equivalent to a 0.5 McFarland standard or its optical equivalent should be used (See Appendix).

Procedure for Performing the Disk Diffusion Test

Inoculum Preparation: Direct Colony Suspension Method

Steps for preparation of the inoculum are as follows:

(1) All organisms need to be subcultured onto blood agar or Sabouraud dextrose agar to ensure purity and viability. The incubation temperature throughout must be 35 °C (±2 °C).

(2) Inoculum is prepared by picking **five distinct colonies** of approximately 1 mm in diameter from a 24-hour-old culture of *Candida* species. Colonies are suspended in 5 mL of sterile 0.145 mol/L saline (8.5 g/L NaCl; 0.85% saline).

(3) The resulting suspension is vortexed for 15 seconds and its turbidity is adjusted either visually or with a spectrophotometer by adding sufficient sterile saline or more colonies to adjust the transmittance to that produced by a 0.5 McFarland standard at 530 nm wavelength. This procedure will yield a yeast stock suspension of 1×10^6 to 5×10^6 cells per mL and should produce semi-confluent growth with most *Candida* species isolates.

Inoculation of Test Plates

(1) Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab is dipped into the suspension. The swab should be rotated several times and pressed firmly against the inside wall of the tube above the fluid level. This will remove excess fluid from the swab.

(2) The dried surface of a sterile Mueller-Hinton + GMB agar plate is inoculated by evenly streaking the swab over the entire agar surface. This procedure is repeated by streaking two more times,

rotating the plate approximately 60° each time to ensure an even distribution of inoculum. As a final step, the rim of the agar is swabbed.

(3) The lid may be left ajar for three to five minutes, but no more than 15 minutes, to allow for any excess surface moisture to be absorbed before applying the drug-impregnated disks.

NOTE: Variations in inoculum density must be avoided. Never use undiluted overnight broth cultures or other unstandardized inocula for streaking plates.

Application of Disks to Inoculated Agar Plates

(1) Antimicrobial disks are dispensed onto the surface of the inoculated agar plate. Each disk must be pressed down to ensure its complete contact with the agar surface. Whether the disks are placed individually or with a dispensing apparatus, they must be distributed evenly so that they are no closer than 24 mm from center to center. Ordinarily, no more than 12 disks should be placed on a 150-mm plate, or more than five disks on a 100-mm plate. Because the drug diffuses almost instantaneously, a disk should not be moved once it has come into contact with the agar surface. Instead, place a new disk in another location on the agar.

(2) The plates are inverted and placed in an incubator set to 35°C (± 2°C) within 15 minutes after the disks are applied.

Reading Plates and Interpreting Results

Examine each plate after 20 to 24 hours of incubation. If the plate was satisfactorily streaked and the inoculum was correct, the resulting zones of inhibition will be uniformly circular and there will be a semiconfluent lawn of growth. The plate is held a few inches above a black, nonreflecting background illuminated with reflected light. Measure the zone diameter to the nearest whole millimeter at the point at which there is a prominent reduction in growth. This is highly subjective, and experience results in greater accuracy (trueness). Pinpoint microcolonies at the zone edge or large colonies within a zone are encountered frequently and should be ignored. If these colonies are subcultured and retested, identical results are usually obtained, i.e., a clear zone with microcolonies at the zone edge or large colonies within the zone. Read at 48 hours only when insufficient growth is observed after 24 hours incubation.

Interpretation of Disk Diffusion Test Results

Zone Diameter Interpretive Standards

Table 9 provides zone diameter interpretive criteria to categorize accurately the levels of susceptibility of organisms to fluconazole.

Interpretive Categories

Susceptible (S): The susceptible category implies that an infection due to the strain may be appropriately treated with the dose of antimicrobial agent recommended for that type of infection and infecting species, unless otherwise contraindicated.

Susceptible-Dose Dependent (S-DD): The susceptible-dose dependent category includes isolates with antimicrobial agent MICs that approach usually attainable blood and tissue levels and for which response rates may be lower than for susceptible isolates. Susceptibility is dependent on achieving the maximal possible blood level. This category also includes a buffer zone, which should prevent small, uncontrolled, technical factors from causing major discrepancies in interpretations, especially for drugs with narrow pharmaco toxicity margins.

Resistant (R): Resistant strains are those that are not inhibited by the usually achievable concentrations of the agent with normal dosage schedules or when zone diameters have been in a range where clinical efficacy has not been reliable in treatment studies.

Zone Diameter Interpretive Criteria

Disk diffusion zone diameters correlate inversely with MICs from standard dilution tests. Table 9 lists the zone diameter interpretive criteria. These categories were developed by first comparing zone diameters to MICs for a large number of isolates, including those with known mechanisms of resistance relevant to the particular drug. MICs and correlated zone sizes were analyzed in relation to the pharmacokinetics of the drug from normal dosing regimens. Whenever possible, the tentative *in vitro* interpretive criteria were analyzed in relation to studies of clinical efficacy in the treatment of specific pathogens.

Of note, these interpretive breakpoints are not applicable to *C. krusei*, and thus identification to the species level is required in addition to MIC determination.

Quality Control

Part of quality management focused on fulfilling quality requirements, which includes operational techniques and activities used to fulfill these requirements.

Reference Strains for Quality Control

To control the precision (repeatability) and accuracy (trueness) of the results obtained with disk diffusion test procedure, several quality control strains should be obtained from a reliable source. The recommended quality control strains include:

- *Candida albicans* ATCC 90028;
- *Candida parapsilosis* ATCC 22019;
- *Candida tropicalis* ATCC 750; and
- *Candida krusei* ATCC 6258.

Storing Quality Control Strains

- The quality control strains should be tested by the standard disk diffusion test procedure described herein using the same materials and methods that are used to test clinical isolates.
- Quality control strains are stored in a way that minimizes the possibility of mutation in the organism.
- There are several methods for prolonged storage of reference strains. For example, yeasts may be grown on slants of potato dextrose agar and then frozen at -70 ℃. Alternatively, strains can be preserved by suspending yeasts into vials containing 50% glycerol solution for freezing and storing at -70 ℃.
- Working quality control cultures are stored on blood agar or Sabouraud dextrose agar at 2 to 8℃ and subcultured each week for no more than three successive weeks. New working cultures should be prepared at least monthly from frozen, freeze-dried, or commercial cultures.
- Frozen or freeze-dried cultures should be subcultured at least twice prior to testing.
- A quality control strain can be used to monitor the precision (repeatability) and accuracy (trueness) of the disk test as long as there is no significant change in the mean zone diameter that cannot be attributed to a faulty methodology. If an unexplained result suggests a change in the organism's inherent susceptibility, a fresh new stock culture of the control strain should be obtained.

Zone Diameter Quality Control Limits

Acceptable zone diameter quality control limits for quality control strains are listed in Table 10. The overall performance of the test system should be monitored using these ranges by testing the appropriate control strains each day the test is performed or, if satisfactory performance is documented, testing may be done weekly.

Frequency of Quality Control Testing

Daily Testing:-When testing is performed daily, for each antimicrobial agent/organism combination, 1 out of every 20 consecutive results may be out of the acceptable range (based on 95% confidence limits, 1 out of 20 random results may be out of control). Any more than 1 out-of-control result in 20 consecutive tests requires corrective action.

Weekly Testing:- Demonstrating Satisfactory Performance for Conversion from Daily to weekly Quality Control Testing

- Test all applicable control strains for 20 consecutive test days and document results.
- To convert from daily to weekly quality control testing, no more than 1 out of 20 zone diameters for each antimicrobial agent/organism combination may be outside the acceptable zone diameter limits in Table 10**.**

Implementing Weekly Quality Control Testing

- Weekly quality control testing may be implemented once satisfactory performance has been documented
- Perform quality control testing once per week and whenever any reagent component of the test (e.g., a new lot of agar plates or a new lot of disks from the same or a different manufacturer) is changed.
- If any of the weekly quality control results are out of the acceptable range, corrective action is required.
- If a new antimicrobial agent is added, it must be tested for 20 consecutive test days and satisfactory performance documented before converting to a weekly schedule. In addition, 20 days of consecutive testing are required if there is a major change in the method of reading test results, such as conversion from manual zone measurements to an automated zone reader.

Corrective Action

Out-of-Control Result Due to an Obvious Error

Obvious reasons for out-of-control results include:

- Use of the wrong disk;
- Use of the wrong control strain;
- Obvious contamination of the strain; or
- Inadvertent use of the wrong incubation temperature or conditions.
- In such cases, document the reason and retest the strain on the day the error is observed. If the repeated result is within range, no further corrective action is required.

Out-of-Control Result Not Due to an Obvious Error

Immediate Corrective Action

If there is no obvious reason for an out-of-control result, immediate corrective action is required.

- Test the antimicrobial agent/organism combination for a total of five consecutive test days. Document all results in question.
- If all five zone diameter measurements for the antimicrobial agent/organism combination are within acceptable ranges, as defined in Table 2, no additional corrective action is necessary.

- If any of the five zone diameter measurements are outside the acceptable range, additional corrective action is required.
- Daily control tests must be continued until final resolution of the problem can be achieved. Additional Corrective Action when immediate corrective action does not resolve the problem; it is likely due to a system error versus a random error. The following common sources of error should be investigated:
    - Zone diameters were measured and transcribed correctly.
    - The turbidity standard has not expired, is stored properly, meets performance requirements, and was adequately mixed prior to use.
    - All materials used were within their expiration date and stored at the proper temperature.
    - The incubator is at the proper temperature and atmosphere.
    - Other equipment used (e.g., pipettes) are functioning properly.
    - Disks are stored desiccated and at the proper temperature.
    - The control strain has not changed and is not contaminated.
    - Inoculum suspensions were prepared and adjusted correctly.
    - Inoculum for the test was prepared from a plate incubated for the correct length of time and in no case was more than 24 hours old.

It may be necessary to obtain a new quality control strain (either from freezer stock or a reliable source) and new lots of materials (including new turbidity standards), possibly from different manufacturers. If the problem appears to be related to a commercial product, the manufacturer should be contacted. It is also helpful to exchange quality control strains and test materials with another laboratory using the same method. Until the problem is resolved, an alternative test method should be used. Once the problem is corrected, documentation of satisfactory performance for another 20 consecutive days is required before returning to weekly quality control testing.

Reporting Patient Results When Out-of-Control Tests Occur

Whenever an out-of-control result or corrective action is necessary, careful assessment of whether to report patient results should be made on an individual basis, taking into account if the source of error, when known, is likely to have affected relevant patient results. Options that may be considered include suppressing the results for an individual antimicrobial agent; retrospectively reviewing individual patient or cumulative data for unusual patterns; and using an alternate test method or a reference laboratory until the problem is resolved.

Limitations of Disk Diffusion Methods

Application to Various Organism Groups

The disk diffusion method described in this document has been standardized for *Candida* species only for other yeasts, consultation with an infectious disease specialist is recommended for guidance in determining the need for susceptibility testing and interpretation of results. Published reports in the medical literature and current consensus recommendations for therapy of uncommon microorganisms may obviate the need for testing. If necessary, a reference dilution method may be the most appropriate alternative testing method, and this may require submitting the organism to a reference laboratory.

Verification of Patient Results

Multiple test parameters are monitored by following the quality control recommendations described in this standard. However, acceptable results derived from testing quality control strains do not

guarantee accurate results when testing patient isolates. It is important to review all of the results obtained from all drugs tested on a patient's isolates prior to reporting the results. Unusual or inconsistent results should be verified by checking for the following: 1) transcription errors; 2) contamination of the test (recheck purity plates); and 3) previous results on the patient's isolates. If a reason for the unusual or inconsistent result cannot be ascertained, repeat the susceptibility test, verify the species identity, or request a new clinical specimen. Each laboratory must develop its own policies for verification of unusual or inconsistent antimicrobial susceptibility test results.

Table-9: Zone Diameter Interpretive Standards and Corresponding Minimal Inhibitory Concentrations (MIC) Breakpoints for *Candida* spp.

Antifungal Agent	Disk Content	Zone Diameter, Nearest Whole (mm)			Equivalent MIC Breakpoints (µg/mL)		
		R*	S-DD*	S*	R*	S-DD*	S*
Fluconazole†	25 µg	≤14	15 - 18	≥19	≥64	16 - 32	≤8
Voriconazole	1 µg	≤13	14 - 16	≥17	≥4	2	≤1

* Susceptible, Susceptible-Dose Dependent (S-DD), and Resistant Interpretive categories

† Isolates of *C. krusei* are assumed to be intrinsically resistant to fluconazole, and their MICs should not be interpreted using this scale.

Table - 10: Recommended Quality Control Zone Diameter (mm) Ranges

Antifungal Agent	Disk Content	*C. albicans* ATCC 90028	*C. parapsilosis* ATCC 22019	*C. tropicalis* ATCC 750	*C. krusei* ATCC 6258
Fluconazole	25 µg	28 - 39	22 - 33	26 - 37	—*
Voriconazole	1 µg	31 - 42	28 - 37	—*	16 - 25

*Quality control ranges have not been established for these strain/antimicrobial agent combinations, due to their extensive interlaboratory variation during initial quality control studies.

Preparation of Mueller-Hinton Agar + 2% Glucose and 0.5 µg/mL Methylene Blue Dye

The medium can be prepared and poured as the complete media with supplements *or* the supplements can be added to commercially prepared Mueller-Hinton agar plates. Using the latter technique enables the use of routine Mueller-Hinton agar plates from the bacteriology laboratory.

Preparation of Supplemented Mueller-Hinton Agar:

(1) Mueller-Hinton agar should be prepared from a commercially available dehydrated Mueller- Hinton agar base according to the manufacturer's instructions.

(2) Dissolve 0.1 gram of methylene blue dye in 20 mL of distilled water and warm gently to dissolve. Do not overheat. Add 100 µL of this solution per litre of agar suspension.

(3) Add 20 grams of glucose per litre of agar suspension.

(4) Autoclave as directed by manufacturer's instructions.

(5) Immediately after autoclaving, allow the agar solution to cool in a 45 to 50 ℃ water bath.

(6) Pour the freshly prepared and cooled medium into plastic, flat-bottomed petri dishes on a level, horizontal surface to give a uniform depth of approximately 4 mm. This corresponds to 67 to 70 mL of medium for plates with diameters of 150 mm and 28 to 30 mL for plates with a diameter of 100 mm.

(7) The agar medium should be allowed to cool to room temperature and, unless the plate is used on the same day of preparation, stored at refrigerator temperature (2 to 8 ℃). The agar medium should have a pH between 7.2 and 7.4 at room temperature

(8) Plates should be used within seven days after preparation unless adequate precautions such as wrapping in plastic have been taken to minimize drying of the agar.

(9) A representative sample of each batch of plates should be examined for sterility by incubating at 30 to 35 ℃ for 24 hours or longer. Plates should undergo quality control testing

RPMI-1640 medium buffered with 0.165mol/L MOPS, 1 L.

10.4 g powdered medium

34.53 g MOPS buffer

Dissolve powdered medium in 900 mL distilled H_2O. Add MOPS to a final concentration of 0.165mol/L and stir until dissolved. While stirring, adjust the pH to 7.0 at 25oC using 1mol/L sodium hydroxide. Add additional water to bring medium to a final volume of 1L. Filter sterilize and store at 4^oC until use.

McFarland 0.5 Barium Sulfate Turbidity Standard

To standardize the inoculum density, a BaSO4 turbidity standard is used (0.5 McFarland standard).

The procedure consists of the following steps:

(1) Prepare this turbidity standard by adding 0.5 mL of 0.048 mol/L BaCl2 (1.175% w/v BaCl2 H2O) to 99.5 mL of 0.18 mol/L H2SO4 (1% v/v) with constant stirring to maintain a suspension.

(2) Verify the correct density of the turbidity standard by using a spectrophotometer with a 1-cm light path and matched cuvette to determine the absorbance. The absorbance at 625 nm should be 0.08 to 0.10 for the 0.5 McFarland standard.

(3) Distribute 4 to 6 mL into screw-cap tubes of the same size as those used in growing or diluting the broth culture inoculum.

(4) Tightly seal these tubes and store them in the dark at room temperature.

(5) Vigorously agitate this turbidity standard on a mechanical vortex mixer just before use.

(6) The barium sulfate standards should be replaced or their densities verified monthly.

Composition of RPMI – 1640 Medium

Constituent	G/L water	Constituent	G/L water
L-arginine (free base)	0.200	Biotin	0.0002
L-asparagine (anhydrous)	0.050	D pantothenic	0.00025
L-aspartic acid	0.020	Cholinechloride	0.003
L-cystine, 2HCl	0.0652	Folic acid	0.001
L-glutamic acid	0.020	Myco-inositol	0.035
L-glutamine	0.300	Niciramide	0.001
Glycine	0.010	PABA	0.001
L-histidine (free base)	0.015	Pyridoxine HCl	0.001
L-hydroxyproline	0.020	Riboflavin 0.000005	0.0002
L-isoleucine	0.050	Thiamine HCl	0.001
L-leucine	0.050	Vitamin B_{12}	0.000005
L-Lysine HCl	0.040	Calcium nitrate, H_20	0.100
L-methionine	0.015	Potassium chloride	0.400
L-phenylalanine	0.015	Magnesium sulfate (anhydrous)	0.04884
L-proline	0.020	Sodium chloride	6.000
L-serine	0.030	Sodium phosphate, diabasic (anhydrous)	0.800
L-threonine	0.020	D-glucose	2.000
L-tryptophan	0.005	Glutathione, reduced	0.001
L-tyrosine, 2Na	0.02882	Phenol red Na	0.0053
L-valine	0.020		

Preservation of Isolates and Slides

STORAGE TECHNIQUES

Various techniques have been found useful for maintaining culture collections. Though storage in liquid nitrogen and lyophilization are the ideal methods but some simple and inexpensive methods are also available.

(i) Periodic subculture and storage at room temperature or refrigeration

- Fungi can be routinely subcultured on a agar slope (medium with low nutrition is preferred).
- Fungi need to be transferred every 3 to 6 months at room temperature and after 6 – 12 months if required.
- Transfer each culture onto at least two new slopes.
- Screw capped or cotton plugged tubes may be used. Chances of infestation with mites in higher with cotton plugged tubes. But screw cap tubes are not suitable for Mucorales as accumulation of carbon-di-oxide inside the tube is toxic to some members of this genus.

(ii) Water Culture Technique

It's probably the simplest and cheapest way of storing fungi.

Method

- Dispense 2 ml of sterile distilled water in small screw cap bottles and sterilize by autoclaving.
- Remove mycelia from the fungal culture and aseptically transfer to the screw-capped bottle.
- Store at room temperature

OR

- To a sporulating culture aseptically add 2ml of sterile distilled water.
- Dislodge the conidia with the help of inoculating wire.
- With a sterile pipette remove the suspension and transfer it into a sterile screw capped bottle.
- Take care not to transfer the agar.
- Store at room temperature, away from light.

(3) Storage under mineral oil

- Autoclave liquid paraffin twice at 121^0C for 15 min.
- Grow fungal culture on short agar slant.
- Add mineral oil to the culture to a depth of 10 mm above the highest point of the agar slants.
- Store at room temperature.

(4) Storing at -20^0C or -70^0C

- Grow the isolate on slopes of suitable agar medium
- Cover the culture with sterile 10% glycerol or 10% dimethyl sulphoxide. *
- Screw the caps firmly and store at -20^0C or -70^0C.

OR

- Take 10% glycerol in small screw capped bottle. Sterilize at 121^0C for 10 minutes.
- Transfer mycelia from fungal culture to bottle containing glycerol.
- Store at -20^0 or -70^0C.

(5) Freeze - drying

This technique is used both for moulds and yeasts. There are many methods of achieving freeze-drying and depends on the equipment available.

The technique of centrifugal freeze drying is used successfully though a technique allowing variation of cooling rate such as shelf freeze – dryer is preferred.

Mounting Medium for Permanent Preparations of Microfungi

The slide culture method of Riddell (8) with lactophenol cotton blue as the mounting fluid is the most popular technique used by medical mycologists to study microscopic morphology of pathogenic and related fungi. Even though this technique provides satisfactory temporary, stained preparations of growth of fungi with minimal dislodgement of conidia, such preparations are not generally long lasting. The slow evaporation of lactophenol starting from the edges of the coverslips eventually causes distortion of conidia due to their loss of turgor. Such preparations have to be discarded and redone again and again for demonstration or teaching.

Sealing the coverslip edges with a clear or an opaque lacquer slows down the process of desiccation, but such sealed preparations, if not done properly, at best provide semi-permanent slide preparations. As pointed out by McGinnis (5, 6), different brands of nail lacquer behave differently with respect to resistance to cracking and ability to adhere to the glass. A variety of brands have to be tried before one can be selected for general use. This is especially true in many countries including India where finding a satisfactory brand of nail lacquer could become quite difficult.

Several alternative mounting fluids, such as polyvinyl alcohol (1, 2), Hoyer's fluid (3), and modified Papanicolaou's staining procedure (7) have been used to make permanent slide preparations of various groups of fungi. Here are a couple of mounting media for permanent preparations of fungi which I have used and have found satisfactory.

Lactophenol cotton blue with Polyvinyl Alcohol (PVA) (Huber's PVA Mounting Medium, modified (Larone D. H. 2002) (4)

Reagents:

PVA: molecular weight 70,000-100,000 (Sigma Chemical Co. catalog no. P-1763)

Phenol: purified grade (Sigma no. P-5566 or Fisher no. A91 1-500)

Lactic acid: ACS reagent (Sigma no. L-1893)

Aniline blue: certified (Aldrich Chemical Co., catalog no. 86, 102-2). This is analogous to cotton blue.

Preparation:

1. Add 7.5 of PVA powder to 50 ml of cold deionized water in a beaker.

2. Transfer beaker to a heated stirring plate; add a magnetic rod for mixing.

3. Place a thermometer in the beaker to monitor temperature.

4. Add 22 g. of lactic acid (BEFORE adding phenol).

5. Add 22 g. of phenol crystals (22 ml of melted phenol).

6. Add 0.05 g. aniline blue.

7. Heat and stir the solution until the temperature reaches 90° C. Do not boil or go over 100°C. Remove from the hot plate.

8. Dispense into small dropper bottles (thoroughly washed and dried ones can be used). Tighten dropper bottle caps, and store at the room temperature.

Procedure

Before mounting your slide cultures, it is important to remember that many fungi of medical importance such as *Aspergillus* species, *Penicillium* species, *Paecilomyces* species produce dry chains of conidia. It is therefore necessary to wet down the growth on the slide culture by adding a "*Wetting Agent"* so that the conidia become wet and release trapped air. Any preparation with trapped air bubbles underneath the coverslip will eventually allow slow evaporation of the mounting fluid eventually causing distortion of conidia and hyphal growth due to their loss of turgor.

Wetting agent (9)

95% Ethanol 50.0 ml, Acetone 25.0 ml, and 85% Lactic Acid 25.0 ml

Let a drop of wetting agent fall gently on the fungal growth on your prepared slide and coverslip to be mounted. Gently roll the slide or coverslip so that all growth is wetted down. Drain the excess wetting agent on a clean filter paper. The preparation is now ready to be mounted in the PVA mounting fluid.

Place 1 drop of PVA mounting fluid on the slide with a fungal growth obtained by the slide culture. Apply coverslip gently so that no air bubbles are trapped. Allow to dry on a flat surface. Similarly prepare second preparation from the growth on the coverslip.

The solution is available in prepared form from Scientific Device Laboratory Inc., Des Plaines, Illinois.

Polyvinyl alcohol (PVA) with acid fuchsin (9)

Polyvinyl alcohol (Sigma Chemicals)	1.66 g.
Lactic acid	10,0 ml
Glycerine	1.0 ml
Cold deionized water	10.0 ml
Acid fuchsin	0.02 g.

Dissolve PVA crystals in water. Dissolve acid fuchsin in lactic acid. Mix the two solutions together and stir until a clear, viscous liquid is obtained. Allow to sit for 24 hours to mature.

The slide preparations mounted in PVA should be left undisturbed on a flat surface at least for 24 to 48 hours before the mounting fluid hardens.

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Annexure A : Media and Stain

Media

1) Ascospore Agar

Potassium acetate	10.0g
Yeast extract	2.5g
Dextrose	1.0g
Agar	30.0g
Distilled water	1000 ml

- Mix thoroughly to give a uniform suspension
- Heat to boiling and boil for 1 min with agitation
- Dispense 8ml in screw top tubes
- Autoclave at 121^0C for 15 min
- Cool in slanted position
- pH – 6.4

2) Brain Heart Infusion Agar (BHIA)

Calf brain infusion	200.0g
Beef heart infusion	250.0g
Peptone	10.0g
Glucose	2.0g
Sodium chloride	5.0g
Disodium phosphate	2.5g
Agar	15.0g
Distilled water	1 litre
OR	
Brain heart infusion agar (dehydrated)	52 gm
Distilled water	1 litre

- Dissolve ingredients by boiling; dispense in tubes.
- Distribute 25ml in 3 Oz bottles
- Autoclave at 121^0C for 15 min.
- Cool in slanting position with one inch butt
- Shelf life is 30 days in test tubes and 14 days in petridishes.
- The medium can be enriched by addition of 5% sheep blood
- Antibiotics cycloheximide (0.5 mg/ml) and chloramphenical (0.05 mg/ml) may be added to the medium.

3) Biphasic medium for blood culture

a) Brain heart infusion agar as described above

b) Brain heart infusion broth.

Brain heart infusion (commercially available)	52g
Distilled water	1 litre

- Suspend and dissolve brain heart infusion in 1 litre water
- Autoclave at 121^0C for 15 min.
- Add 30 ml to each bottle aseptically
- pH (final): 7.4

4) Bird Seed Agar (BSA)[modified by Paliwal and Randhawa, J Clin Microbiol 1978;7:346-8

(Guizzotia abyssinica – is also known as niger seeds)

Bird seed	70 g
Distilled water	1 litre

Boil for 30 mins. Filter through gauze and add the following:

Agar	15 g
Diphenyl	1 g
Chloromphenicol	50 mg

- Autoclave at 121^{0}C for 10 min.
- Dispense in tubes as slants
- Shelf life is 2 wks
- Black coloured colonies of *Cryptococcus neoformans* are due to the caffeic acid extracted from Bird seed.

5) Canavanine glycine bromothymol – blue agar (CGB)

Solution A

Glycine	10 g
Potassium dihydrogen orthophosphate (KH_2PO_4)	1 g
Magnesium sulphate ($MgSO_4$)	1 g
□-Canavanine sulphate	30 mg
Thiamine hydrochloride	1 mg
Distilled water	100 ml

Adjust to pH 5.6 and filter sterilize

Solution B

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

Add 2 ml of solution B to 88 ml of distilled water. Add 2 gm of agar, autoclave at 121^{0}C for 15 min., and cool to about 55^{0}C. Add 10ml of solution-A, mix well and pour in tubes or plates.

6) Caffeic acid agar

Glucose	5.0 g
Ammonium sulphate	5.0 g
Yeast extract	2.0 g
Potassium phosphate	0.8 g
Magnesium sulphate	0.7 g
Caffeic acid	0.18 g
Ferric citrate solution	4.0 ml
Agar	20.0 g
Distilled water	1 litre

- Add 10.0 mg of ferric citrate to 20.0 ml of distilled water. This is ferric citrate solution. Mix reagents, add distilled water 1 litre.
- Bring to boil all the reagents
- Autoclave for 12 mins at 121^{0}C.
- Dispense in petridishes or tubes
- Shelf life 14 days.

7) Casein Agar

Skimmed milk suspension

- Skimmed milk powder 75g
- Distilled water 500ml
- Add powdered milk to distilled water, a little at a time with constant stirring. Do not leave lumps.
- Stir until completely dissolved.
- Sterilize by autoclaving at 115^0C for 20 min
- Cool in water bath to 50 -55^0C.

Agar Solution

- Agar 2g
- Distilled water 500 ml
- Suspend agar in distillled water
- Heat to boiling with frequent agitation until agar is completesly dissolved .
- Sterilize by auto claving at 121^0Cfor 15 min.
- Cool in water bath to 50-55^0C**.**

Complete medium

- Aseptically pour the agar solution in skimmedmilk suspension
- Swirl to mix thoroughly
- Pour in petridish/ tubes.
- pH (final) – 7.0$\pm$0.1

8) Christensen's urea agar

Peptone	1 g
Dextrose	5 g
NaCl	5 g
KH_2PO_4	2 g
Agar	20 g
Distilled water	980 ml
Phenol red	(2%) 5 ml

- Sterilize by autoclaving at 121^0C for 10 min.
- Add 10 ml of 20% Seitz filtered urea solution to 900 ml of base after autoclaving.

9) Corn Meal Agar (CMA)

Corn Meal	50 g
Distilled water	1000 ml
Agar	15 g

- Mix corn meal in 500ml of distilled water.
- Heat for 1 h or autoclave for 10 min at 15 lb
- Filter the suspension through gauze.
- Bring the volume to 1000 ml and add agar.
- Bring to boil
- Autoclave at 121^0C for 15 min.
- Dispense in sterile petridishes
- Shelf life is 14 days.

10) Corn Meal Tween 80 Agar

- To plain Corn Meal Agar, add 10 ml of Tween-80 for 1 litre of medium
- Pour in sterile petri dishes.

11) Corn Meal Tween 80 and 0.2% dextrose

Powedered corn meal agar with 0.2% dextrose(Difco)	17g
Distilled water	1 litre
Tween -80	10ml

- Add powedered corn meal agar with 0.2% dextrose to the distilled water and mix.
- Heat to boiling with frequejnt agitation till the medium is completely dissolved.
- Remove from heat and add 10 ml tween 80; mix thoroughly.
- Sterilize by autoclaving at 121^0C for 15 min.
- Dispense in petridishes.
- Final pH 6.2 at room temperature.

12) Cotton Seed Agar

Pharmamedia	20.0 g
Glucose	20.0 g
Agar	15.0 g
Distilled water	1 litre

- Mix the reagents and boil
- Adjust pH to 6.0 with either 1NHCl or 1N NaOH
- Dispense in tubes
- Autoclave at 121^0C for 15 min.
- Slant the test tubes

13) Converse liquid medium (Levine modification) for *Coccidioides*

Ammonium acetate	1.23g
Glucose	4.0g
Dipotassium phosphate	0.52g
Potassium phosphate	0.4 g
Magnesium sulphate	0.4 g
Zinc sulphate	0.002 g
Sodium chloride	0.014 g
Sodium carbonate	0.012 g
Tamol	0.5 g
Calcium chloride	0.002 g
Agarose (purified)	10.0 g
Distilled water	1000 ml

Autoclave at 121^0C for 15 min. Dispense in petri dishes

14) Czapek-Dox Solution Agar

Magnesium sulphate	0.5 g
Potassium chloride	0.5 g
Dipotassium phosphate	1.0 g
Ferrous sulphate	0.01 g
Sodium nitrate	3.0 g
Glucose	30.0 g
Agar	15.0 g
Distilled Water	1 litre

- Mix reagents and bring to a boil
- Autoclave at 121^0C for 15 min and pour in petridishes

15) Czapek solution Agar for *Aspergillus*

Sodium nitrate	3.0 g
Dipotassium phosphate	1.0 g
Magnesium sulphate	0.5 g
Potassium chloride	0.5 g
Ferrous sulphate	0.01 g
Glucose	30.0g
Agar	15.0 g
Distilled water	1 litre

Mix the reagents by boiling
Autoclave at 121^{0}C for 15 min
Dispense in plates or tubes
Shelf life 30 days in tubes / 14 days on plates.

16) Dermatophyte test medium (DTM)

Phytone peptone	10 g
Dextrose	10 g
Phenol red solution	40 ml

(0.5 gm in 15 ml of 1N NaOH made upto 100 ml with distilled water).

0.8 M HCl	6 ml
Actidione	500 mg
Gentamycin	100 mg
Agar	20 g
Distilled water	1000 ml

- Suspend phyton, dextrose and agar in 100ml of distilled water and mix thoroughly.
- Heat to boiling till all ingredient dissolved remove from heat.
- Add phenol red, HCl, cyclohexamde, gentamicin
- Sterilize by autoclaving at 118^{0}C (112 lb) for 10 min
- Final pH to 5.5
- Pour in test tubes

17) Fermentation broth

Powdered yeast extract	4.5 g
Peptone	7.5 g
Bromothymol blue	0.04 g
Distilled water	1 litre

- Dissolve bromothymol blue in 3.0 ml of 95% ethanol.
- Mix reagents and add bromothymol blue.
- Adjust pH to 7.0
- Dispense 2.0 ml into each tube, opening down.
- Autoclave at 121^{0}C for 15 min
- Allow medium to cool
- To each tube, aseptically add 1.0 ml of 6.0 % aqueous solution of carbon source to be tested, which has been filtered through 0.22 µm filter
- Shelf life: 30 days at 4^{0}C

18) 0.4 % gelatin for differentiation of Nocardia and streptomyces species.

Gelatin	4.0 g
Distilled water	1 litre

- Suspend gelatin in water and heat to boiling
- Dispense in screw – capped tubes

- Autoclave at 121^{0}C for 10 min
- Final pH:7.0

19) 12% gelatin for demonstration of proteolytic activity

Heart infusion broth (Difco)	25 g
Gelatin	120 g

- Suspend powdered broth and gelatin in distilled water and heat to boiling
- Dispense into screw capped tubes
- Autoclave at 121^{0}C for 10 min.
- Final pH: 7.2 – 7.4.

20) Glucose – Yeast Extract Medium

Glucose	10.0 g
Yeast extract	5.0 g
Agar	15.0 g
Distilled water	1 litre

- Mix reagents and bring to boil
- Autoclave at 121^{0}C for 15 min.
- Slant the tubes
- Shelf life is 30 days.

21) Gorodkowa's medium for ascospores.

Glucose	0.63 g
Sodium chloride	1.3 g
Beef extract	2.5 g
Agar	2.5 g
Distilled water	250ml

- Mix reagents and bring to boil
- Autoclave at 121^{0}C for 15 min.

22) KT Medium

Tween 80	0.2 ml
Potassium sulphate	0.5 g
Magnesium citrate	1.5 g
L-Asparagine	5.0 g
Casamino Acids	3.0 g
Glycerol	20.0 ml
Agar	15.0 g
Albumin – Dextrose Solution, Ster	100.0 ml
Albumin, Fraction 5 (Pentex)	5.0 g
Dextrose	7.0 g
Sodium Chloride	0.85 g
ETF Water	9.0 ml

- Dissolve magnesium citrate in 900 ml of ETF water
- While stirring this solution, add the following components and
- Dissolve sequentially: potassium sulphate, asparagines, casamino acids, tween 80, and glycerol. Adjust pH to 6.6.
- Add Agar; Heat to boiling to dissolve.
- Autoclave at 121^{0}C for 15 min. Cool to 56^{0}C in water bath.

- Prepare the sterile solution by dissolving albumin first, then dextrose and sodium chloride in 100 ml of ETF water.
- Adjust pH to 6.6. Inactivate by incubating for 30 min at 56^0C.
- Sterilize by filtering using a 0.22 micron filtration system.
- Add albumin solution to agar aseptically. Mix well.
- Dispense aseptically.

Note: When preparing sterile solution, dissolve albumin completely before adding dextrose and sodium chloride, then adjust pH. Albumin used must be fraction 5.

23) *Histoplasma* Mould to Yeast form Conversion Medium

Brain heart infusion agar base	5.2 g
Glutamine	1.0 ml
Sheep blood	5.0 ml
Distilled water	100.0 ml

- Prepare brain heart infusion agar base
- Autoclave at 121^0C for 15 min
- Add glutamine and sheep blood
- Dispense in test tube
- Slant the test tubes
- Shelf life is 30 days at 4^0C

24) Kelley's Agar

Glucose	10.0 g
Bactopeptone	10.0 g
Sodium chloride	5.0 g
Beef extract	3.0 g
Hemoglobin solution	20.0 ml
Agar	15.0 g
Distilled water	980.0 ml

- Add 5.0 ml of citrated sheep blood to 15.0 ml of distilled water. This is the hemoglobin solution.
- Dispense in tubes and autoclave at 121^0C for 10 min.
- Slant the test tubes.

25) Malt extract agar

Malt extract	20.0 g
Peptone	1.0 g
Glucose	20.0 g
Agar	15.0 g
Distilled water	1 liter

- Mix reagents and bring to boil
- Autoclave at 121^0C for 15 min
- Shelf life 30 days in test tube/ 14 days in petri dishes

26) **Oatmeal Agar (OA)**

Tomato paste	10.0 g
Oatmeal	10.0 g
Magnesium sulphate	1.0 g
Potassium phosphate	1.0 g

Sodium nitrate	1.0 g
Agar	15.0 g
Distilled water	1000 ml

- Adjust pH to 5.6 with sodium hydroxide
- Autoclave at 121^0C for 20 min.
- Dispense in petridishes

27) Pine's Agar Medium for Yeast Phase/*H.capsulatum*

Pine's Citrate medium Salt Solution 1

Potassium phosphate monobasic	8.00 g
Ammonium sulphate	8.00 g
Magnesium sulphate septahydrous	0.86 g
Calcium chloride	0.08 g
Zinc sulphate (for yeast phase only)	0.05 g
Water QS	1000.00 ml

Note : Filter sterilize stock solution

Solution 2

Ferrous sulfate, heptahydrous	5.70 g
Manganese chloride hexahydrous	0.80 g
Sodium molybdate, dihydrate	0.15 g
Hydrochloric acid, conc.	1.00 ml
Water QS	1000.00 ml

Solution 3

Casein hydrolysate (ICN)	40.00 ml

Solution 4

Inositol	0.20 g
Thiamine hydrochloride	0.20 g
Calcium pantothenate	0.20 g
Riboflavin	0.20 g
Nicotinamide (Niacinamide)	0.10 g
Biotin	0.01 g
Water QS	1000.00 ml

Solution 5

Hemin	0.200 g
ETF water QS	1000.00 ml

Solution 6

DL-thioctic acid	0.01 g
Ethyl alcohol, 95%	10.00 ml

Solution 7

Coenzyme A	0.01 g
Water	10.00 ml

Pine's Citrate Medium, Yeast Phase, Part 1

Solution 1 (Salts)	250.00 ml
Solution 2 (Minor elements)	10.00 ml
Solution 3 (Casein hydrolysate)	40.00 ml

Solution 4 (Vitamins)	10.00 ml
Solution 5 (Hemin)	10.00 ml
Solution 6 (Thioctic Acid)	1.00 ml
Solution 7 (Coenzyme A)	1.00 ml
Glucose	10.00 g
□ –Ketoglutaric acid	1.00 g
L-Cysteine hydrochloride	1.00 g
Glutathione (Reduced)	0.50 g
Asparagine	0.10 g
L-Tryptophane	0.02 g
Citric Acid (For Yeast Phase only)	10.00 g
Water, QS	500.00 ml

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

Note: The citric acid is for yeast phase medium only.

Note: This part is added to Pine's Citrate medium, Part 2

Pine's Citrate medium, Yeast Phase, Part 2

Oleic Acid solution., Liquid :	0.10 ml
Oleic Acid	0.10 gm
ETF water	100.00 ml
Starch solution, Liquid :	500.00 ml
Starch, Soluble	1.00 g
ETF Water	500.00 ml
Agar Formula	
Oleic Acid solution, Agar	1.00 ml
Oleic Acid	0.10 g
ETF Water	100.00 ml
Starch solution, Agar	500.00 ml
Starch, Soluble	2.00 g
Agar	10.00 g
ETF Water	500.00 ml

Note Filter sterilize stock solutions

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

28) Potato Dextrose Agar (PDA)

Potatoes	250 g

(Peel, slices and boil potatoes in 100 ml of D.W. for 1 hour, filter and add the following)

Dextrose	20.0 g
Agar	20.0 g

Distilled water to make final volume up to1 litre

- Bring to a boil to dissolve agar
- Autoclave at 121^0C for 15 min.

29 Potato Carrot Agar (PCA)

Potatoes	150 g
Carrot	150 g

(Peel, slice and boil potatoes and carrot in 150 ml of D.W. for 1 hour, filter and add the following)

Agar	15.0 g

Distilled water to make final volume up to1 litre

- Bring to a boil to dissolve agar
- Autoclave at 121^0C for 15 min.

30) Rice Starch Agar (RSA)

Rice grains, uncooked, white, unenriched	10 g
Tween 80	10 ml
Agar	15 g
Distilled water	1 litre

- Add rice to boiling water and continue boiling for 30 seconds
- Add Tween 80 and Agar
- Restore volume to 1000 ml
- Autoclave at 121^0C for 15 min
- Leave in water bath at 80^0C overnight
- Decant clear portion, refilter and again autoclave (pH. 6.2)
- Pour in petri dishes

31) Sabouraud Dextrose Agar (SDA)

Glucose	40 g
Peptone	10 g
Agar	20 g
Distilled water	1000 ml

- Mix reagents by boiling
- Dispense in tubes
- Autoclave at 121^0C for 15 min
- Final pH of 5.5 to 5.6
- Allow tubes to cool at slanted position
- Store at 4^0C
- Shelf life of 30 days in test tubes and 14 days in petridishes

b. Neutral (Emmon's Modification) Sabouraud's Dextrose Agar

Glucose	20 g
Peptone	10 g
Agar	20 g
Distilled water	1000 ml

- Procedure of preparation similar as to SDA
- Final pH of 6.8 to 7.0

c. Sabouraud Dextrose Agar with Cycloheximide and Chloramphenicol and gentamicin

To the molten Sabouraud's, add

Cycloheximide	0.5 mg/ml
Chloramphenicol	0.05 mg/ml
Gentamicin	0.02 mg/ml

- Add 500 mg of cycloheximide in 10 ml of acetone to boiling medium. Mix well. Add 50 mg of chloramphenicol and 20 mg of gentamicin in 10 ml of 95% alcohol to boiling medium.
- Mix the medium thoroughly. Tube and autoclave at 121^0C for 10 min.

d. Modifications of Sabouraud's Agar

i) SDA with thiamine

Thiamine	1 mg%

ii) SDA with yeast extract

(Growth of Dermatophytes)

yeast extract	5 g/litre

iii) SDA without Dextrose

For maintaining stock cultures

32) Soil Extract Agar

Soil	500.0 g
Glucose	2.0 g
Yeast extract	1.0 g
Potassium phosphate	0.5 g
Agar	15.0 g
Tap water	1000 ml

- Mix 500.0 gm of garden soil and 1 litre of tap water.
- Autoclave for 3 hours at 121^0C
- Filter through Whatman No. 2 filter paper. This is the soil infusion.
- Add reagents and bring volume up to 1 litre.
- Dispense in test tubes.

33) Tyrosine agar

Nutrient agar	23 g
Tyrosine	5 g
Distilled water	1 litre

Nutrient agar:

- Add 900 ml distilled water to the nutrient agar mix.
- Apply heat and swirl frequently until the agar is completely dissolved and cool at 55 ^{0}C

Tyrosine:

- Add the tyrosine to 100 ml distilled water and mix
- Be sure that the water is at room temperature or hotter.

Complete medium:

- To the nutrient agat at 55^0C, add the tyrosine suspension and mix.
- Be sure that the tyrosine crystals are evenly distributed.
- Sterilize by autoclaving at 121^0C for 15 min
- Final pH : 7.0 $\pm$0.1

34) Water Agar

Agar	20.0 g
Distilled water	1000 ml

- Dispense in test tubes
- Autoclave at 121^0C for 15 min.

35) Xanthine agar

Nutrient agar	23 g
Xanthine	4 g
Distilled water	1 litre

The method of preparation is same to tyrosine agar except tyrosine is replaced with xanthine.

36) Yeast extract phosphate medium with ammonia for selective isolation of *H. capsulatum* and *B. dermatitidis*.

Yeast extract	1 g
Phosphate buffer	1 g
Bacto agar	20 g
Chloramphenicol	0.5 g
Gentamicin	0.2 g
Distilled water	1 litre

Phosphate buffer preparation

- Dissolve 40 g Na_2HPo_4 in 300 ml distilled water
- Add 60 g of KH_2PO_4
- Adjust pH with HCl or Na OH
- Adjust volume to 400 ml.
- Suspend ingredients in distilled water, heat to boiling with frequent agitation
- Sterilize by autoclaving at 121^0C for 15 min.
- Final pH 6.0
- Use with ammonia
- Specimens are inoculated on to medium in petri dish by spreading 0.5 ml to 1.0 ml on surface.
- Put one drop (~0.5 ml) on agar surface.
- Allow ammonia to diffuse without being spread (Higher concentration of ammonia will inhibit other pathogenic fungi)

37) YPD

Bacto peptone	1 g
Glucose	2 g
Yeast extract	1 g
Distilled water	100 ml

- Sterilize at 121^0C for 15 min.

STAINS

1) Calcofluor white

This substance is available commercially, under a variety of trade names.

Stock Solution

Calcofluor white	1 g
Distilled water	100 ml

Working solution

1:10 dilution of stock solution in 0.05% Evans blue.

2) Gram Stain

1. Crystal Violet Reagents

Crystal violet (85% dye)	2 g
Ethyl alcohol (95%)	10 ml
Distilled water	100 ml

2. Grams Iodine Solution

Iodine	1 g
Potassium iodide	2g

Distilled Water	300 ml

3. Counter Stain

Safranin O	1 g
Ethyl alcohol (95%)	40 ml
Distilled Water	400 ml

4. Decolouriser

Acetone or Alcohol

3) Giemsa Stain

A compound stain formed by interaction of methylene blue and eosin.

Giemsa powder	600 mg
Methyl alcohol	50 ml
Glycerine	50ml

- Grind Giemsa powder in mortar. Pour methyl alcohol and glycerine and decant from top.
- Grind it again with glycerine till whole stain is dissolved.
- Keep the stain at 55^0C for 2 hours; shaking gently at 30 minutes interval.
- Keep it for two weeks for maturation.

Buffer Solution

Na_2HPO_4	6.77 gm
KH_2PO_4	2.50 gm
D.W.	1000 ml
pH	7.2

Dilute the stain with buffer for use.

4) Gomori's methanamine Silver Nitrate Stain (GMS)

a. 10% chromic acid

Chromic acid	10 g
Distilled water	100 ml

b. 1% sodium meta bi sulpfite

Sodium bisulfate	1.0 g
Distilled water	100ml

c. 5% Silver nitrate

Silver nitrate	5,0 g
Distilled water	100ml

(Store in black colored bottle at 4^0C)

d. 3% aqueous methamine (hexaethyltetramine)

Methanamine	3.0 g
Distilled water	100 ml

e. 5% aqueous borax

Borax (sodium borate)	5.0 g
Distilled water	100ml

f. 1% aqueous gold chloride

Gold chloride	15 grain vial
Distilled water	100 ml

g. 5% sodium thiosulfate solution

Sodium thiosulfate	5.0 g
Distilled water	100 ml

h. Light green working solution

1. 0.2% light green (stock lolution)

Light green, SF yellowish	0.2 g
Distilled water	100 ml

2. 10 ml of (1) is added to 50 ml distilled water

i. 70% alcohol
j. 95%alcohol
k. Absolute alcohol

} Used in plastic squirt bottle

l. Xylol, used from dropper bottle

m. Mounting medium

5) Lactophenol Cotton Blue Stain (LCB)

Phenol	20 g (Melt in warm water then weigh)
Lactic acid	20 g
Glycerine	40 g
Cotton blue	0.05 g
Distilled water	20 ml

Add in order→ Lactic acid → glycerine → distilled water → phenol.

Then dissolve with even heat.

6) Mayer's Mucicarmine Stain [For *Cryptococcus* and *Rhinosporidium*]

i). Weigert's Iron Haematoxylin

Solution A

Haematoxylin	1.0 g
Alcohol 95%	100.0 ml

Solution B

Ferric chloride (29%) solution	4 ml
Conc. Hydrochloric acid	1 ml
Distilled water	95 ml

Working Solution

Mix equal parts of A & B solution

Always prepare fresh stain

ii). Metanil Yellow Solution

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

iii). Mucicarmine Stain

Carmine	1 g
Aluminium chloride, anhydrous	0.5 g
Distilled water	2 ml

Mix the stain in test tube – boil for 2 mins. Liquid becomes almost black. Dilute with 100 ml of 50% alcohol and let it stand for 24 hours. Filter. Dilute 1:4 with tap water for use.

7) Nigrosin Staining Solution

Nigrosin (granular)	10.0 g
Formalin (10%)	100 ml

- Place the solution in a boiling water bath for 30 minutes. Add 10% formalin lost by evaporation
- Filter twice through double filter paper (Whatman No.1)

8) Potassium hydroxide (KOH-mounting fluid)

(a) With DMSO

Distilled water	60 ml
Potassium hydroxide	20 g
Dimethyl Sulphoxide (DMSO)	40 ml

(**b) Without DMSO**

Distilled water.	80 ml
Potassium hydroxide	20 g
Glycerine	20 ml

- Addition of DMSO or glycerine prevents rapid drying of the fluid and permits observations of slide for up to 48 hrs.
- DMSO also acts as an excellent cleansing agent.

9) Periodic Acid Schiff (PAS) stain

(a) Formal ethanol mixture

40 % formaldehyde	10 ml
Absolute alcohol	90 ml

(b)Periodic acid solution 1%

Periodic acid	1.0 g
Distilled water	100 ml

(c) Basic fuchsin Solution

Basic fuchsin	0.1 g
Distilled water	200 ml

(i) Warm to 50^0C and filter

(ii) Add 20 ml 1 N HCl (83 ml concentrated HCl / 1000 ml distilled water) cool to 25^0C.

(iii) Add sodium bisulphate 1 g, store in a screw top bottle in the dark for two days

(iv) Add activated charcoal 0.5 g; shake intermittently for one hour

(v) Filter, store in dark – colored tightly closed bottle in refrigerator (5 years). Solution can be used till it turns pink.

(d) Light green working solution

(i) 0.2% light green (stock solution)

Light green, SF yellowish	0.2 g
Distilled water	100 ml

(ii)10 ml of (i) is added to 50 ml distilled water

10) Kinyoun Acid fast stain

Carbol fuchsin

Basic fuchsin (3 g/ml in 100 ml 95% ethyl alcohol)	10 ml
Phenol 5%	90 ml

Decolourizer (0.5% sulfuric acid)

Sulfuric acid (concentrated)	0.5 ml

Distilled water	99.5 ml

Methylene blue

Methylene blue	0.3 g
Distilled water	100 ml

11) Toluidine blue 'O' stain for *Pneumocystitis jiroveci*

Sulfation reagent

Glacial acetic acid	45 ml
Concentrated sulfuric acid	15 ml

- Add concentrated sulfuric acid drop wise to the 250 ml flask containing 45 ml of glacial acetic acid by swirling the flask.
- Pour 40 ml of this reagent into coplin jar and seal with petroleum jelly till used.

Toluidine blue 'O' solution

Toluidine blue 'O' solution (dye content of 50 – 90%)	0.3 g
Distilled water	60 ml
Concentrated sulfuric acid	2 ml

- Mix thoroughly until the TBO is completely dissolved
- Add 140 ml of absolute ethyl alcohol
- Can be store at room temperature for one year

Ethyl alcohol 95%

Absoloute ethyl alcohol	95 ml
Distilled water	5 ml

- Pour in two coplin jar

Xyelene

Pour 50 ml in two coplin jar

12) Alcian blue stain

3% glacial acetic acid

Acetic acid	3.0 ml
Distilled water	100ml

Alcian blue solution

3% glacial acetic acid	100ml
Alcian blue 8GX	1.0 g

- Adjust pH to 2.5 using acetic acid and add a crystal of thymol.

13) Fontana Masson stain

10% Silver nitrate

Silver nitrate	20 g
Distilled water	200ml

0.1 % Gold chloride

1% gold chloride, stock	5.0 ml
Distilled water	45 ml

5% hypo (sodium thiosulfate)

Sodium thiosulfate	5 g
Distilled water	100 ml

Ammonical silver stock solution

- To a 25 ml of 10% silver nitrate, add ammonium hydroxide drop by drop till the solution becomes clear with out any precipitates.
- Add 1 ml of 10 % silver nitrate to it and allow to remain for up to 24 hours.

Ammonical silver nitrate working solution

Ammonical silver nitrate stock solution	12.5 ml
Distilled water	37.5 ml

- Filter the stain before use.

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Annexure – B : Problems Faced in Laboratory

DIFFICULT TO REVIVE CULTURES

i) Revive Culture with Broth

a) To a culture tube aseptically add Sabouraud dextrose broth.

b) Incubate the culture at 30^0C and examine for the presence of new growth. Transfer the new growth.

c) If new growth is not present after 3-4 weeks, discard the culture as non-viable.

ii) Revive Culture with Agar overlays

a) To a culture tube aseptically add a few milliliters of SDA cooled to 48-50^0C. Just cover the top of the colony with molten agar.

b) Incubate the culture at 30^0C and examine for new growth through thin agar overlay. Transfer the new growth to a Potato Dextrose Agar (PDA).

c) If new growth not present after 3-4 weeks, culture considered non-viable.

iii) Revive Culture by Homogenization

a) Transfer the non-viable isolate to 1 ml of sterile distilled water in a test tube and crop the fungus.

b) Pour the entire contents of test tube to a petri dish containing Potato Dextrose Agar (PDA). Seal the edges of petri dish with parafilm.

c) Incubate at 30^0C. If new growth not present after 3-4 weeks; culture discarded as non-viable.

MITES

Mites are minute arachnids that feed on fungi. As they walk from one culture to another they carry on their bodies' bacteria & fungi, which contaminate the stored cultures.

Mites enter primarily due to poor laboratory housekeeping. Thus firstly clean the laboratory with a good disinfectant and discard all the contaminated culture.

For saving cultures from mites several steps can be taken.

i) For cotton plugged tubes

a) Add 1-2 drops of mite poison to the cotton plug.

b) Let the plug dry overnight

c) Label the culture as poisoned

d) Keep the culture for storage

ii) For contaminated cultures

a) Transfer the mite infested fungus to a tube of medium containing hexachlorocylohexane

Hexachlorocyclohexane	0.01 g
Any mycological medium	1 litre

Sterilize as usual.

iii) For Incubators or Refrigerators

For mites present in incubators or refrigerators use either naphthalene or paradichlorobenzene.

a) Clean the incubator or refrigerators with 70% ethanol

b) Place 150 mm petridish containing crystals of naphthalene or para dichlorobenzene. One week will be required to ensure that mites have been killed. [Do not simultaneously conduct biochemical tests of yeast as they might utilize the vapors from these chemicals as a carbon source resulting in false positive biochemical results].

iv) Barrier Technique

Culture tubes can be protected by using parafilm or cigarette paper. Top of the tube is sealed with parafilm, which allows aeration but does not allow mites to enter.

KILLED CULTURE FOR TEACHING

Fungi as *Blastomyces dermatitidis, Coccidioides immitis* and *Histoplasma capsulatum* should be killed before using for teaching purposes.

a) In a screw cap culture tube either replace the cap with 40% formalin soaked cotton plug or insert the cotton plug into the cap and then screw it.

b) Incubate the tube at 37^0C for 48 hrs.

c) Before using the fungus for teaching purposes check the viability of fungi.

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Glossary

Acid-fast — A property of cell walls that, during a staining reaction retain basic dyes when decolorized with mineral acids.

Acropetal — having the youngest conidia at the apex of a chain.

Aerial hyphae — Hyphae that grow above the agar surface.

Aerobic — Having the ability to grow in the presence of oxygen.

Annellide — A conidiogenous cell that gives rise to successive conidia in a basipetal manner. The apex of an annellide becomes longer and narrower as each subsequent conidium is formed and released. An apical ring composed of outer cell wall material, remains as each conidium is released.

Annelloconidium (pl. annelloconidia) — A conidium formed by an annellide.

Annular frill — A ring or skirt like portion of cell wall material at the base of a conidium that remains when the conidium separates from its conidiophore.

Apex (pl. apices) — The tip.

Arthroconidium (pl. arthroconidia) — A conidium formed by the modification of a hyphal cell(s) and then released by the fragmentation-lysis of a disjunctor cell or by fission through a thickened septum.

Arthrospore — See arthroconidium.

Ascospore — A haploid sexual spore that is formed by free-cell formation in an ascus following karyogamy and meiosis.

Ascus (pl. asci) — A saclike cell that gives rise to ascospores. Asci are characteristic of the Ascomycetes.

Assimilation — The utilization of nutrients for growth, with oxygen serving as the final electron acceptor. $C_6H_{12}O_6+6O_2 \rightarrow 6CO_2+ 6H_2O$.

Ballistoconidium (pl. ballistoconidia) — A forcibly discharged conidium.

Balloon form — Pertaining to a large globose conidium formed by some dermatophytes, especially *Trichophyton tonsurans*.

Basidiospore — A haploid sexual spore formed on a basidium following the process of karyogamy and meiosis.

Basidium (pl. basidia) — A specialized cell that gives rise to spores. Basidia are characteristic of the Basidiomycetes.

Basipetal — having the youngest conidia at the base of a chain.

Bipolar budding — The development of conidia at both ends of parent cell.

Biseriate — Having phialides arising from metulae on the vesicles in species of *Aspergillus.*

Black yeast — A dematiaceous, unicellular, budding fungus that typically forms a black, pasty colony.

Blastoconidium (pl. blastoconidia)	A conidium that is blown out from part of its parent cell and is typically released by fission through thickened basal septum.
Blastospore	See blastoconidium.
Budding	A sexual formation of small rounded outgrowths from parent cell. These will become conidia.
Capsule	A gelatinous covering around a cell.
Carry-over	Indigenous substances stored within the cells of *inoculum* nutrients in the original culture medium, or both. These substances support growth of the test isolate in an assimilation study.
Chlamydoconidium (p. chlamydoconidia)	A rounded, enlarged conidium that usually has a thickened cell wall and functions as a propagule.
Chlamydospore	See chlamydoconidium.
Circinate	Coiled into a complete or partial ring.
Clamp connection	A specialized hyphal bridge involved with nuclear division in the Basidiomycetes.
Clavate	Club shaped
Cleistothecium (pl. Cleistothecia)	An enclosed fruiting body that contains randomly dispersed asci.
Collarette	A small collar.
Columella (pl. columellae)	A sterile domelike expansion at the apex of a sporangiophore.
Conical	Cone shaped
Conidiogenous cell.	A cell that gives rise to conidia.
Conidiophore	A specialized hypha upon which conidia develop.
Conidium (pl. conidia)	An asexual, nonmotile, usually deciduous-propagule that is not formed by cytoplasmic cleavage, free-cell formation or by conjugation.
Cottony	See floccose.
Denticle	A peg.
Dimorphic	Having two different morphologic forms.
Disjunctor cell	A cell that releases a conidium by its fragmentation or lysis.
Echinulate	Having a delicate, spiny wall.
Endospore	A spore formed within a spherule by a cleavage process following karyogamy and mitosis.
Erect	Upright.
Exudate	Droplets of fluid formed on the surface of a colony.

Fermentation	The ability to utilize nutrients for growth, with organic compounds serving as the final electron acceptors. $C_4H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$.
Filament	A threadlike element of a bacterium; a hypha of a fungus
Fission	To split into two portions or cells.
Fission arthroconidium	An arthroconidium that is released by fission through a double septum.
Floccose	Having a cottony texture.
Fragmentation	Separation of a hypha into conidia
Fungus (pl. fungi)	A eukaryotic, unicellular to filamentous, achlorophyllous organism having absorptive nutrition. A fungus reproduces by sexual, asexual, or both means.
Fusiform	Tapering at both ends; spindle shaped.
Geniculate	Bent like a series of knees.
Germ pore	An unthickened spot in a spore or conidial wall through which a germ tube may form.
Germ tube	A hypha initially developing from a conidium or spore.
Glabrous	Smooth
Hemispheric	Half of a sphere.
Hilum (pl. hila)	A scar at the base of a conidium.
Hyaline	Without color
Hypha (pl. hyphae)	An individual filament of a fungus
Intercalary	Occurring within a hypha.
Internode	That portion of a hypha that is between two nodes.
Karyogamy	Fusion of two nuclei.
Lanose	Having a wooly texture
Lysis	Dissolution
Macroconiduim (pl. macroconidia).	The larger of two conidia of two different sizes that are produced in the same manner by a single fungus.
Merosporangium (pl. merosporangia)	**A** sporangium having its sporangiospores in a single row.
Metula (pl. metulae)	A sterile branch upon which phialides of some species of *Aspergillus* and *Penicillium* develop.
Microconiduim (pl. microconidia)	The smaller of two conidia of two different sizes that are produced in the same manner by a single fungus.
Moniliform	Having swellings
Mould	A filamentous fungus
Multiple budding	The development of several series of blastoconidia around a parent yeast cell.
Muriform	Having vertical and horizontal septa
Mycelium	The aggregated mass of hyphae making up a fungus.

Mycology	The branch of biology that deals with the study of fungi.
Node	Where a stolon touches a surface
Nodular organ	A knot of hyphae that is often produced by dermatophytes.
Obclavate	Club shaped in reverse.
Obovoid	Egg shaped in reverse.
Olivaceous	Having an olive shade of color
Oval	Egg-shaped
Papilla (pl. papillae)	A small nipple-shaped elevation.
Penicillus	A brush like conidial head produced by members of the genus *Penicillium.*
Percurrent	Developing through a previous apex.
Perithecium (pl. perithecia)	A fruiting body having asci in a basal group or as a layer. Perithecia are usually flask shaped, with an opening through which the asci or ascospores escape.
Phialide	A type of conidiogenous cell that gives rise to successive conidia from a fixed site in a basipetal manner. A phialide does not increase in length as the conidia are formed, and its apex does not become smaller in diameter. A collarette is often present at the apex of the phialide.
Phialoconidium (pl. phialoconidia)	A conidium produced by a phialide.
Pleomorphic	Having several forms. The term is also applied to dermatophyte colonies that become irreversibly sterile.
Polymorphic	Having several forms
Pseudohypha (pl. pseudohyphae)	A series of blastoconidia that have remained attached to each other forming a filament. The blastoconidia are often elongated with the points of attachment between adjacent cells being constricted.
Pseudomycelium	A large amount of pseudohyphae.
Pycnidium (pl. pycnidia)	A saclike fruiting body that gives rise to conidia within its central area.
Pyriform	Pear shaped
Rachis	An extension of a conidiogenous cell-bearing conidia
Racket hyphae (also spelled racquet)	A hypha having a series of cells that are swollen at one end.
Radiating	Spreading from a common center.
Rhizoid	Pertaining to a root like group of hyphae.
Septum (pl. septa)	A crosswall
Simple	Of one piece; unbranched.
Solitary	Separate: alone.
Spherule	A sporangium like structure containing endospores that is produced by *Coccidioides immitis* or *Rhinosporidium seeberi*.

Sporangiolum (pl. sporangiola)	**A** sporangium that contains a small number of sporangiospores. Some sporangiola may contain only one sporangiospore.
Sporangiophore	A specialized hypha that gives rise to a sporangium.
Sporangiospore	A spore that is formed by a cleavage process following karyogamy and mitosis in sporangium.
Sporangium (pl. sporangia)	An asexual saclike cell that has its entire content cleaved into sporangiospore.
Spore	A reproductive propagle that forms either following meiosis or asexually by a cleavage process.
Sterigma (pl. sterigmata)	A pedicel bearing a basidiospore.
Stolon	A runner
Subglobose	Almost round.
Submerge	Within the nutrient agar.
Sympodial	Pertaining to the growth of conidiophore in which new successive lateral, subterminal apices of growth occur following successive conidium formation, Sympodial conidiophores are typically geniculate in appearance.
Synnema (pl. synnemata)	An erect macroscopic structure consisting of united conidiophores that bear conidia terminally, laterally, or in both ways.
Truncate	Ending abruptly.
Tuberculate	Having fingerlike or wart like projections.
Unipolar budding	The development of conidia at one end of the parent cell.
Uniseriate	Having phialides that arise directly from the vesicle in species of *Aspergillus*.
Verrucose	Having warts.
Verticil	A whorl of conidiogenous cells or conidiophores arising from a common point.
Verticillate	having verticals.
Vesicle	A swollen cell; the swollen apices of some conidiophore or sporangiophore.
Villose	Bearing long, hairlike appendages.
Yeast	A unicellular budding fungus that reproduces by sexual, asexual, or both means.
Yeastlike	Pertaining to a unicellular budding fungus that reproduces by asexual means only.
Zygospore	A resting spore in which meioses will occur. Zyospores result from the fusion of two similar hyphal elements. They are characteristic of the Zygomycetes.