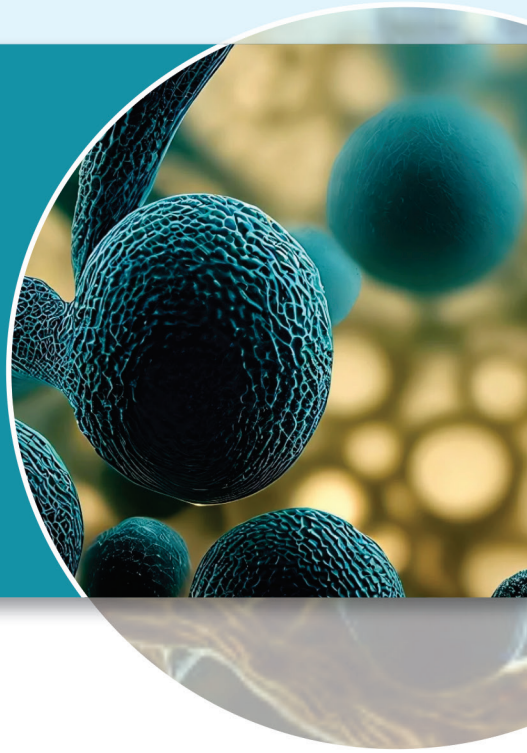
SECTION

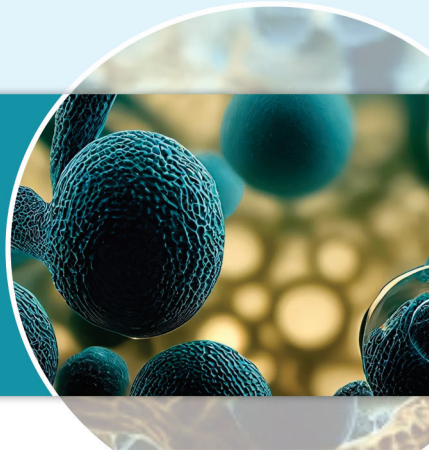
1

General Topics



CHAPTER
1

Introduction, Taxonomy and Classification of Fungi*



Fungi (singular, fungus) are a group of eukaryotic organisms which multiply both sexually and asexually by production of spores. The word ***'fungus'*** is derived from Latin meaning 'mushroom', which, in turn, is derived from Greek word *'spongos'* meaning 'sponge' which refers to the morphology of mushrooms. The term *'**mycology**'* is derived from Greek words '*mykes*' (mushroom) and '*logy*' (study) which means study of fungi. ***Medical mycology*** is the study of fungi that cause disease in humans.

Fungi are eukaryotic, which means, each cell possesses well defined nucleus with nuclear membrane, mitochondria, Golgi apparatus and endoplasmic reticulum. They can be *distinguished from other eukaryotes* by a rigid cell wall composed of chitin, β-glucans, mannan, as well as other polysaccharides, proteins and lipids. Within the cell wall, the cytoplasm is bounded by a cytoplasmic membrane in which the predominant sterol is not cholesterol but ***ergosterol***. Fungi differ from bacteria and other prokaryotes in many ways (Table 1.1).

In 1835, Augustino Bassi observed that muscardine, a disease of silkworm was caused by a fungus, *Baeuveria bassiana*. Shortly thereafter, in 1841, the mycologic etiology of favus was identified by David Gruby. Raymond Jacques Adrien Sabouraud (1864–1938), a dermatologist and mycologist was named as the **'Father of Mycology'** for his pioneering and extensive work on fungal scalp infections (mainly ringworm) and their treatment.

Fungi are ubiquitous, capable of colonizing almost any environment, and generally play an invaluable part in the decomposition and recycling of organic matter. About 1.5 million species of fungi are known. Most of them are found as saprophytes in the soil and on decaying plant material, about 500 species are known to cause human disease, and 50 are capable of causing infection in otherwise normal individuals.

Fungi are now considered as significant cause of morbidity and mortality as they have emerged as important etiological agents of ***opportunistic infections*** in patients with haematological malignancies undergoing chemotherapy, transplant recipients, patients with acquired immunodeficiency syndrome, severe burns, prematurity, and autoimmune diseases. Risk factors like admission to intensive care unit, diabetes, chronic liver and renal diseases are added to the list. Even immunocompetent hosts may occasionally acquire invasive fungal infections by direct introduction of fungi through indwelling

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Table 1.1: Distinguishing features of fungi and bacteria

Features	*Fungi (Eukaryotic)*	*Bacteria (Prokaryotic)*
Cell wall composition	• Rigid, multilayered, chitin, β-glucans, mannan, polysaccharides, proteins and lipids	• *Gram-positive:* Peptidoglycan and teichoic acid • *Gram-negative:* Lipopolysaccharides, proteins, lipoprotein and peptidoglycan • *Acid-fast:* Lipids, proteins, polysaccharides
Cell membrane	• Ergosterol (except in *Pneumocystis*)	• Phospholipids and proteins
Cytoplasmic contents	• Mitochondria, Golgi apparatus and endoplasmic reticulum	• Lack mitochondria, Golgi apparatus and endoplasmic reticulum
Nucleus	• True nucleus with nuclear membrane and paired chromosome	• Single, circular piece of DNA present in cytoplasm attached to mesosome, no nuclear membrane and nucleolus
Ribosomes	• 40S + 60S = 80S	• 30S + 50S = 70S
Replication/ Reproduction	• Mitosis and meiosis	• Binary fission
Spores	• Method of ***reproduction*** (sexual and asexual)	• Method of ***preservation***
Morphology	• *Yeast:* unicellular, *Mold:* multicellular	• Coccal, bacillary, filamentous and spirochaetal forms

devices, trauma or due to large inoculums of spores entering through respiratory tract.

The endemic mycoses prevalent in Asian countries include histoplasmosis, talaromycosis, blastomycosis, and sporotrichosis. Coccidioidomycosis has been occasionally reported as imported mycosis. Among opportunistic mycoses, invasive candidiasis is the commonest disease followed by aspergillosis and mucormycosis. In certain centres, cryptococcosis has been reported at high rate. Other opportunistic fungal infections such as fusiriosis and scedosporiosis are occasionally reported.

MORPHOLOGY OF FUNGI

Broadly, fungi are divided into two morphological forms:

- Yeasts
- Molds

Yeasts are unicellular fungi reproducing asexually by budding (blastoconidia) or by formation of transverse septum known as fission. Fungal spores germinate to produce multicellular branching filamentous structures known as hyphae. All molds are composed of branching hyphae. Most of the fungi exist as hyaline yeast or hyphal form but some of them are darkly coloured known as phaeoid.

Although, all fungi exist in yeast or mold form but traditionally fungi are divided into four morphological groups:

1. Yeasts

Yeasts are round, oval or elongated unicellular fungi. These organisms remain in the yeast form at both, room temperature (25°C) and body temperature (37°C). Most of them reproduce by an asexual process called budding in which the cell develops a protuberance which enlarges and eventually separates from the parent cell (Fig. 1.1A). The buds are termed blastoconidia. Some reproduce by fission. They form moist or mucoid colonies. *Saccharomyces cerevisiae* and *Cryptococcus neoformans* are the examples of non-pathogenic and pathogenic yeasts, respectively.

2. Yeast-like

In some yeasts, like *Candida*, the bud remains attached to the mother cell and elongates, followed by repeated budding forming chains of elongated cells known as **pseudohyphae** (Fig. 1.1B). *C. albicans* and *C. dubliniensis* also produce germ tubes. **Germ tubes are the beginning of true hyphae** and appear as filaments that are not constricted at their points of origin on the parent cell (Fig. 1.1C). **If the filaments are constricted at their points of origin on the parent cell, they are pseudohyphae, not germ tubes** (Fig. 1.1B). True hyphae appear as filaments that are not constricted at their points of origin on the parent cell.

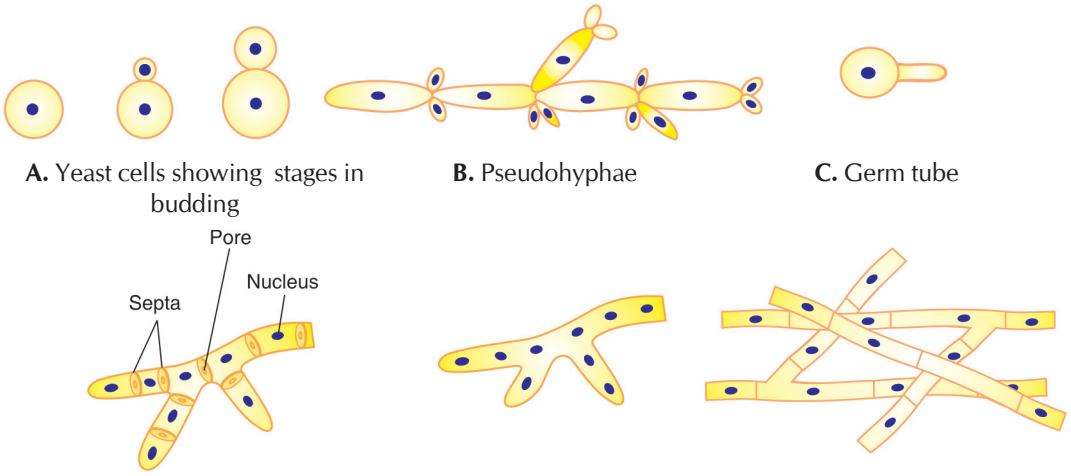
3. Molds

In molds, spores germinate to produce branching filaments called **hyphae** (singular, hypha). They are 2–10 μm in diameter. They may be septate or non-septate (coenocytic). Cells in septate hyphae communicate with each other through pores present in the septa (Fig. 1.1D), whereas in non-septate hyphae, the cells communicate freely as the protoplasm of the cells is continuous (Fig. 1.1E). However, sparse cross-walls or septa may occur. Where septa occur, they are not perforated but serve to isolate reproductive structures or vacuolated regions in the mycelium. The hyphae continue to grow (Fig. 1.1F) and branch to form tangled mass of growth called **mycelium** (plural, mycelia).

In the culture medium, the part of the mycelium which projects above the surface is called **aerial mycelium** and the part growing in the medium is called **vegetative mycelium**. Vegetative mycelium is responsible for absorbing water and nutrients from the medium. Spore or conidia-bearing fruiting bodies arise from aerial mycelium. Therefore, the latter is also known as reproductive mycelium. Molds reproduce by means of spores, produced often in large numbers, by an asexual process (involving mitosis only) or as a result of sexual reproduction (involving meiosis, preceded by fusion of the nuclei of two haploid cells). Dermatophytes, *Aspergillus*, *Talaromyces*, *Mucor* and *Rhizopus* are the examples of molds.

4. Dimorphic fungi

Many fungi pathogenic to man like *Histoplasma capsulatum*, *Sporothrix schenckii*,



A. Yeast cells showing stages in budding
B. Pseudohyphae
C. Germ tube
D. Septate hypha
E. Non-septate (coenocytic) hypha
F. Mycelium

Fig. 1.1: Basic fungal morphology

Blastomyces dermatitidis, *Coccidioides immitis*, *Paracoccidioides brasiliensis*, and *Talaromyces* (*Penicillium) marneffei* have a yeast form in the host tissue and *in vitro* at 37°C on enriched media, and hyphal (mycelial) form *in vitro* at 25°C–30°C.

If a single mycelium is capable of reproducing sexually, it is known as ***homothallic****, and if two mycelia are required to reproduce sexually, they are known as* ***heterothallic*** *fungi.*

Yeasts are the most common fungi isolated in the clinical laboratory. They are ubiquitous in our environment and also live as inhabitants in our bodies, so it is often difficult to determine the clinical significance of an isolate. Implication of yeast as the etiologic agent of infection often requires repeated recovery from the site and direct microscopic demonstration of the yeast in infected tissue. The yeasts and yeast-like organisms are considered opportunistic pathogens, causing disease in patients:

- with a breakdown of the body's immune system;
- on prolonged treatment with antibiotics, corticosteroids or cytotoxic drugs;
- with intravascular catheters;
- with diabetes mellitus; or
- who are intravenous abusers.

REPRODUCTION IN FUNGI

Reproduction in fungi may be asexual or sexual (Fig. 1.2).

Asexual reproduction

Asexual reproduction is a result of mitosis which involves budding or fission, resulting in the production of spores. The spores possess haploid nuclei (polidity *n*). These spores may be present endogenously within **sporangium** and are called **sporangiospores**, or exogenously as conidia.

Sexual reproduction

The sexual stage is characterized by several features that are unique to the kingdom Fungi. Most fungal mating types are morphologically indistinguishable and so, they are not referred to as male or female. In the case of sexual reproduction, compatible mating types fuse in a process that involves **plasmogamy** (fusion of cell membranes, and not the nuclei; polidity is $n + n$). This fusion produces a **heterokaryon** (a mycelium with multiple nuclei from the two mating types; found in the fungi of the order Mucorales) or **dikaryon** (a mycelium with two nuclei from the two mating types; these are found in Ascomycetes and Basidiomycetes), which can divide in the growing mycelium for a prolonged period. The dikaryotic state can last for months, or even years, while the fungus continues to grow and proliferate in its environment. When environmental conditions are suitable, two haploid nuclei fuse (**karyogamy**) to form a highly transient diploid state (polidity becomes $2n$). Meiosis follows, almost immediately, in a specialized spore-producing structure, and genetically distinct haploid spores (polidity n) are produced.

The asexual state of fungi is termed the **anamorphic** state, while the sexual state is termed **teleomorphic** state, e.g. *Histoplasma capsulatum* (anamorph) and *Ajellomyces capsulatus* (teleomorph).

SPORES, CONIDIA AND CONIDIOGENESIS

Spores are the basic unit of fungal reproduction. They may arise from sexual reproduction (meiosis, meiospores), e.g. zygospores, ascospores and basidiospores, or they may arise from asexual reproduction (mitosis, mitospores). The asexual spores are called ***conidia***. The term 'conidia' is derived from the Greek word for dust, *konia*. Sporangiospores contained within a sporangium in Mucorales, and microconidia and macroconidia in dermatophytes are the examples of asexual spores. The conidia are developed from the conidiogenous cells, conidiophores and conidiomata.

Three types of conidia may form from the vegetative mycelium:

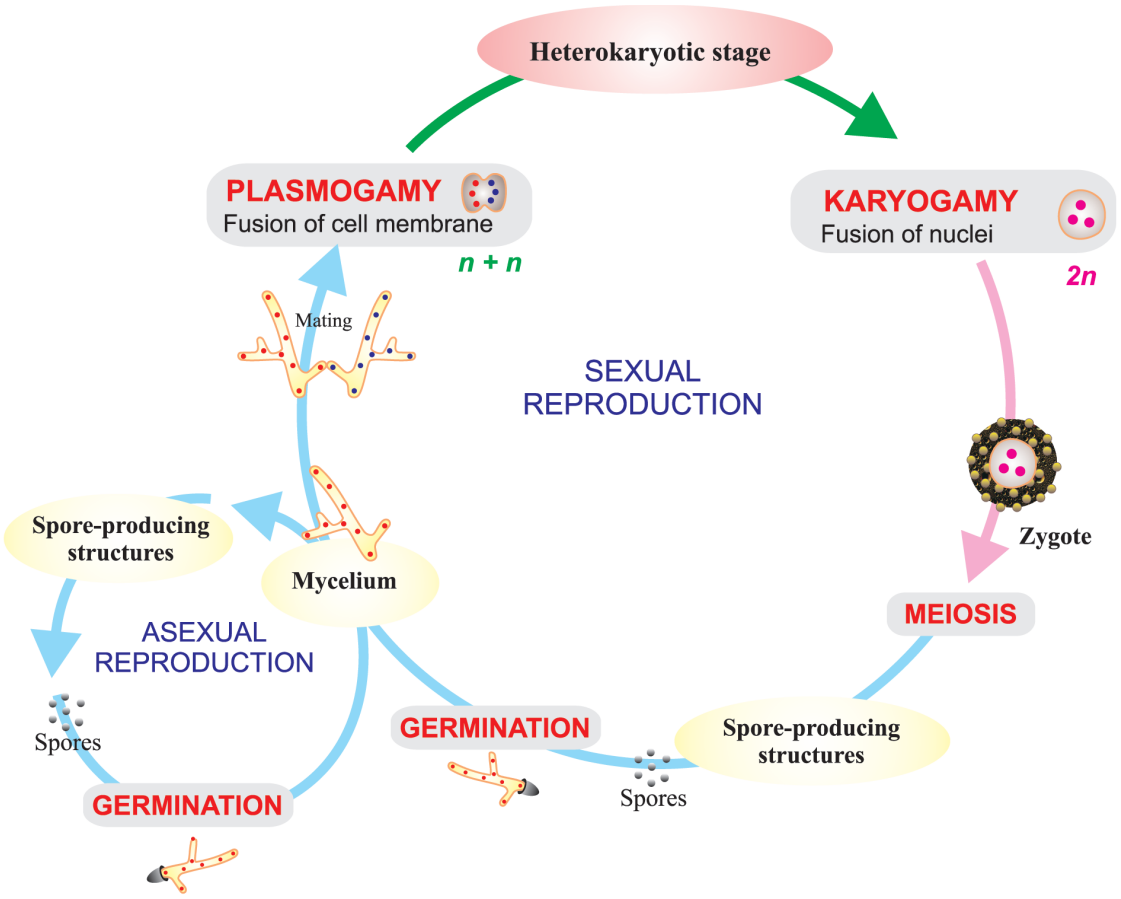


Fig. 1.2: Life cycle of fungi

- **Blastoconidia:** These are the budding forms characteristically produced by yeasts.
- **Chlamydoconidia:** These are formed from preexistent cells in the hyphae, which become thickened and often enlarged. Chlamydoconidia may be found within **(intercalary)**, or at the tip **(terminal)** of the hyphae. This type of conidiation is characteristic of *Candida albicans*.
- **Arthroconidia:** These are formed by the breaking up of a hypha at the point of septation. The resulting cell may be rectangular or barrel-shaped and thick or thin walled, depending on the genus. This type of conidiation is characteristic of the mold form of *Coccidioides immitis* and *Geotrichum* spp.

Method of conidiogenesis

It is mainly of two types—***blastic*** and ***thallic*** (Fig. 1.3).

Thallic conidiogenesis

In this, a septum appears in the hypha before the conidium is initiated. It occurs in molds.

Blastic conidiogenesis

In this, firstly the new conidium is initiated; it swells or thickens, and then is cut off by a septum. It can occur both in molds and yeasts.

When the wall of the conidium is continuous with the cell that produced it, it is called either holothallic (when thallic) or holoblastic (when blastic).

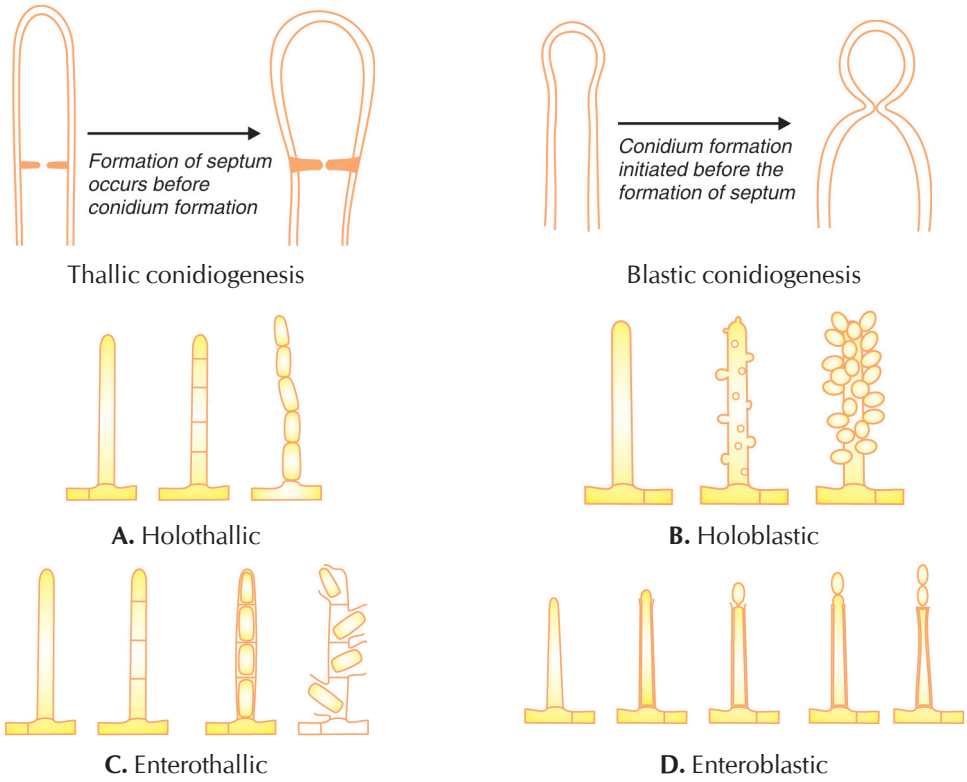


Fig. 1.3: Types of conidiogenesis.

When only the inner walls of the conidium-bearing cell are involved in conidiogenesis, it is called enterothallic and enteroblastic.

Enterothallic types are rare, and enteroblastic types are probably the most common of all and are represented by the ubiquitous phialide. Multiple conidia are produced ***serially*** in enteroblastic, whereas they are produced ***simultaneously*** in rest of the three conidiogenesis types.

Fungi showing different types of conidiogenesis are as follows:

1. Holoblastic – *Trichocladium*
2. Enteroblastic – *Acremonium*, *Bipolaris*, *Talaromyces*
3. Holothallic – *Geotrichum*
4. Enterothallic – *Coccidioides*

CLASSIFICATION OF FUNGI

Robert H Whittaker, an American biologist, in 1969 grouped fungi in a separate kingdom in his Five-Kingdom System, i.e. Monera, Protista, Fungi, Plantae and Animalia. **Kingdom Fungi** includes terrestrial organisms like molds, yeasts and mushrooms, which do not have chlorophyll. Kingdom is the highest taxonomic category. It includes phyla, classes, orders, families, genera and species. The endings that are used for various taxonomic levels in the kingdom Fungi are given below:

Kingdom	Fungi
Phylum	-mycota
Class	-mycetes
Order	-ales
Family	-aceae
Genus	—
Species	—

King Phillip Can Order Five Green Shirts.
(Mnemonics to remember hierarchy of taxonomy)

The classification of kingdom Fungi 2007 is the result of a large scale collaborative research effort. It includes seven phyla within

the kingdom Fungi, out of which Ascomycota and Basidiomycota are contained within a subkingdom Dikarya.

The name of a fungus is binomial composed of a generic name and a specific epithet subject to international code of Botanical Nomenclature. The names are usually derived from Latin or Greek and they are often descriptive of the fungus, source of isolation or derived from names of persons. The generic name is always capitalized while the species name is not capitalized–even if it is derived from a name of a country or a person. The names are always underlined when written and italicized when printed. The name of the author who described the species may be indicated after the binomial (e.g. *Aspergillus fumigatus* Fresenius).

TAXONOMIC CLASSIFICATION

Fungi belong to the kingdom fungi. The largest category of fungi pathogenic for humans belong to the subkingdom Dikarya. It has two phyla—Ascomycota and Basidiomycota. The fungal pathogens previously classed in the phylum Zygomycota are now placed in phylum Glomeromycota, subphyla Mucoromycotina and Entomophthoromycotina. These subphyla contain orders Mucorales and Entomophthorales, respectively (Fig. 1.4). Fungi which lack known sexual state, have been placed in fourth separate taxon called *Deutromycetes* or Fungi Imperfecti.

Holomorph (whole fungus) = Teleomorph (sexual state) + Anamorph (asexual state)

Dual Naming of Fungi

Fungi are classified primarily based on the structures associated with sexual reproduction, which tend to be evolutionarily conserved. However, many fungi reproduce only asexually, and cannot be easily placed in a classification based on sexual characters; some produce both asexual and sexual states. These problematic species are often members of the Ascomycota, but may also belong to the Basidiomycota.

*As per the Article 59 of the **International Code of Botanical Nomenclature (ICBN),** mycologists are allowed to give separate names to asexual state (anamorph) and sexual state (teleomorph) of a fungus but this practice will be discontinued as of January 1, 2013, as the International Botanical Congress held in Melbourne in July 2011 made a change in the International Code of Nomenclature for Fungi that adopted the principle **"one fungus, one name"**. In cases where names are available for both anamorph and teleomorph states of the same fungus, the holomorph either takes the teleomorph name, or it can under some circumstances take the anamorph name only if it is subsequently epitypified with a teleomorph.*

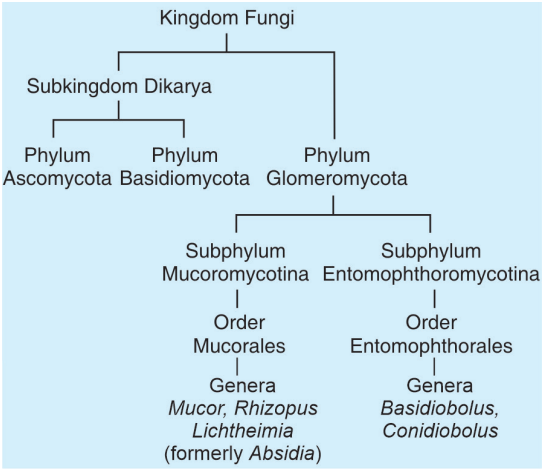


Fig. 1.4: Classification of kingdom fungi

Glomeromycota

These are most primitive classes of fungi. They are fast growing, widely distributed, terrestrial fungi which are largely saprobic on plant debris and soil. Many species are common environmental contaminants, often causing food spoilage, a few are pathogens of plants, insects, and more rarely of humans. They can be differentiated from other classes of fungi by:

- Their ***non-septate*** or ***coenocytic hyphae***; and
- Formation of ***heterokaryotic*** zygosporangium, in contrast to Ascomycota and Basidiomycota in which dikaryotic asci and basidia are produced, respectively.

There are two types of sexual spores— **zygospores** and **oospores**. Two different haploid mating types are often required for sexual reproduction in the fungi. First, gametangia

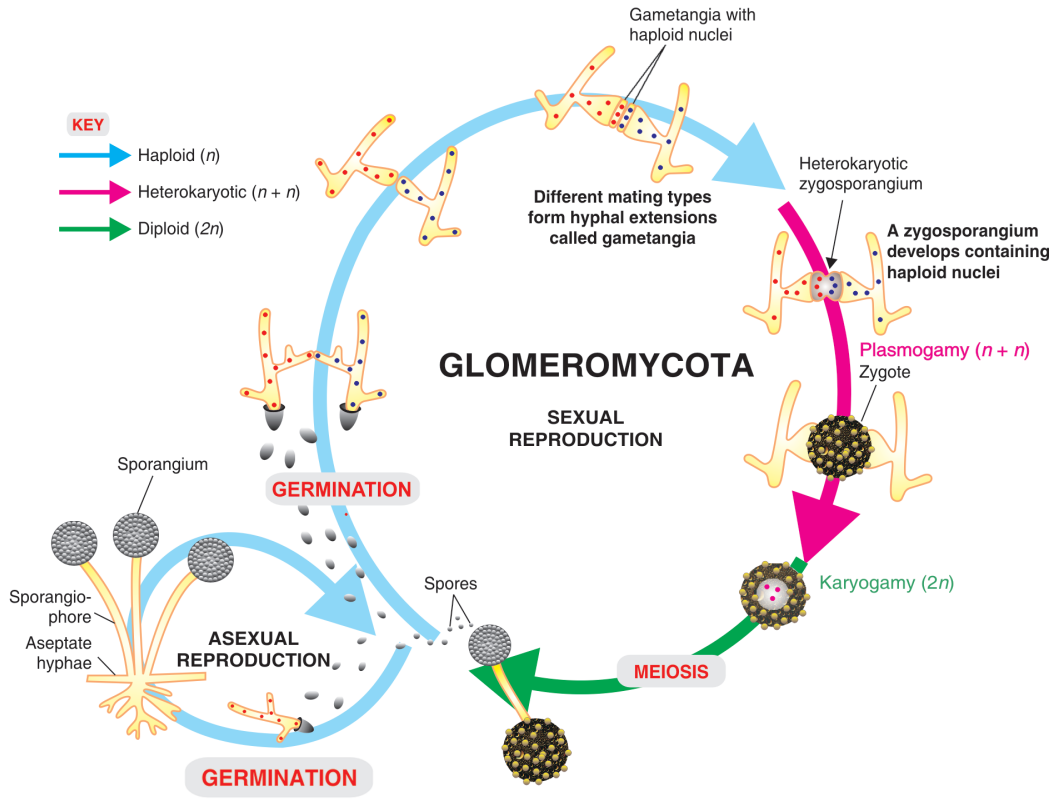


Fig. 1.5: Life cycle of Glomeromycota.

begin to form on hyphae of different mating types (Fig. 1.5). The gametangia then fuse to form the **heterokaryotic** state resulting in the development of heterokaryotic zygosporangium. The zygosporangium develops a rough and thickened cell wall, which renders it resistant to harsh conditions. When conditions become favorable for **zygospore** germination, the nuclei fuse (karyogamy) and a diploid state is briefly formed. Meiosis immediately follows and millions of haploid zygospores are formed in the sporangium by mitosis. The zygosporangium germinates and releases the spores and the cycle begins again.

Oospore result from fertilization of a specialized female structure (**oogonium**), which arises as a side hypha from the main mycelium and contains one or more ova, by the sperm or nucleus of a nearby male structure (**antheridium**) (Fig. 1.6). The fertilization tube of the antheridium penetrates the wall of the oogonium and the male nucleus is discharged into the ovum. A thick wall is formed around this zygote and the **oospore** is formed.

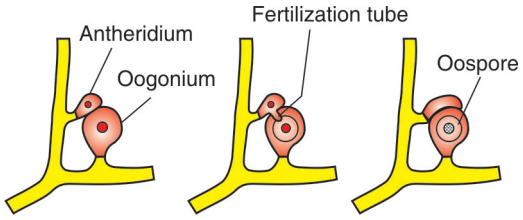


Fig. 1.6: Formation of oospores in Mucoromycota.

The asexual spores are produced inside a swollen structure called **sporangium** (plural, sporangia) by formation of cleavage planes. They develop on the end of branches or hyphae called **sporangiophores** (Fig. 1.5). The spores are known as **sporangiospores** (endoconidia). The sporangium ruptures, dispersing the sporangiospores and leaving the thin walled sporangium in place.

Ascomycota

Ascomycota are the largest, most biologically and morphologically diverse group of fungi.

- The defining morphological character of the phylum Ascomycota is the production of two to eight sexual spores in a microscopic sac-like cell called an **ascus.** Hence, they are sometimes referred to as "**sac fungi**."
- In addition, most Ascomycota bear their asci in macroscopic fruiting bodies called **ascocarps**.
- Ascomycota are also capable of producing enormous amounts of asexual spores called **conidia**, which allow them to propagate without having to undergo sexual recombination.

In the sexual part of the life cycle (Fig. 1.7), two compatible haploid hyphae become intertwined and form an **ascogonium** and an **antheridium**. A very fine hypha, called trichogyne emerges from *ascogonium*, and merges with *antheridium*.The ascogonium acts as a "female" and accepts nucleus from the antheridium after plasmogamy has occurred. The resultant dikaryon is then capable of forming an ascocarp. Asci begin to form on the surface of the ascocarp at the tips of the dikaryotic mycelium, and karyogamy occurs to form the highly transient diploid nucleus. The diploid nucleus immediately undergoes meiosis, yielding four, genetically distinct, haploid nuclei. After an additional round of mitosis, eight haploid nuclei are formed within the ascus. These eight nuclei eventually develop into eight ascospores, which are released from the ascus on the surface of the ascocarp. Finally, haploid mycelia arise from the ascospores as the sexual cycle begins again.

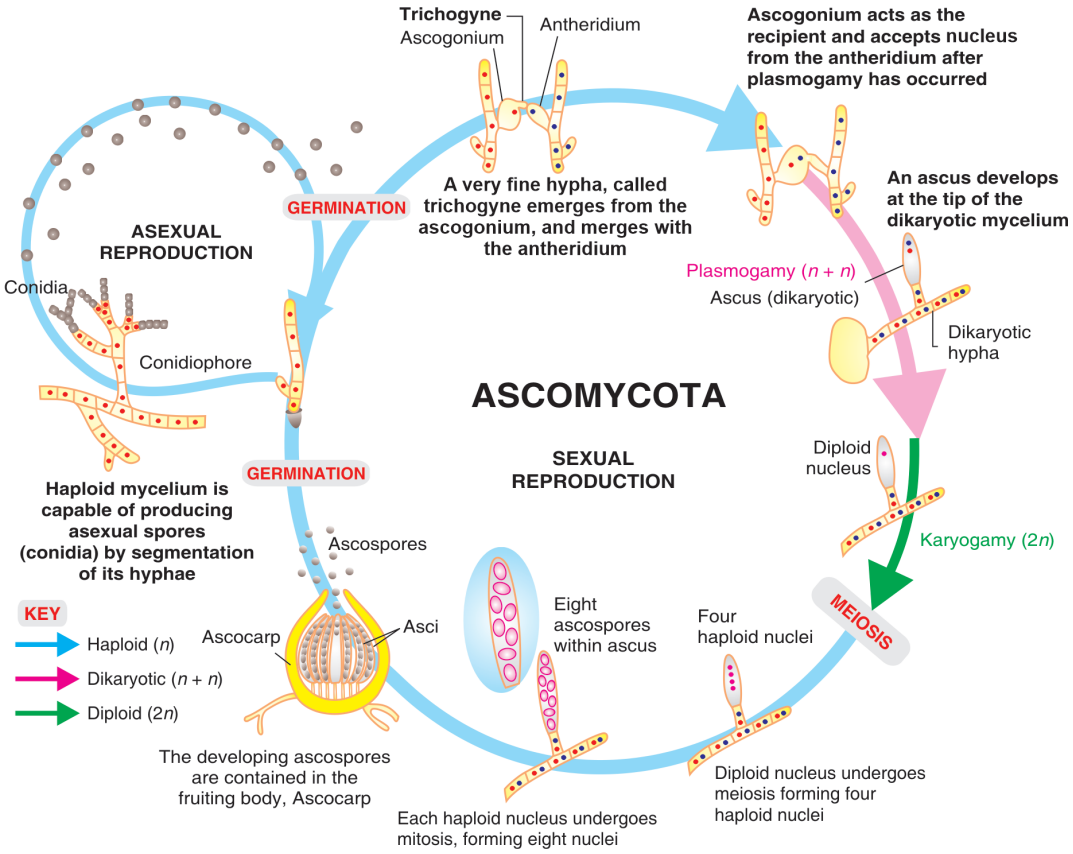


Fig. 1.7: Life cycle of Ascomycota.

In the asexual part of the life cycle, the haploid mycelium is capable of producing asexual spores (conidia) by segmentation of its hyphae. These segments will compartmentalize into conidia, and wind or water dispersal will follow.

The asexually reproducing phase of the Ascomycota life cycle was more or less ignored for many years in favour of teleomorph studies. But when we consider that the anamorph is an important (and sometimes the only) phenotypic expression of many Ascomycota genotypes, we realize that it has much to tell us. Besides, one can get DNA and RNA from anamorphs just as easily as from teleomorphs, so we are beginning to understand the relationships of anamorphs better, even in many cases where no teleomorph is known.

Though they play essentially the same role in the life cycle, the anamorphs of Ascomycota differ from those of Glomeromycota in two very important respects:

1. While in Glomeromycota, sporangiospores originate by free-cell formation inside a sporangium, by cleaving from a single mass of cytoplasm, the conidia of Ascomycota originate either by budding or converted from a whole existing cell.
2. In Glomeromycota, anamorph and teleomorph often develop simultaneously (especially in homothallic species) and always share the same binomial. In Ascomycota, anamorph and teleomorph develop at different time.

Basidiomycota

These fungi are unicellular or multicellular, sexual or asexual, and terrestrial or aquatic. These are characterized by:

- the production of **basidia** (singular, basidium), on which sexual spores are produced, and from which the group takes its name;
- a long-lived **dikaryon**, in which each cell in the thallus contains two haploid nuclei resulting from a mating event; and
- **clamp connections**, a kind of hyphal outgrowths that is unique to Basidiomycota.

Sexual reproduction (Fig. 1.8) is initiated by the fusion of two haploid hyphae of opposite mating types, and a dikaryon is often formed in which each cell contains two haploid nuclei, one from each partner. The hyphae develop **clamp connections** to ensure that each cell maintains the requisite two nuclei. Karyogamy occurs to form a diploid nucleus in each basidium. The paired nuclei eventually fuse within a terminal clavate or club shaped **basidium**, immediately followed by meiosis and each of four genetically distinct, haploid nuclei migrates into appendages and develops into basidiospores. **Basidiospores** are borne externally at the top of the basidium and each is discharged by hydrostatic forces from the tip of a narrow tapering sterigmata.

Formation of clamp connection: Clamp connections are formed by the terminal hypha during elongation. Before the clamp connection is formed this terminal segment contains two nuclei. Once the terminal segment is long enough it begins to form the clamp connection. Simultaneously, each nucleus undergoes mitotic division to produce two daughter nuclei (two red and two blue). As the clamp continues to develop it uptakes one of the daughter nuclei (red) and separates it from its sister nucleus. While this is occurring the remaining nuclei (blue) begin to migrate from one another to opposite ends of the cell. Once all these steps have occurred, a septum forms, separating each set of nuclei (Fig. 1.8).

Medically important fungi belonging to three phyla are given in Table 1.2.

CLASSIFICATION OF MYCOSES

Infection caused by a fungus is known as **mycosis** (plural, mycoses). It can be divided into four categories:

I. Superficial mycoses

These are strictly surface infections involving skin, hair, nail and mucous membrane. These include:

- Infection of skin, hair and nail caused by dermatophytes.

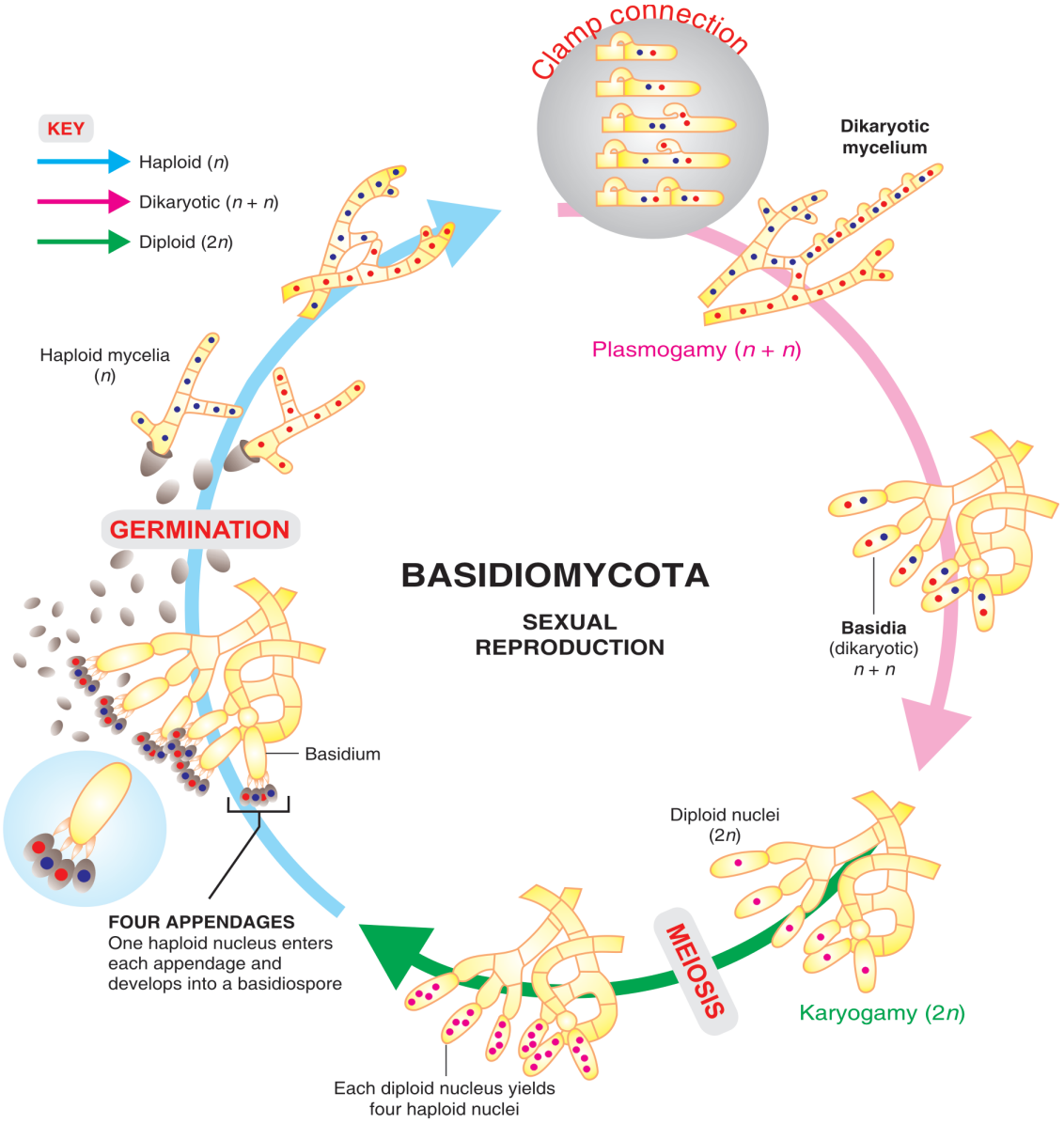


Fig. 1.8: Life cycle of Basidiomycota.

Table 1.2: Medically important fungi

Phylum	*Fungi*
Glomeromycota	*Mucor, Rhizopus, Lichtheimia (formerly Absidia), Rhizomucor, Apophysomyces, Cunninghamella, Saksenaea, Conidiobolus, Syncephalastrum, Basidiobolus*
Ascomycota	*Candida, Pneumocystis, Histoplasma, Blastomyces, Paracoccidioides, Emmonsia, Trichophyton, Microsporum, Arthroderma, Epidermophyton, Chrysosporium, Fusarium, Aspergillus, Talaromyces, Pseudallescheria, Scedosporium, Chladophialophora, Fonsecaea, Exophiala, Hortaea, Rhinocladiella, Bipolaris, Alternaria*
Basidiomycota	*Cryptococcus, Trichosporon, Malassezia*

- Infection of skin, nail and mucous membrane caused by *C. albicans*.
- Infection of skin caused by *Malassezia spp.* (pityriasis versicolor) and *Hortaea werneckii* (tinea nigra).
- Infection of hair caused by *Piedraia hortae* (black piedra) and *Trichosporon* spp. (white piedra).

II. Subcutaneous mycoses

Mycoses of the skin, subcutaneous tissues and bones result from the inoculation of saprophytic fungi of the soil or decaying vegetation leading to progressive local disease with tissue destruction and sinus formation. The lesion may spread via lymphatics. These occur mainly in the tropics and subtropics. The principal subcutaneous mycoses are mycetoma, chromoblastomycosis, phaeohyphomycosis, sporotrichosis and rhinosporidiosis.

III. Systemic mycoses

Systemic mycoses are caused by inhalation of airborne spores produced by the fungi which are present as saprophytes in soil and on plant material. From the lungs the fungus may disseminate to CNS, bone and other internal organs. Systemic mycoses include blastomycosis, histoplasmosis, coccidioidomycosis and paracoccidioidomycosis.

IV. Opportunistic mycoses

The opportunistic mycoses are infections attributable to fungi that are normally found as human commensals or in the environment. Virtually any fungus can serve as an opportunistic pathogen, and the list of those identified as such becomes longer each year. The most common opportunistic mycoses include aspergillosis, talaromycosis (penicilliosis), mucormycosis, candidiasis, cryptococcosis and pneumocytosis.

PATHOGENESIS

Our understanding of the pathogenesis of fungal infections is limited. Relatively few fungi are sufficiently virulent to be considered **primary pathogens**. Primary pathogens are capable of initiating infection in a normal, apparently immunocompetent host. These include *Blastomyces dermatitidis*, *Coccidioides immitis*, *Histoplasma capsulatum*, and *Paracoccidioides brasiliensis*. These fungi possess virulence factors that allow them to actively breach host defences that ordinarily restrict the invasive growth of other microbes.

Healthy immunocompetent individuals have a high innate resistance to fungal infection, despite the fact that they are constantly exposed to the infectious forms of various fungi present as part of the normal commensal flora (endogenous) or in the environment (exogenous). Important opportunistic fungal pathogens include *Candida* spp., *Cryptococcus neoformans*, and *Aspergillus* spp.

Important Questions

1. Discuss classification of fungi.
2. Tabulate differences between fungi and bacteria.
3. Write short notes on:
   (a) Taxonomic classification of fungi
   (b) Morphological classification of fungi
   (c) Classification of mycoses.

Multiple Choice Questions

1. Fungi possess:
(a) A well-defined nucleus.
(b) Mitochondria.
(c) Endoplasmic reticulum.
(d) All of the above.

2. Which of the following statements is incorrect about fungi?
(a) They are prokaryotic organisms.
(b) They are devoid of chlorophyll.
(c) They possess well-defined nuclei.
(d) They reproduce both sexually and asexually.

3. Fungi have been classified under Kingdom:
(a) Plantae.
(b) Animalia.

(c) Fungi.
(d) Protista.

4. Genus *Rhizopus* belongs to which of the following phyla?
(a) Ascomycota.
(b) Glomeromycota.
(c) Basidiomycota.
(d) None of the above.

5. *Talaromyces (Penicillium) marneffei* is a:
(a) Yeast.
(b) Yeast-like.
(c) Mold.
(d) Dimorphic fungus.

6. Which of the following fungi does not possess ergosterol in their plasma membrane?
(a) *Candida albicans*.
(b) *Pneumocystis jirovecii*.
(c) *Blastomyces dermatitidis*.
(d) *Histoplasma capsulatum*.

7. During sexual reproduction the hyphae develop clamp connections in which of the following fungal phyla?
(a) Basidiomycota.
(b) Ascomycota.
(c) Glomeromycota.
(d) Deuteromycota.

8. Which of the following scientists is known as "Father of Mycology"?
(a) Emmons.
(b) Sabouraud.
(c) Conant.
(d) David Gruby.

9. Which of the following fungal diseases is communicable?
(a) Dermatophytosis.
(b) Aspergillosis.
(c) Histoplasmosis.
(d) Sporotrichosis.

10. Which of the following is not true of fungi?
(a) Eukaryotic.
(b) Heterotrophic.
(c) Photosynthetic.
(d) Mitochondria present.

11. The soil-dwelling mold produces conidia, which when inhaled germinate in the lung as a budding yeast. The fungus is said to be:
(a) Allergenic.
(b) Dimorphic.
(c) Homothalic
(d) Thermophilic.

Answers to MCQs

1. (d), 2. (a), 3. (c), 4. (b), 5. (d), 6. (b), 7. (a), 8. (b), 9. (a), 10. (c), 11. (b).

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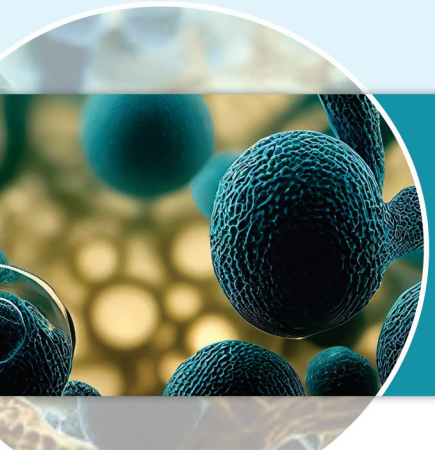

CHAPTER 2

Epidemiology and Laboratory Diagnosis of Mycoses

EPIDEMIOLOGY

Infection in the community

The usual reservoir from which a fungus infects humans is a site in nature where the fungus is growing as saprophyte. Human mycoses are poorly communicable from person-to-person. Mycoses are often endemic but rarely epidemic. Endemicity occurs in areas where a fungus is more frequent in the environment. Invasive infectious diseases are 3–15 times more common in Asia than Western world, because many countries in Asia are located in tropics where fungi thrive due to congenial weather, and solid organ and bone marrow transplant centres are increasing in all countries, but majority of the patients cannot afford to buy medicines post-transplant.

Over the past three decades, the incidence of both nosocomial and community-associated fungal infections has increased dramatically. Factors which have contributed to the increase in fungal infections include a growing population of immunosuppressed or immunocompromised patients whose mechanisms of host defence have been impaired by primary disease states (e.g. AIDS, cancer) and the use of new and aggressive medical and surgical therapeutic strategies, including broad-spectrum antibiotics, cytotoxic chemotherapies, and organ transplantation.

Venereal transmission

Venereal transmission of blastomycosis and histoplasmosis has been reported. With both these mycoses, vulvovaginal and endometrial lesions have resulted from sexual intercourse with a partner who had disseminated disease. Penile lesions have been present in some, but not all, patients suggesting that fungus can be transmitted in seminal fluid.

Transplacental transmission

There is no well-documented example of transplacental transmission of any mycosis.

Infection in the hospital

Patients susceptible to invasive aspergillosis are particularly congregated in oncology, transplantation, and intensive care units of hospitals. Protection against airborne *Aspergillus* conidia can be afforded by high efficiency air filtration, such as occurs in a laminar flow room.

Prevention of candidiasis involves different principle because the usual reservoir is the patient's own body. Decreasing the concentration of *Candida albicans* in the gastrointestinal tract is commonly attempted in the patients with severe and prolonged neutropenia. Such patients are prone to develop haematogenously disseminated candidiasis from numerous small ulcers caused by *Candida* species in the stomach, oesophagus, and intestine. Despite massive oral

doses of nystatin, more than a ten- or hundred-fold reduction in *Candida* colonies per gram of stool has proved difficult; prevention of disseminated candidiasis by nystatin has not been convincingly demonstrated.

Infection in the laboratory

Many ubiquitous fungi, such as *Candida* and *Aspergillus*, can be handled with biosafety level 1 precautions. Working with the mold forms of *Coccidioides immitis* and *Histoplasma capsulatum* requires special precautions. Biosafety level 3 should be observed when handling *Coccidioides immitis* and *Histoplasma capsulatum*.

Precautions with infected laboratory animals

Urine of animals infected with *Cryptococcus neoformans*, *Histoplasma capsulatum* and *Coccidioides immitis* may contain the fungus and contaminate the cage.

Laboratory-acquired mycoses

Accidental cutaneous inoculation of fungi may occur in laboratory workers as a result of needle sticks, scalpel cuts, or scratches containing infectious material. Local cutaneous granulomata due to the agents of cryptococcosis, blastomycosis, coccidioidomycosis, or histoplasmosis generally resolve spontaneously. Laboratory workers may acquire clinical ringworm while working with dermatophytes or handling guinea pigs experimentally infected with *Trichophyton mentagrophytes*. These lesions are unlikely to resolve spontaneously and usually require treatment.

Tissue reactions to fungal infection

The most common tissue reactions to fungal infection are necrotizing granulomatous reactions. Usually, the infectious agent is present where the inflammation is the most pronounced. However, if the tissue is healed with little inflammatory component remaining, the organisms are less likely to be detected. Patients who are immunocompromised, due to immunosuppressive drug or an immune deficiency disease, often do not produce a pronounced cellular response to fungal infection.

Mucormycetes and *Aspergillus* spp. have a tendency to invade blood vessels (angioinvasion), blocking the lumen of the vessel, stopping the flow of blood, and causing death of the tissue deprived of blood supply. This process is known as infaraction.

Splendore-Hoeppli reaction is indicative of localized immunologic host response to antigens of a variety of infectious agents, including some fungi and bacteria. It is characterized by Splendore-Hoeppli material coating or bordering the microorganisms. Histologically, it appears as radiating homogenous, refractile, eosinophilic (pink with H & E) club-like material surrounding a central eosinophilic focus. Fungi associated with this phenomenon are *Sporothrix schenckii*, *Conidiobolus coronatus* and those causing mycetoma. Actinomycetes, causing actinomycetoma and actinomycosis, and bacteria causing botryomycosis also generate this phenomenon.

A fungus ball results when filamentous fungi (most commonly, but not exclusively, *Aspergillus*) colonize a previously formed cavity that has access to oxygen. There is minimal or no invasion of the tissue unless the host is immunocompromised. Hyphae and somtimes conidal heads complete with phialides and conidia develop. Fungal masses are seen lying free within the cavity. The hyphae are not usually attached to the cavity wall.

LABORATORY DIAGNOSIS OF MYCOSES

Laboratory diagnosis of mycoses depends on:

- Recognition of the pathogen in tissue by microscopy.
- Isolation of the causal fungus in culture.
- Use of serological tests.
- Detection of fungal DNA by the polymerase chain reaction.

Successful laboratory diagnosis of mycoses is dependent not only upon the mycologic expertise of the clinical laboratory workers but also heavily upon the quality of the specimens provided for laboratory analysis. Specimens for the diagnosis of mycoses include skin scrapings, oral scrapings, vaginal scrapings, corneal scrapings, hairs, nails, bone marrow, blood, cerebrospinal fluid, urine, sputum, bronchial lavage specimens, mycetoma grains, pus and tissue.

Specimen should be collected under aseptic conditions, i.e. after appropriate cleaning and decontamination of collection site in a leak-proof sterile container and processed as soon as possible. Anaerobic transport media or anaerobic containers should never be used for fungi. Specimens that contain normal bacterial flora should be transported as soon as possible because bacterial overgrowth can inhibit slower-growing fungi as well as inhibit fungal viability.

If the lesion has a definite edge, the material should be taken from the active margin, otherwise a general scraping is adequate. When blisters are present, a pair of fine scissors may be used to cut off a blister roof for microscopic examination and culture; such samples are often packed with hyphae. The scrapings should be collected and transported in folded paper, which keeps the specimen dry, thus preventing contamination. Hairs to be examined for the presence of black or white piedra may be simply cut off at skin level. If dermatophytosis is suspected, the hairs should be removed with roots intact; cut hairs are unsuitable.

Isolation of the pathogen in culture from nail material is more difficult to achieve than for skin and hair samples. In the majority of nail infections, the material for examination is taken from the distal end of the nail, despite the fact that the infection is advancing proximally. The hyphae at the distal end of the nail are less likely to be viable. Debris from under the nail is a fruitful source of material, which may be scraped out using the flat end of a dental probe. The mouth or vagina may be sampled using a blunt scalpel or by using swabs. Scraping from the external ear canal may be supplemented with swab samples.

When thrush is suspected, the lesions should be scraped gently with a wooden spatula and material transferred to a clean glass slide for wet mount microscopy. With oral lesions, a tongue depressor works well. With vaginal smears, the cervical cytology spatula is useful. Procedure for collection of sputum, bronchial brushing, biopsy and bronchial lavage fluid, cerebrospinal fluid, urine, prostatic secretions and exudates is given in Table 2.1.

Culturing the centrifuged sediment from 50–100 ml urine may yield *Cryptococcus neoformans*, *Blastomyces dermatitidis*, *Histoplasma capsulatum* and *Coccidioides immitis* in the presence of disseminated infection, even without clinical evidence of genitourinary infection.

The clinical information is very important in guiding the laboratory in terms of specimen processing and interpretation of results. This is especially important when dealing with specimens from non-sterile sites such as sputum, bronchial washings and skin. Furthermore, it alerts the laboratory personnel that they may be dealing with potentially dangerous pathogen such as *Coccidioides immitis*. If delay in processing is unavoidable, the specimen for fungal culture may be stored at 4°C for a short time. Specimens not likely to contain contaminating microorganisms, e.g. **cerebrospinal fluid should not be refrigerated**. Causative agents of mycoses can be identified by following methods:

I. Direct microscopic examination of specimens

The direct microscopic examination of clinical specimen may be as simple as placing a drop of liquid specimen onto a clean glass slide and examining it with light microscope, or it may involve more complex procedures, including

Table 2.1: Procedure for collection of specimen for fungus culture

Specimen	*Procedure*
Sputum	The first early-morning sample should be collected after vigorously rinsing mouth with water followed by coughing of sputum into a sterile, screw-capped container.
Broncho-scopy	Bronchial brushing, biopsy or bronchoalveolar lavage fluid should be transported promptly to the laboratory in a sterile, sealed container.
Cerebro-spinal fluid (CSF)	As much CSF as possible should be used for the culture of fungi. If processing is to be delayed, the sample should be left at room temperature in an adequate fluid culture medium in which fungal elements can survive until subcultured.
Urine	Mid-stream urine specimen should be collected aseptically in sterile screw-capped container. If a delay in processing beyond 2 hours is anticipated, the urine sample should be refrigerated at 4°C to inhibit overgrowth of rapidly growing bacteria.
Prostatic secretions	The bladder is first emptied, followed by prostatic massage. Secretions should be inoculated directly into appropriate fungal culture media. If secretions are not obtained then 5–10 ml of urine be collected and processed.
Exudates	The skin over pustular lesions should be disinfected and exudates aspirated using a sterile needle and syringe. Hair, nails, and skin scrapings may be stored up to 72 hours at room temperature before culture, and they may be shipped by mail.

staining of tissue. Although, the Gram stain performed in the routine microbiology laboratory often gives the first evidence of infection with yeast, other direct stains give more specific information concerning a mold infection. The types of direct examination used in identification of fungal infections include wet preparation such as KOH preparation, KOH with calcofluor white, India ink, and tissue stains such as periodic acid-Schiff (PAS) stain, Gomori methenamine silver (GMS) stain, Giemsa stain, and haematoxylin and eosin (H&E) stain.

KOH preparation

A 10–20% solution of KOH is useful for detecting fungal elements in skin, hair, nails, and tissue. In this procedure, KOH is mixed in equal proportions with the specimen on a slide and the specimen material is teased with two inoculating needles. A coverslip is placed over it and heated gently. Preparation with KOH clears the tissue and cellular debris from all types of clinical specimens without damaging the fungal cells. This clearing process requires only 5–10 minutes, after which one can observe the fungal morphology as well as the pigment of the fungal cell wall under a phase-contrast or bright-field microscope, using low-power followed by high-power objectives.

Nail specimens take longer to clear, but if small pieces and debris are taken, they will usually soften within 10 minutes. In those instances, where the nails do not soften satisfactorily, the slide may be put in a 37°C incubator for one hour, and the material then be flattened. In contrast to skin and nail samples, infected hairs are very delicate, and if heated or left in mounting fluid for more than a few minutes tend to disintegrate, obscuring the characteristic arrangement of the arthroconidia. They should, therefore, be examined as soon as possible after mounting.

Reaction of KOH with pus, sputum, and skin may produce artifacts that superficially resemble hyphae or budding forms of fungi. Therefore, experience is required in interpreting the results. Moreover, crystals can form on standing, so that reading of the smear becomes difficult.

With KOH preparation, a definitive diagnosis of blastomycosis, paracoccidioidomycosis, coccidioidomycosis, mycetoma,

phaeohyphomycosis, lobomycosis or rhinosporidiosis can be made. Tentative diagnosis can be derived from the presence of fungal elements compatible to the etiologic agents of aspergillosis, mucormycosis, dermatophytosis, candidiasis, sporotrichosis, or cryptococcosis. **To confirm such a diagnosis, however, culture proof is necessary.**

KOH with calcofluor white

A drop of 0.1% calcofluor white solution (fluorescent reagent) can be added to the KOH preparation prior to placing coverslip over it. Calcofluor white binds to polysaccharide present in the chitin of the fungus or to cellulose. Fungal elements fluoresce apple green or blue-white, depending on the combination of filters used. The actual fungal structure must be seen before a positive preparation is reported.

On wet preparations of specimens or on smeared and dried material fluorescent calcoflour white stain with or without KOH, is far superior to the traditional KOH alone. Fluorescent calcofluor white stain is also useful in detecting sparse amounts of fungi in deparaffinized tissue sections.

India ink

India ink preparations may be used for detecting encapsulated yeast *Cryptococcus neoformans* in cerebrospinal fluid (CSF). A drop of India ink is mixed with a drop of centrifuged deposit of CSF, and the preparation is examined under high power. With the negative stain, budding yeast surrounded by a large clear area against a dark background is presumptive evidence of *C. neoformans*. White blood cells and other artifacts may resemble encapsulated organisms; therefore, careful examination is necessary.

The morphologic characteristics of fungi seen on direct microscopic examination include budding yeasts, hyphae and pseudohyphae. *Rhizopus*, *Mucor* and *Lichtheimia* (*Absidia*) characteristically show broad, ribbon-like, non-septate hyphae. Dematiaceous fungi show darkly pigmented yeast-like and hyphal forms.

Tissue stains

The diagnosis of fungal diseases should preferably be established on the basis of histopathologic evidence combined with cultural evidence, because detection of fungi in tissue and confirmation of tissue invasion are required in diagnosing many opportunistic fungal infections. For example, isolation of common fungi such as *Aspergillus*, *Penicillium*, and *Rhizopus* species from sputum does not establish pulmonary infection by these fungi unless there is also histopathological evidence. Moreover, certain mycoses, e.g. rhinosporidiosis, lobomycosis, and *Pneumocystis jirovecii* infection can only be diagnosed by histologic studies since growth conditions of the fungi causing these infections have not been defined.

Histopathologic procedures are rapid and relatively inexpensive. Because of the size, characteristic morphology, and staining properties of many fungi, histopathologic studies often yield a presumptive diagnosis. There are several mycoses in which etiologic agents can be identified to the generic level because the morphologic characteristics are distinctive to the genus involving several species, e.g. aspergillosis and candidiasis.

Haematoxylin and eosin (H&E) stain used routinely in the pathology laboratory is often not adequate for detecting fungal elements. Many fungi stain poorly and some fungi do not stain at all with H&E. However, with this stain the tissue response can be demonstrated better than with any special stain and the innate colour of the fungal elements, whether dematiaceous or hyaline, can be determined.

No specific inflammatory reaction is characteristic of any particular mycotic agent. A single etiologic agent can elicit more than one type of tissue response and many different fungi can cause identical tissue responses. H&E procedure stains *Leishmania donovani*,

Toxoplasma gondii, and *Trypanosoma cruzi*, which can be confused with *Histoplasma capsulatum*. This problem can be avoided by using special stains for fungi.

Special stains used in the histologic section for detection of fungal elements are Gomori methenamine silver (GMS), Gridley fungus (GF), periodic acid-Schiff (PAS), Giemsa, Mayer's mucicarmine and alcian blue stains.

- **The GMS staining procedure** provides better contrast between the fungi and background tissue. This procedure results in the brownish black colouration of all forms of viable and non-viable fungal cells. Extracellular capsule and intracellular details are not visible by this method. The GMS stain is the best special fungal stain for screening, and H&E is the best for studying the tissue response to etiologic agents.
- **The GF stain** colours fungal cells purplish red with a yellow background. Mucin and elastic tissue are also stained purplish red. Non-viable fungi at the time of fixation may not be stained.
- **The PAS stain** is one of the most widely used stains for fungal histopathology. Aldehydes produced by the oxidation of fungal polysaccharide react with periodic acid and colour the fungi pinkish red. In old caseous foci of histoplasmosis, yeast cells may be stained by GMS but not by PAS.
- **Giemsa stain** is used primarily to detect *Histoplasma capsulatum* in blood or bone marrow.
- **Mayer's mucicarmine and alcian blue procedures** stain the mucopolysaccharide capsule of *Cryptococcus* spp. red and blue, respectively. These stains, therefore, are useful in differentiating cryptococci from the other fungi of similar size and appearance, although, these stains are not specific for cryptococci. *Rhinosporidium seeberi* and some cells of *Blastomyces dermatitidis* are also variably stained with mucicarmine. *R. seeberi*, however, produces sporangiospores (endospores) within the large sporangia and should not be mistaken for *B. dermatitidis*.
- **Acid-fast staining** is useful for detecting *Nocardia* spp. and for differentiating them from other aerobic actinomycetes. *Nocardia* spp. exhibit partial acid-fastness. Some of the filaments stain red with carbol-fuchsin staining, while others may appear blue because of a counterstain effect. *Nocardia asteroides* and *Streptomyces* spp. are used as positive and negative controls, respectively.

Applications and limitations of staining techniques for demonstrating fungi and related pathogens in tissue sections are given in Table 2.2.

Table 2.2: Applications and limitations of staining techniques for demonstrating fungi and related pathogens in tissue sections

Stain/Method	*Applications*	*Limitations*
Haematoxylin and eosin (H&E)	• Tissue response can be demonstrated better than with any other stain. • Innate colour of the fungal elements whether phaeoid (pigmented) or hyaline, can be determined. • Haematoxylin stains nuclei of most yeast-like cells. • Some fungi, e.g. the aspergilli and Mucorales are haematoxylinophilic and are readily delineated with H&E.	• Does not stain many fungi or stains poorly. Even in the instance of poor staining careful examination often reveals the outlines of unstained fungal elements, which suggest the existence of fungal infection. • Inadequate to screen for sparse fungal elements.
Gomori methenamine silver (GMS)	• Provides better contrast between the fungi and background tissue. • This procedure results in the brownish black colouration of all forms of viable and non-viable fungal cells.	• GMS may overstain fungi and obscure internal details. • Does not allow proper study of host response.

Contd.

Table 2.2: Applications and limitations of staining techniques for demonstrating fungi and related pathogens in tissue sections

Stain/Method	*Applications*	*Limitations*
Gridley's fungus (GF)	• Colours fungal cells purplish red with a yellow background. • Mucin and elastic tissue are also stained purplish red.	• Non-viable fungi at the time of fixation may not be stained. • Does not allow proper study of host response. • Fungi stained with GF stain tend to fade with prolonged storage.
Periodic acid-Schiff (PAS)	• Colours the fungi pinkish red.	• In old caseous foci of histoplasmosis, yeast cells may be stained by GMS but not by PAS.
Mayer's mucicarmine, and Alcian blue staining	• Stain mucopolysaccharide capsular material of *Cryptococcus neoformans*.	• Not specific for *Cryptococcus neoformans*. They may also stain *Rhinosporidium seeberi* and some cells of *Blastomyces dermatitidis*.
Gram stain (Brown and Brenn)	• Demonstrates Gram-positive filaments of actinomycetes, e.g. *Actinomyces*, *Nocardia*, *Actinomadura*, etc. • Demonstrates causative agents of botryomycosis. • Some fungi, especially the yeast forms of *Candida* spp. and the conidia of *Aspergillus* spp. are usually Gram-positive.	• Does not selectively stain most fungi (those that do stain are Gram-positive).
Modified Ziehl-Neelsen stain	• Useful to detect *Nocardia asteroides*, *N. brasiliensis* and *N. otitidiscaviarum*. • As the *Nocardia* spp. are weakly acid-fast and non-alcohol-fast in tissue sections, these filamentous bacteria can be distinguished from the agents of actinomycosis with modified Ziehl-Neelsen stain that uses a weak aqueous solution consisting of 0.5 or 1% sulphuric acid for decolourization. The nocardiae are weakly acid-fast, whereas the agents of actinomycosis are not acid-fast. The cytoplasm of certain fungi with yeast-like tissue forms, especially *Blastomyces dermatitidis* and *Histoplasma capsulatum* var. *capsulatum* is also variably acid-fast.	• Most fungal cells and the agents of actinomycosis are not acid-fast.
Calcofluor white	• Stains cell walls of most fungi including *Pneumocystis jirovecii*.	• Requires a fluorescence microscope. • May not stain degenerated fungi.
Fluorescent monoclonal antibody treatment	• Examination of respiratory specimen for *Pneumocystis jirovecii*.	• Sensitive and specific method for detecting the cysts of *Pneumocystis jirovecii*. Does not stain trophozoites or trophic forms.

Disadvantages of special stains

1. They mask the innate colour of fungal elements, making it impossible to determine whether a fungus is hyaline or phaeoid. Such a determination may be crucial to the diagnosis of a mycosis caused by pigmented fungi, e.g. phaeohyphomycosis, chromoblastomycosis, and black grain eumycotic mycetomas. Therefore, duplicate H&E stained or unstained tissue sections, should always be examined to look for brown pigmentation of fungal cell walls.
2. They do not allow adequate study of the host reaction to fungal invasion. To avoid this limitation, H&E can be used as the counterstain for the GMS procedure. This combination of stains (GMS-H&E) readily colours fungal elements brownish-black while staining background tissue components as expected. Thus, it is possible simultaneously to detect the fungus and to evaluate the host's inflammatory response and its relationship to the fungus. When only a single unstained section from a suspected lesion is available for examination, GMS-H&E is the best stain combination for attempting to make a diagnosis. Tissue sections stained with Giemsa, PAS, and GF procedures can be decoulorized in acid alcohol after the coverslip has been removed and then restained with GMS.

II. Culture

For some of the superficial mycoses such as pityriasis versicolor and, to a lesser degree, black piedra, white piedra and tinea nigra, the appearance of the fungal elements observed on direct examination is so characteristic that culture is not strictly necessary for the diagnosis of infection. On the other hand, for otomycosis, culture is essential, as the range of organisms that cause infection is enormous, and the specific identification of the pathogen will have profound effect on the therapy selected. In case of dermatophyte infections, on direct examination of skin and nail scrapings different species are indistinguishable. Generally, all the dermatophyte species are believed to respond similarly to the major systemic and topical antifungals available, and treatment is initiated on the basis of direct examination. However, this may not be true with some of the more recent antifungals developed.

Optimal recovery of medically important fungi from clinical specimens is related to multiple factors. The first is the specimen itself, which must be freshly collected and appropriate for the mycotic infection being considered. In general, there are fewer fungal cells at the site of an infection than there are bacterial cells in a bacterial infection. Therefore, enough specimen must be cultured to ensure optimal recovery. **Fluids**, e.g. peritoneal fluid, pericardial fluid and CSF in volumes of >1–2 ml should be concentrated by centrifugation and the sediment should be inoculated on the culture media. Alternatively, the specimen may be filtered through a 0.45 μm pore size membrane filter, and the filter can then be cultured. Most fungi will not survive the 2% NaOH treatment used for recovery of mycobacteria.

Most pathogenic fungi are easy to grow in culture. **Sabouraud dextrose agar** (SDA) is most commonly used. This may be supplemented with chloramphenicol (50 mg/l) to minimize bacterial contamination and cycloheximide (500 mg/l) to reduce contamination with saprophytic fungi. Cycloheximide should not be added to all media because the growth of *Cryptococcus* spp., *Candida* spp., *Trichosporon* spp., *Aspergillus* spp., Mucorales, hyalohyphomycetes, yeast phase of *Histoplasma capsulatum*, *Blastomyces dermatitidis* and *Paracoccidioides brasiliensis* are completely or partially inhibited by it. Chloramphenicol is likely to prevent the growth of aerobic actinomycetes; therefore, if *Nocardia* or any other filamentous bacteria are suspected, it is necessary to inoculate media lacking it. Special (differential) media may be used for isolation and to help rapid identification when the identity of a particular fungus is

strongly suspected. For example, *C. neoformans* develops black colonies on **bird seed agar**.

Specimens for the isolation of fungi are inoculated on slants or agar plates. For isolating discrete colonies, culture plates are more useful than slants, because the plates have a larger surface area. However, the plates are more susceptible to laboratory contamination and must be taped with an oxygen-permeable tape. When slants are used, the culture tube should be large enough (2 × 15 cm) to provide a wide surface area for growth. The screw caps should be left loosened during incubation to permit an adequate supply of oxygen.

Many fungal pathogens have an optimum growth temperature below 37°C. For molds, the temperature of incubation should be 25–30°C, and for *Candida* spp., the temperature of incubation should be 37°C. With some dimorphic pathogens, enriched media such as **brain heart infusion agar** or **blood agar** are used to promote growth of yeast phase. Many fungi grow relatively slowly and cultures should be retained for at least 2–4 weeks before being discarded.

Growth of *Candida*, *Aspergillus*, *Mucor* and *Rhizopus* species appears within 24–72 hours. Therefore, fungal cultures should be examined for growth daily for the 1st week, three times for the 2nd week, twice for the 3rd week, and once for the 4th week rather than being evaluated once at the end of 4 weeks. Plates must be opened inside a certified biological cabinet to prevent contamination of plate and exposure of personnel to potentially dangerous fungi. Tubed media have a smaller surface area but offer maximum safety and resistance to dehydration and contamination.

It is important to use media with and without inhibitory agents. Specimens from normally sterile sites can be inoculated on media without inhibitory substances. Selective media should be tested with strains known to be sensitive and resistant to the inhibitory agent in the media, while the differential media should be evaluated with fungi that produce both positive and negative reactions.

Once an organism has grown, it is examined for characteristic gross and microscopic structures, so that identification can be made. Pigment on the reverse side of the colony or in aerial mycelium is noted. For microscopic examination **slide mounts** should be made in **lactophenol cotton blue** (LPCB). On occasion, a **slide culture** may be prepared (*see* Appendix C) when the initial isolate fails to show conidial morphology. Many molds begin as white mycelial growths, colouration occurs at the time of conidiation or sporulation. Characteristics that should be observed are septate versus non-septate hyphae, hyaline or dematiaceous (phaeoid) hyphae, and the types, size, shape, colour and arrangement of conidia. Identification of medically important fungi is given in Table 2.3.

III. Serological tests

Positive fungal cultures can be difficult to obtain—the yield being low, growth often slow, and taking biopsy from a deep infected site may be difficult or impossible. Therefore, serological tests have been developed for the diagnosis of fungal infections. Tests for antibody have an established diagnostic use in coccidioidomycosis, paracoccidioidomycosis, aspergillosis, blastomycosis, and in some patients with histoplasmosis.

Serological tests have been developed commercially for detecting cryptococcal and *Candida* antigens. Detection of polysaccharide cryptococcal antigen in serum and cerebrospinal fluid by latex agglutination is a method of choice for the rapid diagnosis of meningitis and disseminated infection caused by *C. neoformans*. The most widely used is the assay for the detection of *Histoplasma* antigen. This test has proven useful for the detection of antigenemia in patients having disseminated disease, particularly those with acquired immunodeficiency syndrome.

Serological tests for the detection of fungal antibodies and fungal antigens are given in Table 2.4.

Table 2.3: Identification of medically important fungi

Hyaline		*Mold colonies*		*Dematiaceous (phaeoid)*		*Yeast colonies*	*Yeast-like colonies*
Growth in < 3 days	***Growth in 3–5 days***	***Growth in 3–5 days***	***Growth in > 5 days***	***Growth in 3–5 days***	***Growth in > 5 days***	***Growth in 2–5 days***	***Growth in 2–5 days***
Hyphae broad and non-septate **Mucorales** • *Mucor* • *Rhizopus* • *Lichtheimia (Absidia)* • *Syncephalastrum* • *Cunninghamela*	Hyphae hyaline and septate **Agents of hyalohyphomycosis** ***Conidia in chains:*** • *Aspergillus* • *Penicillium* • *Paecilomyces* • *Scopulariopsis* ***Conidia in clusters:*** • *Acremonium* • *Fusarium* • *Trichoderma* • *Gliocladium* ***Conidia borne singly:*** • *Scedosporium apiospermum* • *Scedosporium prolificans* • *Chryosporium* • *Sepedonium*	Colonies often granular and pigmented; hyphae hyaline and septate **Dermatophyte** • *Trichophyton* spp. • *Microsporum* spp. • *Epidermophyton floccosum*	Hyphae hyaline and slender Growth on cycloheximide agar Yeast forms when incubated at 37°C **Dimorphic fungi:** • *Blastomyces dermatitidis* • *Coccidioides immitis* • *Histoplasma capsulatum* • *Sporothrix schenckii* • *Paracoccidioides brasiliensis*	Dark colony; black reverse; hyphae yellow-pigmented and septate **Agents of phaeohyphomycosis** ***Conidia have both transverse and longitudinal septa:*** • *Alternaria* spp. ***Conidia divided by transverse septa only:*** • *Curvularia* spp. • *Bipolaris* spp. • *Exserohilum* spp.	Dark colony; black reverse; hyphae yellow-pigmented and septate **Agents of chromoblastomycosis** ***Cladosporium-type sporulation:*** • *Cladophialophora carrionii* • *Cladophialophora bantiana* ***Phialophora-type sporulation:*** • *Philalophora verrucosa* ***Acrotheca-type sporulation:*** • *Fonsecaeca pedrosoi* • *Fonsecaeca compacta* **Agent of mycetoma** ***Phialophora-type sporulation:*** • *Exophiala jeensemei*	Smooth, pasty or mucoid colonies **Yeast and yeast-like** • *Candida albicans* • *Cryptococcus neoformans* • *Rhodotorula* • *Malassezia furfur*	Yeast-like colonies with low aerial mycelium ***Arthroconidia produced:*** • *Geotrichum candidum* • *Trichosporon* spp.

Table 2.4. Serological tests for detection of fungal antibodies and fungal antigens	
Tests for detection of fungal antibodies	***Tests for detection of fungal antigens***
• Immunodiffusion	• Latex agglutination
• Counterimmunoelectrophoresis	• ELISA
• Whole cell agglutination	
• Complement fixation	
• Enzyme-linked immunosorbent assay (ELISA)	

Molecular testing

- ***In situ* hybridization:** One of the simplest approaches used has been ***in situ*** hybridization using specific nucleic acid probes for identification of organisms in patient specimens.
- **Polymerase chain reaction:** Amplification assays using the polymerase chain reaction allow for the detection of small amounts of target DNA in clinical specimens. Specific primers with or without specific probes have been used with some success. Assays have been developed to detect DNA of *Candida*, *Aspergillus*, *Fusarium*, *Cryptococcus*, *Histoplasma*, *Blastomyces*, *Paracoccidioides*, *Pneumocystis jirovecii* and *Talaromyces (Penicillium) marneffei*.

MICROBIOLOGICAL SAFETY CABINETS

Microbiological safety cabinets provide a barrier between the worker and the infective material and are designed to prevent infection by splashing or aerosol.

Class 1

These cabinets are open-fronted. These rely on the walls, glass upper front and integral tray to contain spills and splashes. An inward airflow provides a protection factor of at least 1.5×10^5; this factor represents the number of particles which, if liberated into the air of the cabinet, will not escape into the room. The air-borne particles are contained within the cabinet and filtered from the exhaust air through a HEPA (high efficiency particulate air) filter.

Class 2

These cabinets are also open-fronted, but are designed to prevent air-borne contamination of the work materials and reduce exposure of the operator to particles dispersed within the cabinet. These objectives are achieved by recirculating filtered air over the work area while maintaining an inflow of air through the working aperture. Some of the air is exhausted through a HEPA filter.

Class 3

These cabinets are totally enclosed and separate the operator from the work by an airtight barrier. These are scavenged by air entering and leaving through HEPA filters, the air pressure in the cabinet being kept less than that in the room.

Biosafety level 2 (BSL 2) practices are recommended for handling and processing clinical specimens, identifying isolates, and processing animal tissues suspected of containing pathogenic fungi. BSL 2 is also sufficient for mold cultures of *Blastomyces dermatitidis*, *Cryptococcus neoformans*, *Talaromyces marneffei*, *Sporothrix schenckii*, *Bipolaris* spp., *Cladophialophora bantiana*, *Wangiella dermatitidis*, *Exserohilum* spp., *Fonsecaea pedrosoi*, *Ochroconis gallopava*, and *Scedosporium prolificans*.

BSL 3 conditions should be observed when working with mold-form cultures identified as *Coccidioides* spp. and *Histoplasma capsulatum*.

Important Questions

1. Discuss in detail laboratory diagnosis of mycoses.
2. Discuss briefly staining techniques used for identification of fungi in tissue sections. Give merits and demerits of each.
3. Discuss applications and limitations of staining techniques for demonstrating fungi and related pathogens in tissue sections.

Multiple Choice Questions

1. Which of the following fungi is potentially dangerous to the laboratory workers?
(a) *Candida albicans*.
(b) *Cryptococcus neoformans*.
(c) *Coccidioides immitis*.
(d) *Trichophyton rubrum*.

2. India ink preparation may be used for detecting
(a) *Cryptococcus neoformans*.
(b) *Candida albicans*.
(c) *Trichosporon*.
(d) *Geotrichum*.

3. For the culture of molds the temperature of incubation should be
(a) 20°–22°C.
(b) 25°–30°C.
(c) 30°–32°C.
(d) 35°–37°C.

4. Which of the following statements is incorrect for haematoxylin and eosin staining for the diagnosis of fungal infections?
(a) Tissue response can be demonstrated better than with any other stain.
(b) Does not stain many fungi or stains poorly.
(c) Innate colour of the fungal elements whether phaeoid or not can be determined.
(d) Adequate to screen for sparse fungal elements.

5. How will you process CSF specimen for the diagnosis of fungal infection?
(a) Centrifuge and place a drop of sediment in India ink preparation and examine under microscope.
(b) Centrifuge and inoculate sediment on Sabouraud dextrose agar.
(c) Inoculate a drop of sediment on bird seed agar.
(d) All the above.

6. Venereal transmission may occur in:
(a) Blastomycosis.
(b) Histoplasmosis.
(c) Both above.
(d) None of the above.

7. Which biosafety level (BSL) precautions should be observed when working with mold-form culture of *Coccidioides immitis*?
(a) BSL-4
(b) BSL-3
(c) BSL-2
(d) BSL-1

8. Which biosafety level (BSL) precautions should be observed when working with mold-form culture of *Blastomyces dermatitidis*?
(a) BSL-4
(b) BSL-3
(c) BSL-2
(d) BSL-1

9. Which biosafety level (BSL) precautions should be observed when working with culture of *Candida albicans*?
(a) BSL-4
(b) BSL-3
(c) BSL-2
(d) BSL-1

Answers to MCQs

1. (c), 2. (a), 3. (b), 4. (d), 5. (d). 6. (c), 7. (b), 8. (c), 9. (d).

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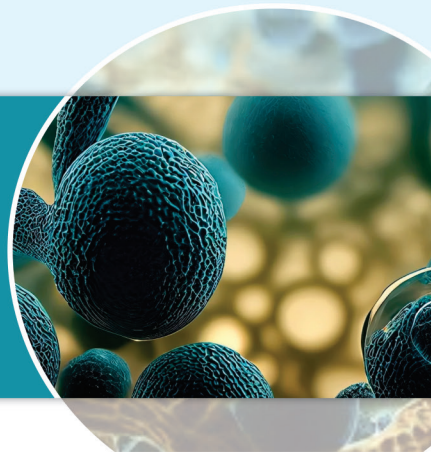
CHAPTER

3

Antifungal Drugs*



Fungal infections (mycoses) are widespread and there has been a steady increase in the incidence of serious secondary systemic fungal infections since 1970s. The major contributory factors which predispose patients to invasive fungal infections are the use of broad-spectrum antibiotics, corticosteroids, anticancer/immunosuppressive drugs, denture, indwelling catheters and implants, and emergence of AIDS. As a result of breakdown of host defence mechanisms, saprophytic fungi easily invade the living tissue. Elderly people, diabetics, burn wound victims and pregnant ladies are particularly at risk of developing fungal infections such as candidiasis.

Fungi are also important nosocomial pathogens causing severe morbidity and mortality in hospitalized patients with a combination of a variety of risk factors and immunosuppression. Systemic infections caused by *Candida* spp. other than *C. albicans*, *Aspergillus* spp., and other filamentous fungi (molds) are being reported more frequently. As fungal infections became an important public health problem and resistance to established antifungal agents began to emerge, pharmaceutical companies developed new agents with either a broader spectrum or different targets of activity.

Unlike the development of antibacterial agents, to date relatively few drug targets in fungi have been exploited in the development of currently available antifungal agents. Antibacterial agents have taken advantage of multiple targets available in bacteria that are not present in mammalian cells. Fungi have similarities to mammalian cells that have made the search for antifungal targets difficult. Nevertheless, hosts and fungal cells do have some significant differences, and effective therapeutic agents have been discovered or developed to exploit them.

To date three targets—plasma membrane sterols, nucleic acid synthesis and cell wall constituents, have been exploited with varying degree of success. There is poor penetration of drugs in tissues because fungi infect relatively poorly vascularized areas. Furthermore, slow growth of fungi and granulomatous response of host tissue also decrease drug penetration into the target sites. In addition, poor absorption from gastrointestinal tract demands the use of parenteral route entailing an increased toxicity of these drugs.

The number of agents available to treat fungal infections has increased by 30% since 2000. Despite the development of many new

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antifungal agents, the mortality of invasive fungal infections (IFIs) has not come down significantly. This is largely due to delay in diagnosis of IFIs. The diagnosis of fungal infection still relies on conventional techniques including microscopy and culture. The conventional techniques have poor sensitivity and growth of fungi takes long time. Differences in antifungal spectrum of activity, bioavailability, formulation, drug interactions and side effects necessitate a detailed knowledge of each drug class.

Classification of antifungal drugs

Fungal infections have been divided into two distinct classes, systemic and superficial. Therefore, the majority of the antifungal agents are discussed under two main headings – systemic and topical, although this division is becoming arbitrary as many antifungal agents may be used both systemically and topically, and many superficial infections can be treated either systemically or topically (Table 3.1).

Table 3.1: Systemic and topical antifungal drugs

A. Systemic Antifungal Drugs
1. **Antibiotics**
• *Polyene:* Amphotericin B* • *Heterocylic benzofurans:* Griseofulvin
2. **Azoles**
• *Imidazoles:* Clotrimazole*, Ketoconazole • *Triazole:* Fluconazole, Itraconazole, Voriconazole, Posaconazole
3. **Echinocandins:** Capsofungin, Anidulafungin, Micafungin
4. **Allylamines:** Terbinafine*
5. **Antimetabolite:** Flucytosine
B. Topical Antifungal Drugs
1. **Polyene antibiotics:** Nystatin, Hamycin, Natamycin
2. **Azoles:** Clotrimazole, Miconazole, Econazole, Oxiconazole
3. **Allylamines:** Butenafine, Naftifine, Amorolfine
4. **Other topical agents:** Tolnaftate, Undecylenic acid, Ciclopirox olamine, Benzoic acid, Salicylic acid and Sodium thiosulphate

* Used topically as well

SYSTEMIC ANTIFUNGAL DRUGS

Antibiotics

Amphotericin B

It is an amphoteric polyene macrolide which remains the most effective antifungal agent for severe systemic mycoses. It is a fungicidal antibiotic without antibacterial activity. It is derived from culture of aerobic actinomycete, *Streptomyces nodosus*. Discovered by Gold in 1956, it truly represents a gold standard in the treatment of fungal infections.

Mechanism of action

Ergosterol, a key component of the fungal cell membrane, is critical to the integrity of the membrane and functions by regulating membrane fluidity and symmetry. It is not present in mammalian cells and thus it is an ideal target for antifungal activity.

Amphotericin B binds to ergosterol in the cell membrane of fungi and thus increases its permeability and induces cell lysis. It has probably more than one mechanism of action but its most important property is probably its ability to form large pores in the cell membrane. This causes gross disturbances in ion balance, including loss of intracellular K^+.

In addition to direct antifungal activity, amphotericin B stimulates release of cytokines such as tumour necrosis factor and interleukin-1 from mammalian phagocytic cells and also stimulates release of macrophage superoxide ion, all of which augment antifungal activity. Cholesterol, present in host cell membranes, closely resembles ergosterol. The polyenes bind to it as well, though with lesser affinity. Thus, the selectivity of action of polyenes is low, and amphotericin B is one of the most toxic systemically used antibiotics.

Resistance may develop from changes in ergosterol structure and decreased amounts of ergosterol in the fungal cell membrane which makes it less susceptible to the drug.

Antifungal spectrum

It has broad-spectrum of activity against *Candida* spp., *Cryptococcus neoformans*,

Coccidioides immitis, *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Paracoccidioides brasiliensis*, *Sporothrix schenckii*, *Aspergillus* spp., *Talaromyces (Penicillium) marneffei*, and the Mucorales. It has limited activity against the protozoa *Leishmania brasiliensis* and *Naegleria fowleri*. It has no antibacterial activity.

Pharmacokinetics

It is not absorbed from gastrointestinal tract which makes intravenous administration necessary. It can be given orally for intestinal amoebiasis. Administered intravenous as a suspension made with the help of deoxycholate, it gets widely distributed in the body, but penetration in CSF is poor. Therefore, only intrathecal administration can be effective for treatment of fungal meningitis.

It binds to sterols in tissues and to lipoproteins in plasma and stays in the body for long periods. The terminal elimination $t_{½}$ is 15 days. About 60% of amphotericin B is metabolized in the liver. Excretion occurs slowly both in urine and bile, but urinary concentration of active drug is low.

Uses

1. Amphotericin B is the most effective drug for various types of systemic mycoses, viz disseminated candidiasis, cryptococcal meningitis, coccidioidomycosis, histoplasmosis, blastomycosis and extracutaneous sporotrichosis.
2. It can be applied locally for oral, vaginal and cutaneous candidiasis and otomycosis.
3. It is the most effective drug for resistant cases of kala-azar and mucocutaneous leishmaniasis.

Adverse effects

Amphotericin B is highly toxic.

a. **Acute reaction:** This consists of chills, fever, aches and pain all over the body, nausea, vomiting and dyspnoea lasting for 2–5 hours.

b. **Long-term toxicity:** Nephrotoxicity is the most important complication which can manifest as hypokalemia and renal tubular acidosis. It can be minimized by adequate hydration. The lipid based amphotericin B formulations have shown lower incidence of nephrotoxicity. Most patients develop slowly progressive anaemia which is due to bone marrow depression. It is largely irreversible. CNS toxicity occurs only on intrathecal injection leading to headache, vomiting, nerve palsies, etc.

Drug interactions

1. Amphotericin B has synergistic effect with flucytosine in the treatment of systemic candidiasis and cryptococcosis.
2. It should not be co-administered with other drugs with nephrotoxic potential.

Griseofulvin

It is a narrow spectrum antifungal agent isolated from cultures of *Penicillium griseofulvum*. It is a fungistatic drug. It acts by interacting with fungal microtubules and interfering with mitosis. It can be used to treat dermatophyte infection of skin or nails when local treatment is ineffective but treatment needs to be very prolonged.

Pharmacokinetics

The absorption of griseofulvin from gastrointestinal tract is erratic. Fatty meals and microfining the drug particles can improve its oral absorption. It is metabolized in the liver with plasma $t_{½}$ of 24 hours. Griseofulvin gets deposited in keratin forming cells of skin, hair and nails. It is especially concentrated and retained in tinea infected cells. Because it is fungistatic and not fungicidal, the newly formed keratin is not invaded by fungus, but the fungus persists in already infected keratin, till it is shed off. Therefore, the duration of treatment is dependent upon the site of infection, thickness of infected keratin and its turnover rate. It has largely been superseded by other drugs.

Adverse effects

Toxicity of griseofulvin is low. It may cause allergic reactions, headache and gastrointestinal disturbances.

Azoles

The azoles were introduced in 1980s and have become the most widely used antifungal agents presently. The azole antifungals include two broad classes, imidazoles and triazoles, according to the number of nitrogen atoms in the azole ring. Both the classes have same antifungal spectrum and mechanism of action. Clotrimazole, econazole, miconazole and oxiconazole from the imidazoles are used topically, while ketoconazole is used both orally and topically. Triazoles especially fluconazole and itraconazole have largely replaced ketoconazole for systemic mycoses because of better efficacy, longer $t_{½}$, fewer side effects and drug interactions.

Spectrum of activity

The azoles are a group of fungistatic agents which have broad-spectrum antifungal activity against common fungal pathogens, e.g. *Candida* spp., *Cryptococcus neoformans*, *Blastomyces dermatitidis*, *Histoplasma capsulatum*, *Coccidioides immitis*, *Paracoccidioides brasiliensis*, *Sporothrix schenckii*, *Aspergillus* spp. and dermatophytes. *Candida krusei* and agents of mucormycosis are resistant. These drugs also have antiprotozoal effect against *Leishmania major*.

Mechanism of action

These cause blockades of fungal cytochrome P450 mediated synthesis of ergosterol from lanosterol, thus inhibiting fungal growth. These inhibit the fungal cytochrome P450-3A enzyme, lanosine 14-β-demethylase, which is responsible for converting lanosterol to ergosterol, the main sterol in the fungal cell membrane. The fluidity of the membrane is altered due to ergosterol depletion which interferes with the action of membrane associated enzymes. The net effect is an inhibition of replication.

There is, however, cross reactivity with human cytochrome P450 enzymes which explains their potential for inhibition of steroid synthesis in humans and for various interactions with other hepatically metabolised drugs. Development of fungal resistance to azoles has been noted among *Candida* spp. infecting advanced AIDS patients.

Ketoconazole

It was the first successful orally used azole antifungal agent in 1980. It is available in both oral and topical formulations. It is an effective broad-spectrum antifungal drug, useful in both dermatophytosis and deep mycoses. However, for systemic mycoses, ketoconazole has been superseded by fluconazole and itraconazole due to their improved pharmacokinetics, tolerability and efficacy. Topical formulations of ketoconazole are useful for treating seborrheic dermatitis. It is widely distributed in tissues but concentration in CSF and urine is low. It is not effective in fungal meningitis. The most common side effects are nausea, vomiting, anorexia, headache, paresthesia, hair loss, rashes, gynaecomastia, oligospermia and menstrual irregularities. It has a greater potential for drug interactions which is one of its main limitations.

Fluconazole

Fluconazole possesses the most desirable pharmacological properties including high bioavailability, low degree of protein binding, wide distribution into body tissues and long half-life. It displays a high degree of water-solubility and good CSF penetration. Oral bioavailability is not affected by food or gastric pH. It can be administered orally as well as intravenously. It is indicated for the treatment of cryptococcal meningitis, systemic and mucocutaneous candidiasis in both normal and immunocompromised patients, coccidioidal meningitis and histoplasmosis.

Generally, recommended dosages of fluconazole are 50–400 mg once daily for either oral or intravenous administration. It is ineffective in aspergillosis and mucormycosis. Side effects are nausea, vomiting, abdominal pain, rash and headache.

Itraconazole

It has a broader spectrum of activity than ketoconazole and fluconazole. It is fungistatic and is effective in immunocompromised patients. Oral absorption is enhanced by food and gastric acid. It gains extensive distribution in all tissues, but penetration into CSF is poor. It is used for the treatment of histoplasmosis, blastomycosis, sporotrichosis, paracoccidioidomycosis and chromoblastomycosis. It affords some relief in aspergillosis.

Voriconazole

It has potent broad-spectrum activity against various fungi. It is used for difficult to treat fungal infections like invasive aspergillosis, disseminated infections caused by fluconazole-resistant *Candida* and *Fusarium* infections. It has become the azole of choice in aspergillosis. It is available for oral and intravenous administration. Pharmacokinetics, indications and adverse effects of commonly used azoles–ketoconazole, fluconazole, itraconazole and voriconazole are given in Table 3.2.

Posaconazole

Posaconazole is a novel lipophilic antifungal triazole with broad range of activity against *Candida* spp., *Aspergillus* spp. and Mucorales. After oral administration, it is absorbed within three to five hours. It is predominantly eliminated through the liver, and has a half life of about 35 hours. Oral administration of posaconazole taken with a high-fat meal exceeds 90% bioavailability. Posaconazole has been approved by USFDA and other regulatory authorities for the treatment of oropharyngeal candidiasis and for the prophylaxis of invasive aspergillosis and *Candida* infections in severely immunocompromised patients. It is generally well tolerated.

Table 3.2: Pharmacokinetics, indications and adverse effects of ketoconazole, fluconazole, itraconazole and voriconazole

Drug	*Pharmacokinetics*	*Indications*	*Adverse effects*
Ketoconazole	• Oral bioavailability 75% • Protein binding 99% • CSF penetration < 10% • Elimination $t_{½}$ 7–10 hours	• Replaced by fluconazole and itraconazole for most fungal infections	• Hepatotoxicity, gastrointestinal disturbances, pruritis, gynaecomastia and adverse drug interactions
Fluconazole	• Oral bioavailability > 80% • Protein binding 11% • Excellent CSF penetration > 70% • Elimination $t_{½}$ 22–35 hours	• Fungal meningitis (cryptococcosis, coccidioidomycosis) • Candidiasis (deep and superficial) • Prophylaxis in immunocompromised host	• Nausea, headache, pain abdomen, exfoliative skin lesions (in AIDS patients), hepatitis (rare), and drug interactions seen only at higher doses > 400 mg
Itraconazole	• Oral bioavailability > 70% • Protein binding > 99% • Poor CNS penetration < 1% • Elimination $t_{½}$ 24–42 hours	• Non-meningeal infections (e.g. histoplasmosis) • Reserved for oropharyngeal candidiasis in case of unresponsiveness to fluconazole	• GIT disturbances, headache, dizziness, allergic skin reactions and drug interactions
Voriconazole	• Oral bioavailability 96% • Protein binding 56% • Elimination $t_{½}$ 6 hours	• Invasive aspergillosis • Initial treatment of candidiasis	• Transient abnormal vision, skin rash and hepatotoxicity (rare)

ECHINOCANDINS

The echinocandins comprise a ring of six amino acids linked to a lipophilic side chain.

Mechanism of action

All echinocandins have the same mechanism of action but differing pharmacologic properties. They act by inhibiting the synthesis of 1,3-β-glucan, a glucose polymer, a vital component of cell wall that is necessary for maintaining the structure of cell walls of fungi. Fungal cells lose integrity in the absence of this polymer and lysis quickly follows.

Antifungal spectrum

They are fungicidal against some yeasts (most species of *Candida*, but not against *Cryptococcus*, *Trichosporon* and *Rhodotorula*), fungistatic against some molds (*Aspergillus*, but not *Fusarium* and *Rhizopus*), and modestly or minimally active against dimorphic fungi (*Blastomyces* and *Histoplasma*). *In vitro* resistance can be conferred in *C. albicans* by mutation in one of the genes that encodes 1,3-β-glucan synthase. Azole-resistant isolates of *C. albicans* remain susceptible to echinocandins.

The first approved echinocandin was caspofungin, and later micafungin and anidulafungin were approved. All these preparations so far have low oral bioavailability, so must be given intravenously only. Echinocandins have now become one of the first line of treatment for *Candida*, and even as antifungal prophylaxis in haematopoietic stem cell transplant patients.

Adverse effects

These include fever, headache, nausea, vomiting, diarrhoea, abdominal pain, itching, and pain and redness around injection site. Altered liver enzyme levels are also reported. Compared to amphotericin B, caspofungin seems to have a relatively low incidence of side effects.

ALLYLAMINES

The allylamines are a group of synthetic antifungal compounds effective in the topical and oral treatment of dermatophytoses. The various drugs included in this group are terbinafine, butenafine, naftifine and amorolfine.

Terbinafine

It is the most commonly used antifungal drug from the allylamine group.

Mechanism of action

It acts by preventing ergosterol synthesis of fungal cell by inhibiting the key fungal enzyme squalene epoxidase with resultant ergosterol deficiency and squalene accumulation which results in cell death. Ergosterol is the principal sterol in the membrane of susceptible fungal cells. It is orally and topically active drug against dermatophytes and *Candida*. It is less effective against cutaneous and mucosal candidiasis. In contrast to azoles which are primarily fungistatic, terbinafine is fungicidal. Shorter courses of therapy are required and relapse rates are low. It is available as oral and cream formulation. It is applied topically as 1% cream or administered orally 250 mg once a day. It does not seem to affect cytochrome P450 system and has demonstrated no significant drug interaction.

Adverse effects

Side effects of oral terbinafine are gastric upset, rashes and taste disturbance. Some cases of hepatic dysfunction, haematological disorder and severe cutaneous reactions are reported. Topical terbinafine can cause erythema, itching, dryness, irritation, urticaria and rashes.

Butenafine, naftifine and amorolfine are discussed under topical antifungal agents.

ANTIMETABOLITE

Flucytosine

Flucytosine is a fluorinated pyrimidine analogue that is used orally to treat severe fungal

infections. To exert its effect, flucytosine is taken up in susceptible fungi by the transport enzyme cytosine permease. Once inside the fungal cell, flucytosine rapidly undergoes intracellular conversion to 5-fluorouracil via cytosine deaminase, and subsequently converted to 5-fluorouridine triphosphate, which is incorporated into fungal RNA and interferes with protein synthesis. 5-Fluorouracil intermediate also inhibits thymidylate synthetase, and interferes with DNA synthesis. Mammalian cells do not convert flucytosine to fluorouracil which is responsible for the fungal selectivity of this drug.

Antifungal spectrum

It has narrow spectrum of activity. It is active against *Cryptococcus neoformans*, *Candida* spp., and chromoblastomycosis. It is fungistatic in action.

Therapeutic uses

It is primarily used in the treatment of cryptococcal meningitis and serious systemic candidiasis in combination with amphotericin B. Drug resistance develops rapidly due to altered drug permeability. For this reason amphotericin B and flucytosine are given in combination due to their synergistic effects.

Pharmacokinetic

It is absorbed rapidly and well from gastrointestinal tract. It is actively secreted and concentrated into the urine with an elimination $t_{½}$ of 2.5–6 hours. It has wide distribution including CSF.

Adverse effects

Flucytosine may depress the bone marrow and lead to leucopenia and thrombocytopenia. Patients are more prone to this complication if they have underlying haematological disorders. Other untoward effects include rash, nausea, vomiting and severe enterocolitis. Toxicity is more severe in patients with AIDS and when plasma concentration exceeds 100 μg/ml.

TOPICAL ANTIFUNGAL AGENTS

Topical treatment is useful in many superficial fungal infections, i.e. those confined to the stratum corneum, squamous mucosa, or cornea. Such diseases include dermatophytosis, candidiasis, pityriasis versicolor, piedra, tinea nigra and fungal keratitis. A plethora of topical agents is available for the treatment of superficial mycoses. The systemic agents used for the treatment of superficial mycoses have been discussed above.

POLYENE ANTIBIOTICS

Nystatin

Nystatin is a polyene macrolide antibiotic, obtained from *Streptomyces noursei*. It is similar to amphotericin B in antifungal action and other properties. However, it is too toxic for parenteral administration and is used only locally in superficial candidiasis including vaginal candidiasis and oral thrush. Given orally, it is not absorbed. It can be used for diarrhoea caused by *Candida* spp. Nausea and bad taste in mouth is the only side effect. It is effective (but less than azoles) in vaginal candidiasis and oral thrush. It is also used for corneal, conjunctival and cutaneous candidiasis in the form of an ointment. It is ineffective in dermatophytosis.

Hamycin

Hamycin is isolated from *Streptomyces pimprina*. It is similar to nystatin, but more water soluble. Its use is restricted to topical application for oral thrush, cutaneous candidiasis, otomycosis by *Aspergillus* spp., vaginal candidiasis and *Trichomonas* vaginitis.

Natamycin

Natamycin is isolated from *Streptomyces natalensis*. It is similar to nystatin. It has a broader spectrum of action, and is used only topically. A 1% ointment or 5% suspension have been used particularly in *Fusarium solani* keratitis. Vaginal keratitis and *Trichomonas* vaginitis are also amenable to natamycin.

TOPICAL AZOLES

A number of topical azoles is available for the treatment of superficial mycoses such as clotrimazole, econazole, miconazole, oxiconazole, etc.

Clotrimazole

It is the most commonly used azole for the treatment of superficial mycoses. It acts by interfering with amino acid transport into the fungus by an action on cell membrane. It is effective in the topical treatment of dermatophytosis, otomycosis and oral/cutaneous/vaginal candidiasis. The standard regimens for vaginal candidiasis are one 100 mg vaginal tablet once a day inserted at bedtime for 7 days or one 200 mg tablet once a day for 3 days. For oropharyngeal candidiasis, 10 mg troche of clotrimazole is allowed to dissolve in the mouth 3–4 times a day, or can be applied as lotion/gel. Local irritation occurs in some patients. However, no systemic toxicity is seen after topical use.

Econazole

It is similar in activity to clotrimazole. It penetrates superficial layers of the skin and is highly effective in dermatophytosis, otomycosis and oral thrush. It is somewhat inferior to clotrimazole for the treatment of vaginitis. With the exception of local irritation in a few cases, no adverse effects have been reported.

Miconazole

This imidazole derivative has a broad-spectrum of activity. Topical formulations are highly effective for the treatment of tinea, pityriasis versicolor, otomycosis, and cutaneous and vulvovaginal candidiasis. No systemic adverse effects are seen, however, it may lead to vaginal irritation and even pelvic cramps.

Oxiconazole

It is a new topical imidazole, effective in tinea and other dermatophytic infections, as well as vaginal candidiasis. In some cases local irritation may occur.

TOPICAL ALLYLAMINES

Butenafine

Butenafine is a benzylamine derivative of terbinafine with a mechanism of action similar to that of terbinafine. Efficacy in tinea cruris, tinea corporis and tinea pedis is similar to that of topical terbinafine. It displays superior activity against *C. albicans* than terbinafine. Butenafine achieves high concentrations in skin and remains in skin tissue for prolonged periods. It is mostly distributed in epidermis. A small amount is detectable also in dermis, probably due to transport via sebaceous glands and hair follicles. It exerts antiinflammatory as well as antifungal activity. This property is particularly beneficial in dermatophytic infections that are accompanied by a marked inflammatory reaction in the infected tissue.

Adverse reactions

Topical butenafine is well tolerated and adverse reactions are rare. Mild burning sensation at the application site has been observed in some patients.

Naftifine

Naftifine is a topically active allylamine antifungal drug. It is used as 1% cream for the topical treatment of tinea pedis, tinea cruris, and tinea corporis. Twice daily application is recommended. The drug is well tolerated although some patients may develop local irritation.

Amorolfine

Amorolfine is a morpholine derivative which is used topically as an antifungal agent. It acts by inhibiting two separate enzymes, reductase and isomerase, in the pathway of ergosterol synthesis resulting in depletion of ergosterol in the fungal cytoplasmic membrane. It has a broad spectrum of activity, including dermatophytes, various filamentous and

dematiaceous fungi, yeasts and dimorphic fungi. Its activity is fungicidal for most species. Amorolfine 5% nail lacquer is applied once or twice weekly for up to 6 months for the treatment of onychomycosis.

MISCELLANEOUS TOPICAL AGENTS

Tolnaftate

Tolnaftate is a thiocarbamate. It is effective in the treatment of cutaneous mycoses caused by dermatophytes and *Malassezia furfur*, but it is ineffective against *Candida*. It is available in a 1% concentration as a cream, gel, powder and topical solution. The preparations are applied locally twice a day. Symptomatic relief occurs early, but if applications are discontinued before the fungus bearing tissue is shed, relapses are common.

Undecylenic acid

Undecylenic acid is primarily fungistatic. It is used topically in combination with its zinc salt. It is available as foam, ointment, cream, powder, soap, and liquid. Undecylenic acid preparations are used in the treatment of various dermatophytoses, especially tinea pedis. The preparations as formulated are usually not irritating to tissue, and sensitization to them is uncommon.

Ciclopirox olamine

Ciclopirox olamine is broad-spectrum antifungal agent used for the treatment of superficial mycoses. It is fungicidal to *C. albicans*, *Epidermophyton floccosum*, *Microsporum canis*, *Trichophyton mentagrophytes*, *T. rubrum* and *Malassezia furfur*. After application to the skin, it penetrates superficial layers and reaches hair follicles and sebaceous glands, but systemic absorption is negligible. It can sometimes cause hypersensitivity. It is available as cream and lotion for the treatment of cutaneous candidiasis, and for tinea corporis, tinea cruris, tinea pedis, and pityriasis versicolor. It is available as 1% shampoo for the treatment of seborrheic dermatitis of the scalp, and 8% solution for the treatment of onychomycosis.

Benzoic acid and salicylic acid

An ointment containing benzoic acid and salicylic acid is known as Whitfield's ointment. It contains benzoic acid and salicylic acid in a ratio of 2 : 1 in which fungistatic action of benzoic acid is combined with the keratolytic action of salicylic acid. It is used mainly in the treatment of tinea pedis and sometimes it is also used to treat tinea capitis. Since benzoic acid is only fungistatic, eradication of infection occurs only after the infected stratum corneum is shed, therefore, medication is required for several weeks to months. The salicylic acid accelerates desquamation. Mild irritation may occur at the site of application

Sodium thiosulphate

Sodium thiosulphate is weak fungistatic. It is active against *Malassezia furfur*. A 20% solution applied twice daily for 2–3 weeks is effective in pityriasis versicolor. However, normal pigmentation of skin takes longer to return.

Antifungal drugs used for the treatment of important superficial and systemic mycoses are given in Table 3.3.

MECHANISMS OF RESISTANCE TO ANTIFUNGAL AGENTS

Expanded use of antifungal drugs has accelerated the development of resistance to these compounds. There are two types of resistance—**intrinsic resistance**, which is an inherited characteristic of a species or strain, and **acquired resistance**, which occurs when a previously susceptible isolate develops resistant phenotype, usually as a result of prolonged treatment with antifungals. In certain genera of fungi, some species are susceptible and other species are resistant to specific antifungals.

Intrinsic azole resistance

Candida krusei is intrinsically resistant to azoles. In addition, many strains of *C. glabrata*

Table 3.3: Antifungal drugs used for the treatment of important superficial and systemic mycoses

Mycoses	*Drugs*
I. Systemic mycoses	
Aspergillosis	Voriconazole, Amphotericin B, Caspofungin, Itraconazole, Posaconazole, Anidulafungin, Micafungin
Blastomycosis	Amphotericin B, Itraconazole, Fluconazole, Posaconazole, Voriconazole
Candidiasis	Amphotericin B, Fluconazole, Voriconazole, Caspofungin, Itraconazole, Posaconazole, Anidulafungin, Micafungin
Coccidioidomycosis	
Mild to moderate	Itraconazole, Fluconazole
Severe	Amphotericin B
Meningitis	Fluconazole, intrathecal Amphotericin B
Cryptococcosis	
Meningitis	Amphotericin B + Flucytosine followed by Fluconazole
Non-meningeal infection	Fluconazole, Amphotericin B
Histoplasmosis	
Moderate disease	Itraconazole, Fluconazole
Severe disease and meningitis	Amphotericin B
Mucormycosis	Amphotericin B, Posaconazole
Sporotrichosis	Itraconazole, Amphotericin B (extracutaneous)
Paracoccidioido-mycosis	Amphotericin B, Fluconazole, Itraconazole, Posaconazole, Voriconazole
II. Superficial mycoses	
Candidiasis	
Cutaneous	Amphotericin B, Clotrimazole, Ciclopirox, Econazole, Miconazole, Nystatin (topical)
Oropharyngeal	Clotrimazole, Nystatin (Topical) Fluconazole, Itraconazole (oral)
Vulvovaginal	Clotrimazole, Miconazole, Nystatin (Topical) Fluconazole (oral)
Dermatophytosis (Ringworm infection)	Clotrimazole, Miconazole, Butenafine, Terbinafine, Naftifine (topical), and Itraconazole, Griseofulvin, Terbinafine (systemic)

are intrinsically resistant and other strains can quickly acquire resistance during therapy. Therefore, *C. krusei* and *C. glabrata* are increasing in frequency in oral and systemic candidiasis in patients that use azole drugs for treatment or prophylaxis.

Acquired azole resistance

Acquired azole resistance in fungi develops when long-term azole therapy is used. Oral candidiasis was a common opportunistic infection in human immunodeficiency virus (HIV)-infected patients, occurring in over 90% of all patients with low CD4 counts. Azole drugs were commonly prescribed at relatively low doses for long-term suppressive therapy that was administered intermittently. Therefore, azole resistance in oral candidiasis became a significant problem. In addition to *C. albicans*, *C. dubliniensis* also has the ability to develop resistance to azole drugs.

Acquired resistance to azoles has also developed in isolates of *Cryptococcus neoformans* from AIDS patients who have

been on maintenance azole therapy to prevent cryptococcal meningitis, and in isolates of *A. fumigatus* from patients who have received repeated treatment with itraconazole or voriconazole.

Intrinsic amphotericin B resistance

Intrinsic resistance to amphotericin B has been seen in yeasts *C. krusei* and *C. lusitaniae*, and the molds *Aspergillus terreus* and *Scedosporium* spp.

Acquired amphotericin B resistance

Acquired resistance to fungicidal amphotericin B is rare; it occurs mostly in yeasts from cancer patients who have received repeated doses of the drug to treat recurring systemic fungal infections.

Mechanism of azole drug resistance

Mechanism of azole drug resistance in fungi may be due to overexpression of efflux pump. Drug resistance in fungi is not normally associated with gene amplification. Unlike studies with prokaryotes, studies with fungi have not yet identified any plasmids or episomes containing resistance markers. There is no report of transfer of drug resistance from one fungal isolate to another.

MECHANISM OF NONAZOLE DRUG RESISTANCE

Flucytosine resistance

Up to 10% of clinical isolates of *C. albicans* have intrinsic resistance to flucytosine, and 30% of the susceptible isolates develop acquired resistance during drug therapy. Resistance can be associated with mutation.

Echinocandin resistance

Echinocandin-resistant clinical isolates are limited in number, because the echinocandins have not yet been in clinical use for as long as the other agents. The mechanism of resistance to the echinocandins that has been characterized in laboratory strains of *Candida albicans*, *C. glabrata*, *C. tropicalis*, *C. krusei* and *C. lucitaniae* is one of an altered glucan synthesis enzyme complex that shows a decreased sensitivity to inhibition by agents within the class.

Though lot of advancement has been made in the treatment of fungal infections but still there is a need for novel drugs due to the problem of drug resistance and toxicity with current drugs. Cytokine therapy (such as interferon-α) and use of growth factors such as granulocyte macrophage-colony stimulating factor (GM-CSF) have been shown in animal studies to increase clearance of fungi and result in better clinical outcomes and are being evaluated in human disease. Development of an antifungal vaccine is also being considered with advances in antibody technology.

Important Questions

1. Classify antifungal drugs. Discuss their mechanism of action and indications.
2. Write short notes on:
   (a) Systemic antifungal drugs
   (b) Topical antifungal drugs
   (c) Antifungal drug resistance.

Multiple Choice Questions

1. Which of the following targets has/have been exploited in the development of antifungal drugs?
(a) Plasma membrane sterols.
(b) Nucleic acid synthesis.
(c) Cell wall constituents.
(d) All of the above.

2. Which of the following is systemic antifungal drug?
(a) Nystatin.
(b) Griseofulvin.
(c) Hamycin.
(d) Natamycin.

3. Amphotericin B is an:
(a) Amphoteric polyene macrolide
(b) Azole.
(c) Echinocandin.
(d) Antimetabolite.

4. Which of the following is the most toxic systemically used antifungal drug?
(a) Griseofulvin.
(b) Amphotericin B.
(c) Flucytosine.
(d) Fluconazole.

5. Which of the following antifungal drugs can be used both orally and topically?
(a) Clotrimazole.
(b) Miconazole.
(c) Ketoconazole.
(d) Econazole.

6. Which of the following antifungal drugs is fungicidal?
(a) Amphotericin B.
(b) Griseofulvin.
(c) Flucytosine.
(d) Itraconazole.

7. Which of the following antifungal drugs can be used for the treatment of diarrhoea caused by *Candida* spp.?
(a) Hamycin.
(b) Natamycin.
(c) Nystatin.
(d) Miconazole.

8. Which of the following antifungal drugs is not fungicidal?
(a) Undecyclinic acid.
(b) Amphotericin B.
(c) Amorolfine.
(d) Ciclopirox olamine.

9. Amphotericin B can be used for the treatment of:
(a) Aspergillosis.
(b) Candidiasis.
(c) Blastomycosis.
(d) All of the above.

10. Which of the following drugs can be used for the treatment of dermatophytosis?
(a) Griseofulvin.
(b) Clotrimazole.
(c) Terbinafine.
(d) All of the above.

11. Which of the following antifungal drugs acts by inhibiting synthesis of plasma membrane ergosterol?
(a) Nystatin.
(b) Amphotericin B.
(c) Clotrimazole.
(d) Ciclopirox olamine.

12. Flucytosine is used in combination with which of the following antifungal drug for the treatment of cryptococcal meningitis?
(a) Ketoconazole.
(b) Amphotericin B.
(c) Caspofungin.
(d) Griseofulvin.

13. Which of the following antifungal drugs is an antimetabolite?
(a) Griseofulvin.
(b) Nystatin.
(c) Voriconazole.
(d) Flucytosine.

14. Griseofulvin is effective against dermatophyte infection of:
(a) Skin.
(b) Hair.
(c) Nail.
(d) All of the above.

15. Which of the following azole antifungal drugs has the lowest protein binding?
(a) Fluconazole.
(b) Itraconazole.
(c) Ketoconazole.
(d) Miconazole.

16. Which of the following statements about amphotericin B is not correct?
(a) It is active against yeast-like, dimorphic and filamentous fungi.
(b) In high concentrations it is fungicidal.
(c) It acts by binding to ergosterol of fungal cell membrane.
(d) It is least toxic antifungal drug.

17. Which of the following antifungal drugs is obtained from *Streptomyces nodosus*?
(a) Amphotericin B.
(b) Nystatin.

(c) Griseofulvin.
(d) Flucytosine.

18. Flucytosine is effective against which of the following fungal infections?
(a) Cryptococcosis.
(b) Candidiasis.
(c) Chromoblastomycosis.
(d) All of the above.

19. Nephrotoxicity is the most serious long-term toxicity of:
(a) Griseofulvin.
(b) Amphotericin B.
(c) Butenafine.
(d) Clotrimazole.

20. The absorption of which of the following drugs is greatly decreased in the absence of gastric acidity?
(a) Flucytosine.
(b) Fluconazole.
(c) Nystatin.
(d) Itraconazole.

21. Which of the following antifungal drugs enters the CSF in adequate concentration in cryptococcal meningitis in HIV-infected patients?
(a) Miconazole.
(b) Ketoconazole.
(c) Fluconazole.
(d) Clotrimazole.

22. Which of the following is the drug of choice for the treatment of aspergillosis?
(a) Griseofulvin.
(b) Ketoconazole.
(c) Nystatin.
(d) Voriconazole.

23. Which of the following azole antifungal drugs is most effective in the treatment of *Candida* bloodstream infection?
(a) Clotrimazole.
(b) Fluconazole.
(c) Itraconazole.
(d) Miconazole.

24. Which of the following statements is/are true about amphotericin B?
(a) Acute adverse effects may include chills, fever, headache, nausea, loss of appetite.
(b) Chronic adverse effects include kidney toxicity.
(c) It binds to ergosterol in the cell membrane of fungi and thus increases its permeability and induces cell lysis.
(d) All of the above.

25. Flucytosine combination therapy is preferred primary therapy with:
(a) Amphotericin B to treat cryptococcosis.
(b) Fluconazole to treat systemic candidiasis.
(c) Itraconazole to treat aspergillosis.
(d) Micafungin to treat mucormycosis.

26. For which disease is oral terbinafine therapy recommended?
(a) Black piedra.
(b) Oral thrush.
(c) Pityriasis versicolor.
(d) Dermatophyte infection (tinea unguim).

Answers to MCQs

1. (d), 2. (b), 3. (a), 4. (b), 5. (c), 6. (a), 7. (c), 8. (a), 9. (d), 10. (d), 11. (c), 12. (b), 13. (d), 14. (d), 15. (a), 16. (d), 17. (a), 18. (d), 19. (b), 20. (d), 21. (c), 22. (d), 23. (b), 24. (d), 25. (a), 26. (d).

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