Mycological Examination

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Key Points
- Mycoses are diseases caused by fungi, present in the soil, air, contaminated food, animals, and humans. Fungi are classified according to the microscopy appearance and by the method of reproduction in culture. Therefore, laboratory testing is necessary to confirm the diagnosis in all conditions.
- Superficial localized infection can be diagnosed rapidly by taking fresh unstained material (skin, nails, hairs) to be examined by *direct microscopy* after potassium hydroxide (KOH) clarification. This procedure is usually rapid and easy to handle even in general practice, if provided with a light microscopy.

- *Fungal culture* is necessary to identify the fungus and to confirm the vitality of the strain. When a local or systemic antifungal treatment is performed, culture is necessary to assess effective recovery or current infection. It requires specific training and great expertise, reserved to specialized mycology laboratories.
- False-negative results are possible; therefore, *biopsy and histology* are indicated if direct microscopy and culture are negative and clinical suspicion is high.
- *Wood's lamp examination* has very limited indication, detecting the natural luminescence of only few pathogens, mainly *Microsporum spp.*, *Malassezia furfur*, and *Corynebacterium minutissimum*.
- *Molecular techniques*, such as DNA hybridization and PCR, are useful to prompt diagnosis, detecting even small amounts of fungi, but are expensive procedures, limited to the research or reference laboratories for the diagnosis of rare fungal infections, which are difficult or dangerous to cultivate in vitro.
- Great simplification of the fungal identification process is expected from the introduction of the *assay mass spectrometry* in routine laboratory.

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General Principles

Mycological examination is the diagnostic procedure necessary to confirm a fungal disease, from the Greek word "mykes" meaning mushroom. The mycological test is also necessary to document the complete cure response to treatment, as clinical recovery is often followed by a relapse if the fungus has not been completely eliminated from the skin (mycological cure). The diagnosis of a fungal infection is considered simple from a clinical point of view, but the report of unusual presentations, misdiagnosed or sometimes neglected, has increased worldwide causing extensive and long-standing disease. General immunosuppressive treatment for inflammatory chronic diseases and acquired immune depression syndrome are frequently complicated with fungal superinfections, both from common strain and rare or opportunistic fungal species, such as cryptococcosis, histoplasmosis, sporotrichosis, blastomycosis, and aspergillosis. In the immunocompetent patient, dermatophytes are the main fungal infection, being the primary pathogen. Clinical presentation has also changed with time, from the classic ringworm appearance (tinea) to more veiled eczematous undefined patches, simulating more frequent diseases, such as atopic or contact eczema, seborrheic dermatitis, impetigo, rosacea, and lupus erythematosus. A general common attitude to prescribe combination treatments and/or corticosteroid topics before confirming the diagnosis ("ex adjuvantibus") is one of the possible causes of further dermatophyte pathomorphosis and misdiagnosis, named tinea incognita or tinea atypica.

The etiology of a fungal infection is rapidly proved with laboratory testing and possible in a general office if provided with very basic light microscopy. Besides, few dermatologists and laboratory physicians are trained to recognize dermatophytes. Final identification of the species requires culture facilities and further expertise, which should be referred to a mycology laboratory.

False-negative results are possible, especially if the specimen is not correctly taken, and more invasive assessment should be performed before ruling out the diagnosis of a fungal disease. Biopsy specimens for histopathology examination with PAS or silver staining are indicated when clinical suspicion is high. Sensitivity of culture alone is about 32 %, while histology reaches 85 %, especially in onychomycosis.

A specific fluorescent dye named calcofluor-white might be used, because it binds selectively to the fungal cell wall chitin (apple green fluorescence), but a fluoroscope is necessary.

A molecular approach has been proposed, but the tool is expensive; a plethora of different methods has been proposed without conclusive validation and therefore has never gained much popularity. Direct staining with monoclonal antibody labeled with fluorescent dyes has been proposed to detect rare and small amounts of fungi in the clinical specimens, mainly *Pneumocystis carinii* in bronchoalveolar lavage.

The perspective in clinical mycology laboratories is the fast fungal identification by assay mass spectrometry (matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (MS)) from clinical cultures. Spectral databases (biotype library) of different fungal species have been realized by several independent researchers, and preliminary results confirm the usefulness of the tool, with a sensitivity of 91.9 % on skin isolates, which is hoped to become routine in the immediate future.

Biology and Morphology of the Fungi

Fungal microscopy identification requires an extensive knowledge of fungi biology as a whole, whose principles are magisterially described in the reference book by Grigoriu et al. (1987) and are somewhat synthesized as follows.

Fungi are classified with the lower plants (category, cryptogams; phylum, thallophytes), although devoid of chlorophyll and incapable of photosynthesis, and they are:

- *Heterotrophic*, meaning they subside on organic substances for survival and are unable to directly elaborate mineral elements, such as green plants. They are provided with several

enzyme systems able to digest organic substances (keratin) and to de novo synthesize toxins and antibiotics.

- *Aerobic*, developing at different temperatures varying from one species to another, but usually ranging between 20 and 37 °C, preferring neutral pH and several degrees of humidity, while dryness is poorly tolerated. Direct light exposure has different effects among species, fostering or inhibiting growth.

- *Eukaryotes*, with well-differentiated cytoplasm and nuclei. They consist of a thallus or aerius mycelium, which is a matted network of branching tubes called hyphae, filled with the living substance, continuously expanding with growing tips. The thallus accomplishes both vegetative and reproductive functions: from the central starting point of the colony, the hyphae develop centrifugally in all directions obtaining nourishment from the environment, with the young elements at the periphery and aging dead elements at the center. When the vegetative expanding apparatus has accumulated sufficient nourishment and the environment is favorable, the reproductive phase might take place, which can be either sexual (*perfect fungi*) or asexual. A limited number of fungal species do not have a documented sexual way of reproduction and for this reason are called *imperfect fungi*.

A number of *classifications* have been proposed on the basis of the thallus' different characteristic and sexual reproduction ways, to consent a precise nomenclature and identification, whose simplest ones are illustrated in Table 123.1.

- On the basis of thallium morphology, it is possible to distinguish among septate and not septate fungi. A single cell generates a type of thallus with intercommunicating filaments (hyphae), named coenocytic filaments, without subdivision and therefore called aseptate hyphae. Other thalli are made of septated hyphae, which indicate the presence of regular periodical septal division in the filaments, provided with central pores allowing the passage of nutrients from one segment to the other. Another type of thallus consists of isolated cells, solely and elongated or attached one to the other, forming fragile, eventually branching chains but without intercommunication, therefore called pseudomycelium.

- The principal way of fungal reproduction in pathologic material is asexual, producing a variety of spores (Table 123.2) responsible for fungal dissemination in the environment. The aspect of pathogenic fungi might differ from the parasitic state to the culture optimal saprophytic state. In fact, mycelia fungi do not usually deserve fruiting bodies in the pathologic materials, and species identification is possible only in culture specimens. Yeasts might bud in vivo, while the so-called dimorphic fungi are typically found as yeast cells in pathologic material, producing mycelia filaments in cultures.

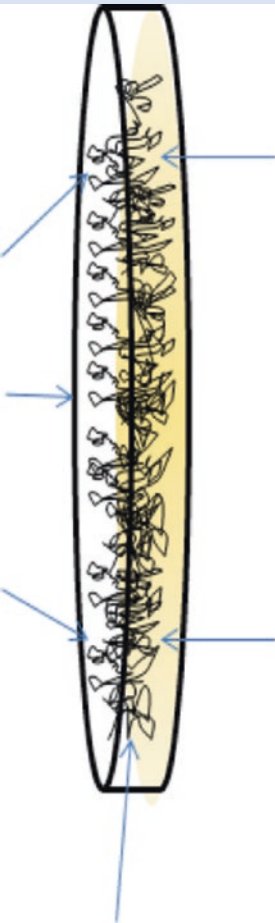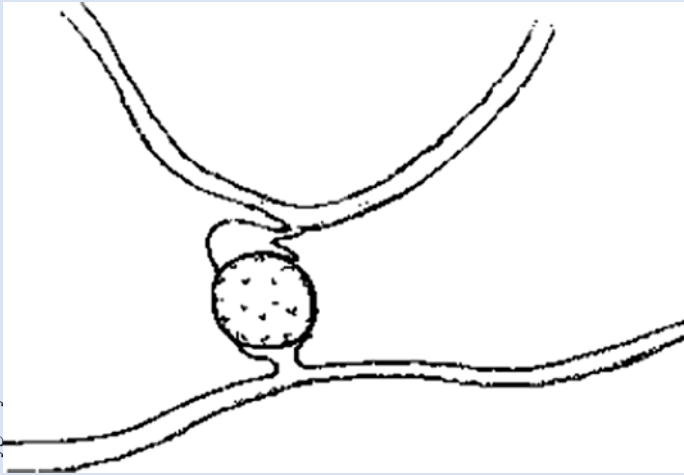## Basic Concepts Related to the Pathogens

From a practical medical point of view, the pathogenic fungi for humans are:

- Dermatophytes (whose mycological diagnosis is extensively treated in this chapter)
- Yeasts and yeastlike fungi (whose laboratory diagnosis requires specialized equipment and expertise, and only basic concepts are expressed in this chapter)
- Some dimorphic and opportunistic fungi, becoming pathogen under peculiar circumstances (whose diagnosis is not developed in this chapter)

Dermatophyte Infections

Dermatophytes are actually classified among the ascomycetes and represent a closely related group of fungi, whose main pathogens for humans are the *Trichophyton*, *Microsporum*, and *Epidermophyton* genera. They are ubiquitous and are characterized by the ability to use keratin as a nutrient source, therefore evolving a dependency on humans and/or animals for the survival and dissemination of their species. They are noninva-

Table 123.1 Fungal classification based on the thallus characteristics and type of sexual reproduction
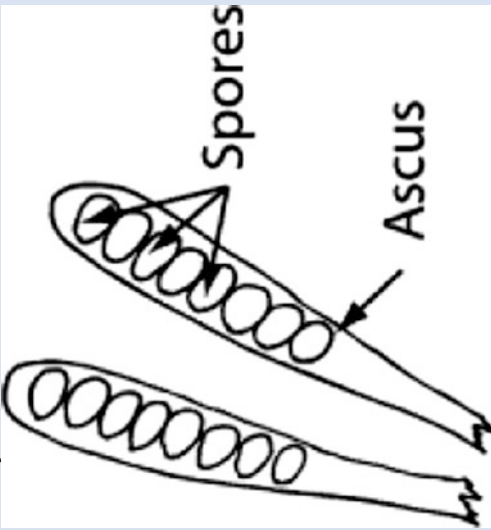
Fungal classification		Type of sexual reproduction
	Thallus reproductive form with fertile hyphae or spores	
	Aerial mycelium	
	Thallus vegetative apparatus with submerged hyphae	
Zygomycetes		The name comes from the type of sexual reproduction, taking place in a specialized organ named zygosporangia, forming resistant spherical spores. They live in soil, on decaying plant, or on animal material
Nonseptate hyphae		The thallus is characterized by continuous filaments, without any septa, forming the coenocytic thallus or coenocytes. The cellular multiple nuclear divisions are not followed by the cytokinesis, resulting in single coordinated unit composed of multiple cells linked structurally and functionally

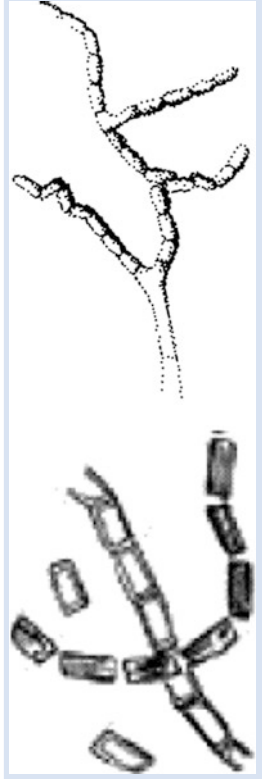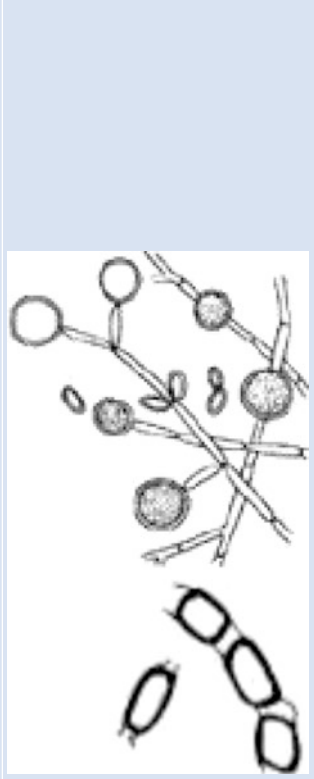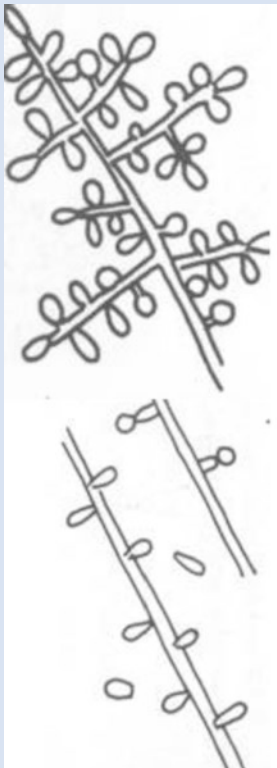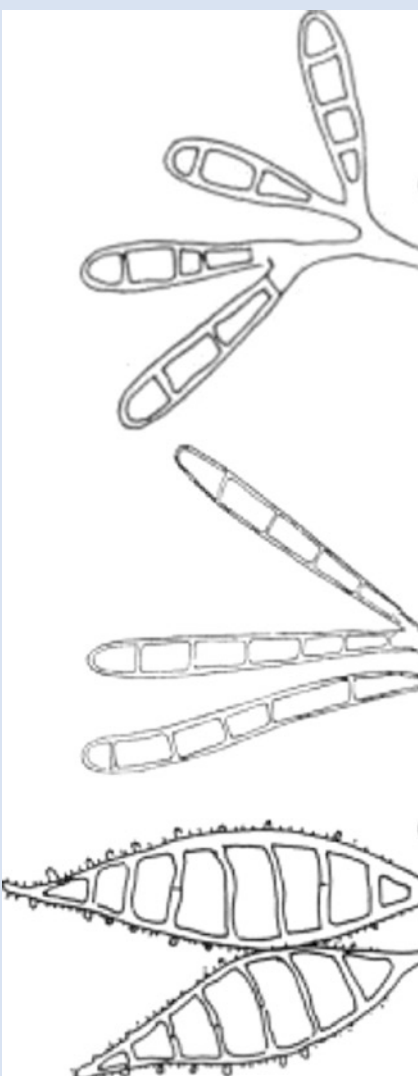|		
Ascomycetes		The sexual reproduction takes place in a specialized ovum structure (ascus), formed by the fusion of a male and female gamete, inside which successive divisions of the zygote produce unicellular endospores (ascospores)
Basidiomycetes		Similar sexual reproductive process, but the ovum makes peculiar extrusion towards the external space, called basidiospores, keeping them attached to the central core by minute arm called sterigmata
Imperfect fungi		The thallus produces septate hyphae in the vegetative state, but the sexual reproduction modality has never been documented
Septate hyphae		The thallus is characterized by regular interruption of the filaments (hyphae), called septate provided with central pores allowing the passage of nutrients from one segment to the other
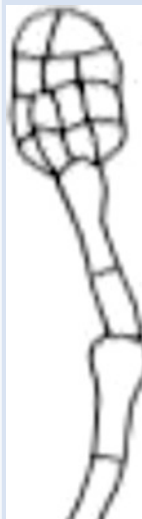
Table 123.2 Distinctive morphological characteristics of fungal spores

Arthrospores	Formed by septated hyphae fragmentation, at the tip of the filaments, maintaining a rectangular shape	
Blastospores	Budding cells, the bud separating from the parent cell or keeping the linkage for a certain time and yet continue budding to produce extensive branching (pseudomycelia)	
Chlamydospores	Swollen portion of the hypha or pseudohypha, with a very thick double wall (intercalary or terminal), which ensures resistance to many adverse conditions	
Aleuriospores	Lateral budding of the hypha, disposed like a Cross of Lorraine or bunches, which remains attached to the hypha axis	

Microconidia	They are identical to aleuriospores but are deciduous and detach from the hypha immediately after their formation	
Macroconidia or fuseaux	Very voluminous and elongated septated budding, with the same significance of microconidia	
Dictyospores	Multicellular digitiform or globular elements	

sive, as they colonize the keratinized stratum corneum, which is a nonliving tissue. However, the presence of the fungus and its metabolic products usually induces an inflammatory eczematous response in the host, counteracting the parasitism. More severe reactions might be related to the species and strain of dermatophyte causing the infection, but also from host predisposing condition, such as skin barrier alterations or previous skin diseases.

- The superficial dermatophyte infections affect the outer layers of the skin, the nails, and hairs. Growing fungi produce typically branched and compartmented septate filaments (hyphae) and arthrospores, which break off from the hyphae at the septate level and represent the main ways of propagation and species conservation. Each segment forms a physiological unit.
- In culture, the characteristic morphology of the reproductive spores (micro- and macroconidia) and accessory features consent species identification.

Guidelines for Proper Specimen Collection in Dermatophyte Infections

Specimen collection is a delicate moment and is the first cause of false-negative results. It should be adapted to the site affected and type of material, including skin scrapings, broken hairs, nail clippings, corneal scraping, and body fluids such blood, CSF, urine, sputum, as well as discharge or pus from lesions.

All specimens must be transported to the laboratory without delay to prevent bacterial overgrowth. Some laboratories suggest refrigeration if a long delay is expected, especially for body fluids and discharged materials, but it is recommended only for a short period.

Skin scales scrapings are best taken from the leading edge of the rash, after the skin has been cleaned with soaks of 0.9 % saline solution to remove cream residuals. Disinfection with

alcohol or ether is often advocated but is not necessary and might remove the upper scales which are the most infested. Gently remove the surface skin using a blade or curette and place the material in a sterile container or a black paper envelope (Fig. 123.1).

Hairs should be gently pulled out with a pair of tweezers, choosing those broken off at the surface (black dots) or few millimeters from the follicle, discolored and lusterless, surrounded by a whitish sheath, or covered by scales (Fig. 123.1). Parasitized hairs are removed easily and painless. A toothbrush can be used to collect scales from the infected scalp.

Nail clippings are usually taken from the ventral surface scaly deposits, using a blunt curette or a blade. If the nail plate is widely detached and full of debris, the distal end should be cut with a pair of scissors and discarded to find the fresh infected nail. Depending on the site of involvement, the lateral and/or proximal surface of the nail plate should also be scraped (Fig. 123.2).

Examination by Direct Microscopy

The most common and first step laboratory examination is to examine fresh unstained materials (*native preparation*), such as scales, hairs, and nails. Part of the material is placed on a clean slide and eventually split to very fine dust with the aid of a blade. Body fluids, content of vesicles, and pustules can be also directly placed on the smear. Keratinized material and cell walls are opaque at microscopy, and some chemical solutions (Table 123.3) must be added to dissolve keratinocytes and make the material transparent, a process that is called "clearing." Certain laboratories add glycerin to delay the preparation drying out and have more time to perform the slide lecture. One or two drops of the clearing agent is put on the collected material on the slide and then covered with a cover slip (Fig. 123.1). The most used clearing solution is potassium hydroxide (KOH) in 10–40 % concentrations. It is very cheap and stable on light and does not
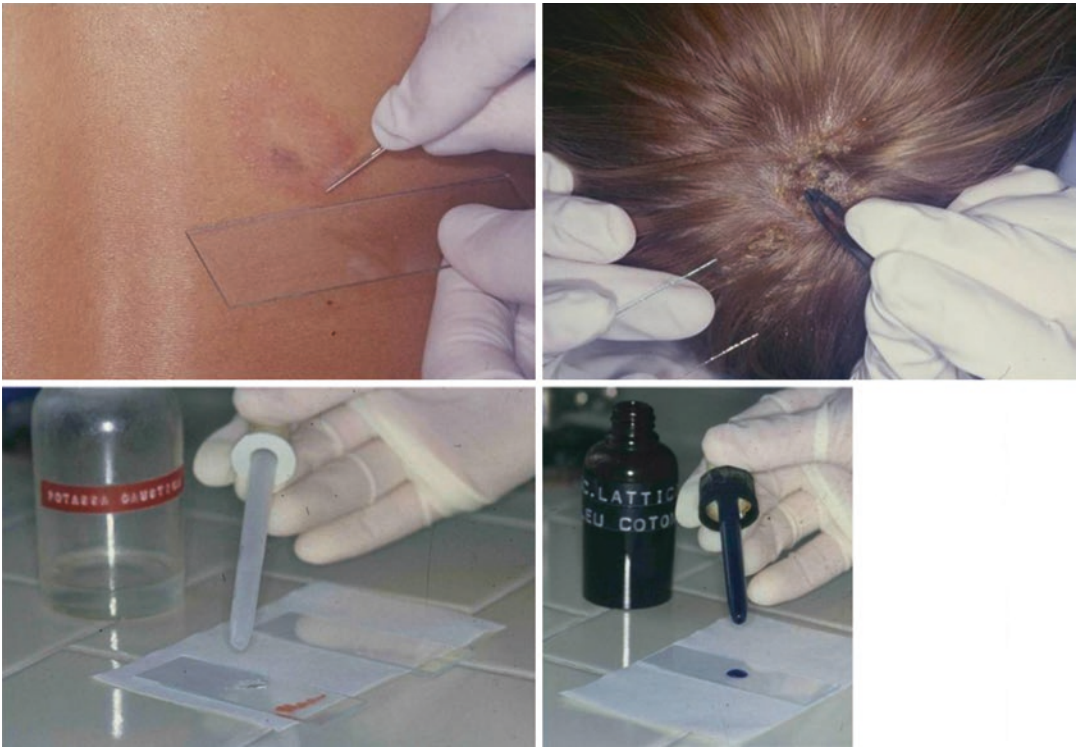
Fig. 123.1 Correct execution of the scales sample from the active borders of an erythematous scaling round-shaped lesion (*top-left* inset); correct execution of the hair sample from discolored and few millimeter broken-off hairs plucked with a pair of eyebrow tweezers (*top-right* inset). The native preparation is put on a smear and a drop of a clearing solution is added (*down-left* inset); staining with blue or black ink preparations might be useful to highlight hyphae and arthrospores or yeasts presence (*downright* inset)

need particular conservation or caution, and the preparation is rapidly ready. Time to clearing depends on the material type and thickness, the hairs being rapidly destroyed by KOH and therefore examined in 20 min, 1 h at most; the skin scales usually are examined within 3 h, while the nails are more resistant, sometimes requiring 24 h and addition of new clearing solution or physiologic solution, because one of the major disadvantages with KOH is precipitation and formation of crystals. Additional staining with methylene blue, Parker blue, or black ink do not replace direct examination (Fig. 123.1), but it is sometimes useful to highlight the chitin in the fungal cell walls. Fixation and staining with lactophenol cotton blue (LPCB) is preferred to observe yeasts, fungal accessories, and fruiting structures, especially from culture specimen.

The microscopic examination is first performed under a low-power lens (100×) run carefully along the slide to find fields of interests (Fig. 123.3). Once the fungus is detected, the high-power lens (200 or 400x) observation allows for true hyphae confirmation and distinction from pseudohyphae, as well as arthrospores selection from yeasts.

Fungal elements are sometimes difficult to find, and a negative result does not rule out fungal infection. Microscopy should be repeated, possibly with fresh material or giving more time to clearing and repeating the observation with great perseverance.

Distal subungueal onychomycosis

Proximal subungueal onychomycosis
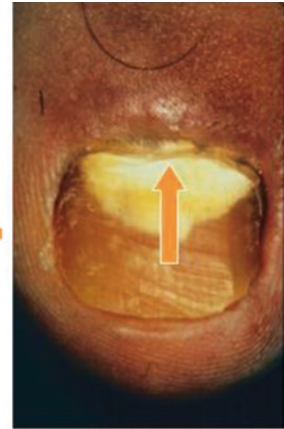
Distal lateral subungueal onychomycosis

Total subungueal onychomycosis

White superficial onychomycosis

Fig. 123.2 Correct site of sampling depending on the clinical form of onychomycosis

Table 123.3 Clearing solutions for direct microscopic examination

Chemical solution	Preparation
KOH	Potassium hydroxide 10–40 % concentration
Amann's chlorallactophenol	Equal parts of chloral hydrate + lactic acid + phenol
Sodium sulfide	10 % sodium sulfide in a solution made of ¾ distilled water and ¼ 80 % alcohol
Sodium lauryl sulfate	5 % in distilled water

Direct Microscopic Examination Pitfalls

Specific training is necessary to avoid the many possible sources of errors in mycology, as in any other laboratory procedure.

- Mistakes in sample collection: insufficient material, inadequate site of sampling, and contamination.
- Insufficient clearing and hasty observation yield most false-negative results.
- Artifacts (Fig. 123.4): the mycelia filaments and arthrospores have to be distinguished from:
  - Fungus mosaics: a network made of refractive small filaments or crystals, irregular in shape and diameter, whose chain arrangement is very similar to the mycelia and might mislead a nonexpert investigator. They are cell debris containing cholesterol crystals due to a reaction of the intercellular substance with potassium hydroxide. Usually the mosaics are located at the limits of the cells and disappear if a drop of
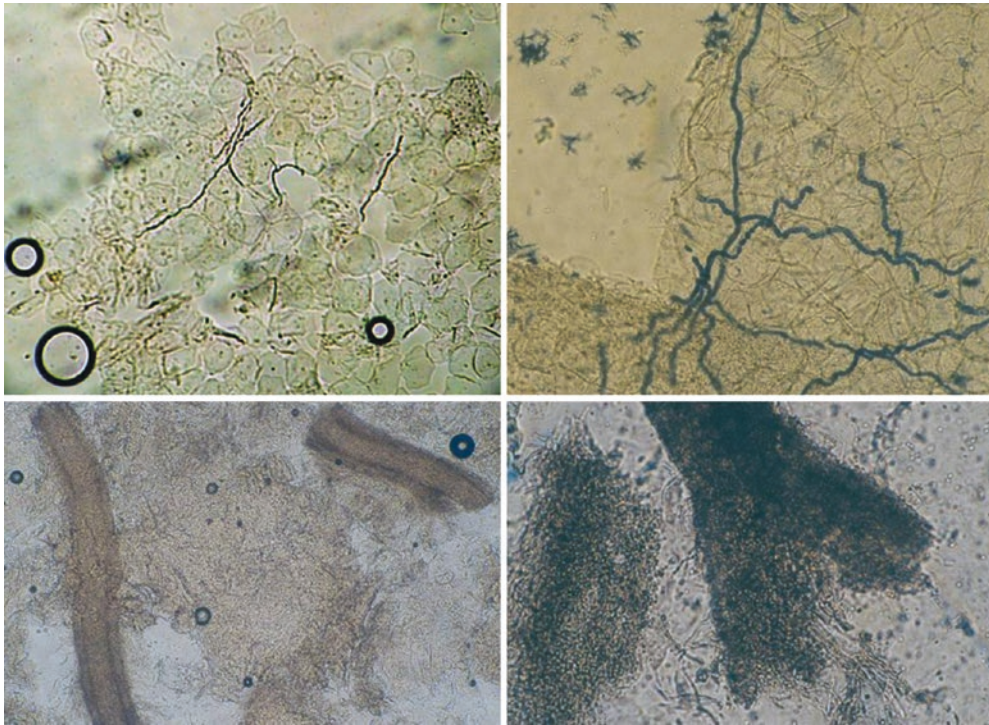
Fig. 123.3 Skin scraping: under low-power light microscopy, selection of a field of interest (*top-left* inset; 100×); major definition of hyphae and arthrospores characteristics at higher power observation (*top-right* inset; 400×). Parasitized hairs: sheath of arthrospores surrounding the hair shaft in an ectothrix arrangement and hyphae presence in the scales (*bottom-left* inset; 100×); hyphae and arthrospores inside the hair root and shaft in endothrix parasitism (*bottom-right* inset; 400×)
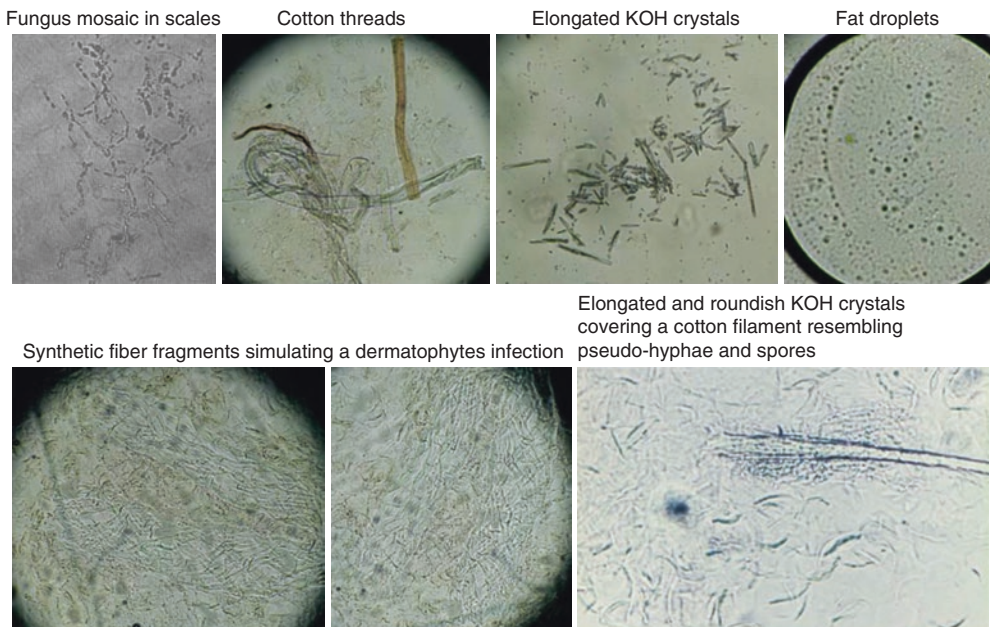
Fig. 123.4 Microscopic examination artifacts

glycerol or physiologic solution is added to the preparation. The higher-power observation (100×) enables the distinction from the fungal hyphae, which are regular in caliber and shape, septated, connected together continuously.

- Cotton threads, other vegetal filaments and fur brittles, which are usually woven or twisted on their axis and irregular in caliber, tufted at the extremities.
- Polyhedral or elongated crystals: refractive bodies differing in appearance one from the other and not connected together. It is the dehydrated clearing agent that usually causes this artifact, and a drop of distilled water added directly to the smear makes them to disappear.
- Fat droplets, refractive roundish or oval bodies, enclosed by a double membrane frequently due to the previous use of cosmetic creams, persisting on the skin although disinfected. Cleaning with water and soap before examination is advisable.

• Filamentous bacteria: some *Actinomycetales* bacteria and particularly the *Streptomyces* genus are able to produce a network of branching filaments very similar to the fungal mycelia, but smaller in dimensions (0.5–0.8 μm).

Yeast Infections

Yeasts and yeastlike are a subtype of fungal species characterized by clusters of round or oval cells. They divide and propagate budding out similar cells from their surface. Some yeasts are capable of producing filaments, which remain part of the single original cell, not connected with other elements and called pseudohyphae. They are usually saprophytic, but a pathogenic potential might arise in the presence of several favoring conditions. The main yeasts causing human superficial skin infections, whose diagnosis might be confirmed in a general laboratory provided with a light microscopy, are:

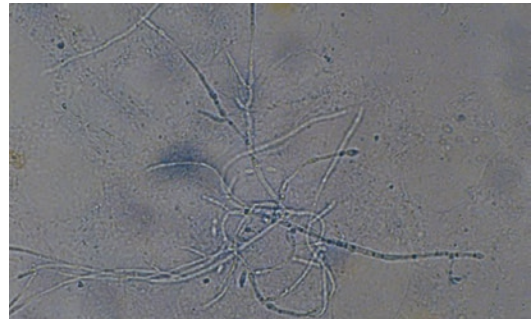• *Candida* species and *Torulopsis glabrata*
• *Malassezia* species

Fig. 123.5 *Candida* yeast cells and pseudohyphae in a groin intertrigo (400×)

Specimen Collection and Examination by Direct Microscopy

Basic concepts and procedures are similar to dermatophyte mycological examination, and only useful additional information for the general practice is addressed in the following paragraphs.

Candida spp. *infection* can be confirmed by swabs and by scrapings. The pathologic material (scales, pustules, white deposits, and secretions) is cleared with KOH between the slide and the cover slip. Urine can be examined without clarification, and blood is seeded directly on the medium for culture. Under the microscope, the presence of pseudohyphae and round, oval yeasts forms might be sufficient to confirm the type of infection (Fig. 123.5), but when dealing with opportunistic infections, culture execution is recommended to confirm pathogenicity instead of simple colonization. Detection of yeasts should be interpreted in close cooperation with clinicians, and equivocal or unusual findings should be verified by repeated laboratory tests. Superinfection of skin lesions in the course of several diseases is very frequent, and *Candida* detection is not sufficient to ascribe its etiologic role. Histology is sometimes necessary to distinguish primary infection from superinfection.

Malassezia spp. *infection* is better documented by stripping off the skin surface with an adhesive tape (scotch test), as the scales are very subtle and superficial. The scotch is then stuck on

a glass slide and examined using potassium hydroxide (KOH), eventually stained with blue or black ink preparation. The abundant presence of the fungal filamentous form budding from the yeast cells in a pattern called "spaghetti with meatballs" is very peculiar and diagnostic (Fig. 123.6).
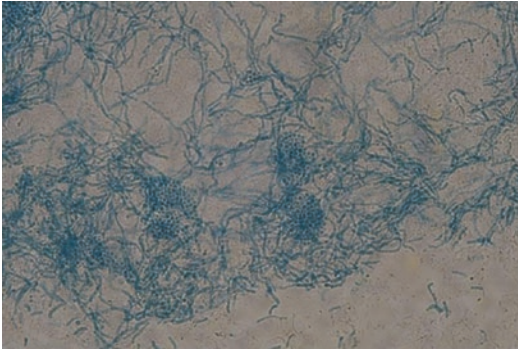

Fig. 123.6 *S*kin scotch test after KOH + Parker's blue clarification positive for *Malassezia furfur* pseudohyphae and clusters of yeastlike cells (400×)

Fungal Culture

General Principles of the Correct Procedure and Fungal Identification

Culture is necessary to correctly identify most of the fungal species but requires great expertise, and only very specialized laboratories are reliable.

Specimen collection is similar to the previously described technique for direct examination, but sterile materials should be used, because the transferring process of the pathologic samples (scales, hairs, nails) is a delicate moment, at high risk of contamination. Every subsequent passage should be performed under sterile conditions. The specimen must be placed on a sterile plate, subdivided into small particles with the aid of a sterile dissecting needle or a razor blade and collected with a previously flamed platinum loop to be seeded on the culture medium (Fig. 123.7). A distance of about 1 cm should be maintained
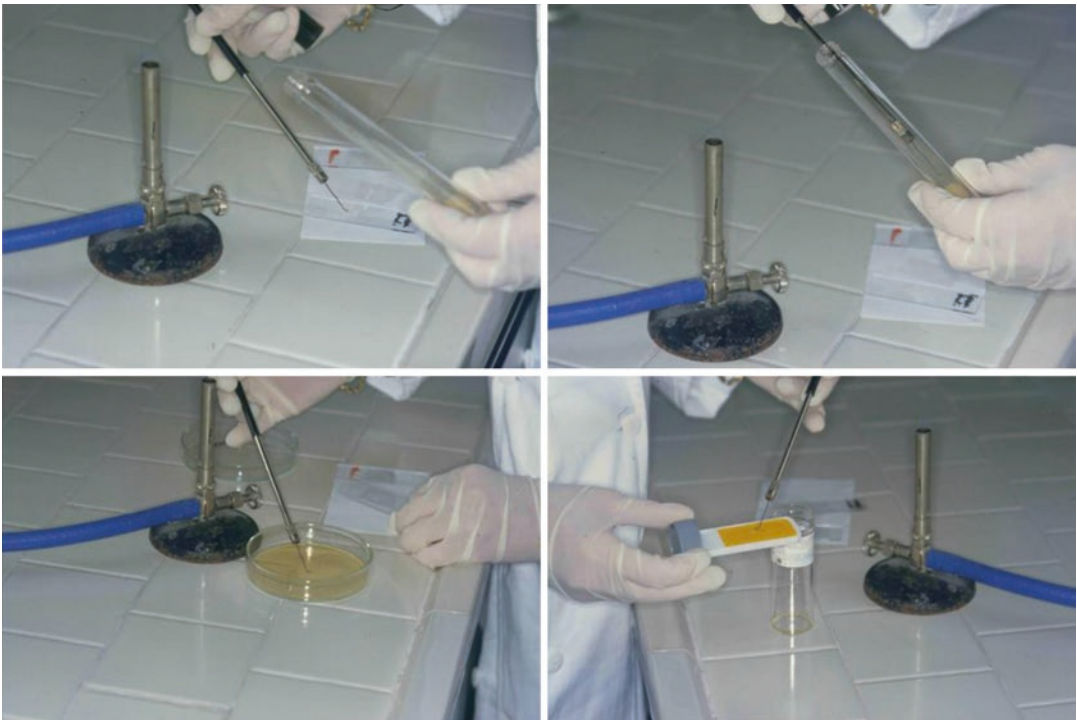

Fig. 123.7 Culture technique: transferring process of the pathologic material on different medium containers

between each inoculum on the medium surface. The test tubes or Petri dishes must be taken from fridge cool to room temperature, and the mouth of the tube or the dish plate must be flamed with a Bunsen burner to sterilize it.

The most commonly used culture medium is Sabouraud's dextrose agar added with cycloheximide and chloramphenicol to avoid mold contamination. Time to colony growth on Sabouraud's agar is a first discrimination of the type of fungus: filamentous fungi grow very slowly, along several weeks (3 weeks or more), incubated at 25–30 °C. Yeasts produce fast growing colonies (24–48 h). A number of other organic and synthetic culture media have been proposed (Table 123.4), whose laboratory preparation has been simplified by the use of prompt dehydrated media, which only need the addition of a fixed amount of distilled water, sterilization, and portioning on test tubes or Petri dishes.

A selective dermatophytes medium (DTM) is available for practitioner's use in the office, which does not require special conservation or care. Dermatophyte growth is indicated from the medium's color change to orange. Unfortunately, this kind of test is expensive and does not allow for subsequent fungal identification.

Table 123.4 Some examples of fungal culture media composition

Medium denomination	Composition	
Agar Sabouraud's medium	Crude glucose	40 g
	Granulated peptone	10 g
	Agar	20 g
	Tap water	1,000 ml
Emmons' variant	+ chloramphenicol	0.5 g
	+ cycloheximide (actidione)	0.5 g
Sabouraud's liquid medium	Same composition without agar	
Vanbreuseghem's variant	20 % earth + agar + tap water	
Rice medium (to favor dermatophytes fruiting)	Gelose	20 g
	Husked rice	20 g
	Peptone	2 g
	Tap water	1,000 ml
Potato-carrot medium (to favor *Candida albicans* chlamydospores formation)	Chopped potatoes 20 g	
	Chopped carrots 20 g	
	Macerated in 1 l tap water for 1 h; boiled for 5 min, filtered, and added with powdered agar g 20; the whole brought up to 1,000 ml	
Marcelou-Kinti's medium (used for the rapid sugar fermentation test)	Agar	9 g
	Peptone	10 g
	Bromocresol purple	0.04 g
	Chloramphenicol	0.5 g
	Tap water	1,000 ml
	+10 % sodium bicarbonate solution to adjust the pH	
	To this base, several sugars are added in separated 30 % solution: glucose, maltose, raffinose, galactose, lactose, saccharose, trehalose. Each sugar final solution is used to impregnate a disk of blotting paper, placed in hemolysis tubes and finally added with the fungal culture inoculum suspended in physiological solution. After 24–48-h incubation, the fermented tubes have discolored medium	
Lodder's medium (to study the assimilation of sugar and nitrogen)	Potassium dihydrogen phosphate	1 g
	Magnesium sulfate	0.5 g
	Ammonium sulfate	5 g
	Agar	20 g
	Distilled water	1,000 ml

Once the colonies have grown, identification relies on both gross and microscopic features. Colony morphology and growth dynamics may vary considerably with the culture medium used (type of glucose, peptone, pH) and other conditions such as the degree of humidity and temperature. It is therefore essential to maintain constant conditions in the laboratory and to use the same components for the media preparation. Well-established and reference features are based on Sabouraud's medium, and it is advisable to always perform at least one culture on that medium when other culture media are used.

Candida species cannot be identified either by direct microscopy or by culture macro- and microscopic features. The final species determination relies on the germ tube tests (rapid production of chlamydospores) and on sugar fermentation and assimilation tests, as well as nitrogen utilization (Table 123.5).

Candida strains resistant to antifungals have been detected (35–39); therefore, the antifungal susceptibility test should be performed, especially in nosocomial settings and in immunocompromised patients.

Culture of *Malassezia species* is not easy to obtain and usually not necessary. The Sabouraud's agar medium is added with a layer of olive oil to favor *Malassezia* caramel-colored colonies growth.

Gross Morphology Examination

The colony morphology examination has a relative value, because gross features are changeable and similar to related fungi (Figs. 123.8 and 123.9). Observation includes the reverse side of the tubes or Petri dishes, because some features may differ greatly.

Surface of the colony: convex or flat; elevated with a central button, convoluted with regular folds or vermiculate, with radial branching.

Aspect of the colony: plaster-like or waxy, glabrous or wooly, granular, dusty, creamy.

Color: grayish or creamy, orange, red, purple, pink, cafè au lait, dark brown, black, dark or olive green. The pigmentation density usually varies as

it diffuses in the medium and might greatly change on the reverse side of the colony.

Consistency: soft and friable or so hard that it is difficult to detach with the platinum loop.

Culture Microscopic Examination

Once the gross morphology observation is completed, a specimen of the culture is taken with a platinum loop and put on a slide, cleared with one or two drops of KOH or stained with lactophenol cotton blue, which stains the fungal elements in blue, and covered with a cover slip.

The first step of the culture microscopic examination is to reveal if the specimen contains hyphal fungi (septate or not septate) or yeasts (pseudohyphae, budding cells). In the case of yeasts, it is not usually possible to complete identification under the microscope, and therefore a switch to other techniques is necessary, as previously mentioned (implantation on selective medium, sugar fermentation and assimilation test, nitrogen utilization).

The *second step* is the detailed description of the mycelia elements configurations (Tables 123.6 and 123.7). Some hyphae are very straight, others sinuous, slender, or thick, with periodical enlargement of single enchained elements and/or swelling at the tips of the filaments. These specialized, ornamental or accessory structures, arising from a single cell modification into a thick-walled resting cell, with a very peculiar morphology aid in species identification: spiral and racquet hyphae are commonly seen in *Trichophyton mentagrophytes*; pectinate body occurs in *Microsporum audouinii*; chandelier hyphae occur in *Trichophyton schoenleinii* and *Trichophyton violaceum* infections, and nodular organ characterize *Trichophyton mentagrophytes* and *Microsporum canis*.

Although suggestive, the accessory structures are changeable, and the most important elements to note are the presence of fruiting bodies (spores, chlamydospores, asci, aleuriospores, etc.). In the case of dermatophytes, the micro- and macroconidia configurations, arrangement, and shape make identification certain. Additional biochemical tests

Table 123.5 Additional tests to discriminate the main pathogen *Candida species*

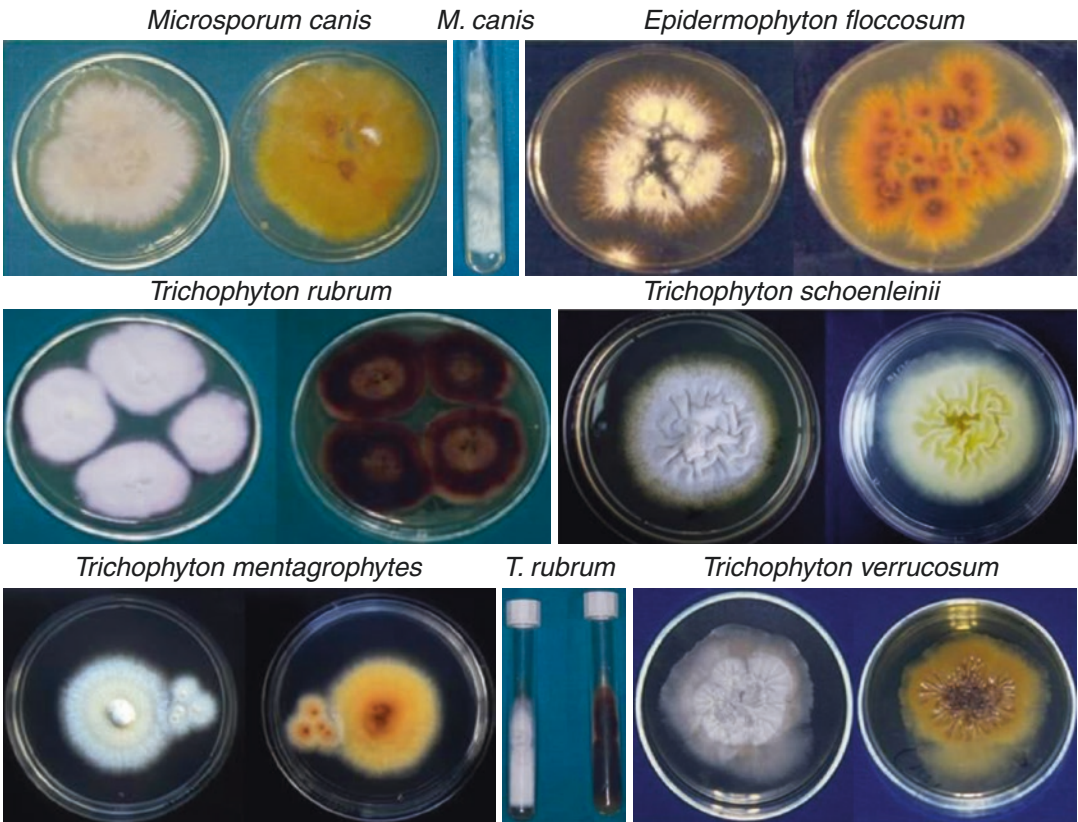| Species	Fermentation test (zymogram)						Assimilation test (auxanogram)						
	Glucose	Galactose	Saccharose	Maltose	Lactose	Raffinose	Glucose	Galactose	Saccharose	Maltose	Raffinose	Trehalose	KNO$_3$
C. albicans	+	±	–	+	–	–	+	+	+	+	–	+	–
C. pseudotropicalis	+	+	+	+	–	+	+	+	+	+	–	/	–
C. parapsilosis	+	+	+	+	–	+	+	+	+	+	–	/	+
C. tropicalis	+	+	+	+	–	–	+	+	+	+	–	/	–
C. guilliermondii	+	±	+	–	–	+	+	+	+	+	–	/	–
C. stelloidea	+	–	–	+	–	–	+	+	–	+	–	/	–
C. krusei	+	–	–	–	–	–	+	–	–	–	–	–	–
Torulopsis glabrata	+	–	–	–	–	–	+	+	–	–	–	+	/

Fig. 123.8 Gross morphology of *dermatophyte* colonies

Fig. 123.9 Gross morphology of *Candida* colonies on Sabouraud's agar

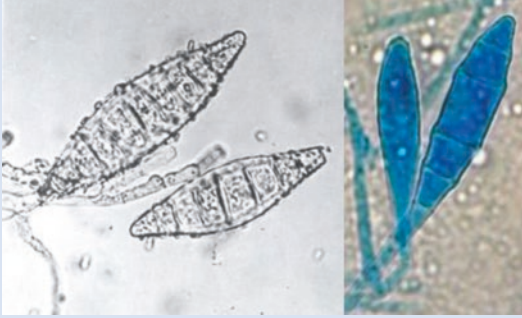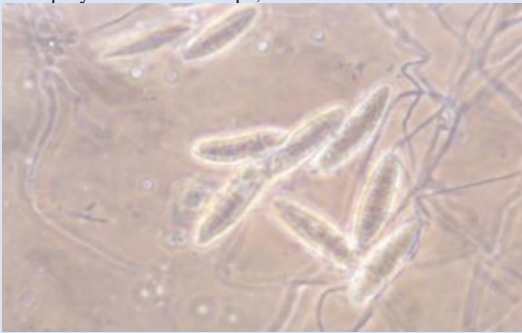**Table 123.6** Some examples of the main dermatophyte species characteristics on culture identification process

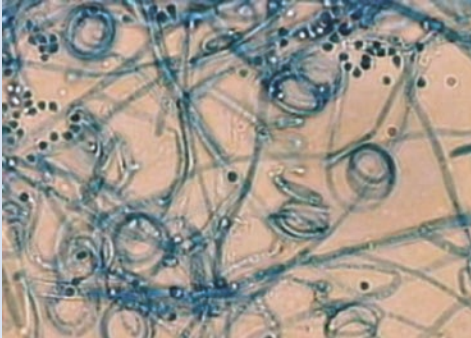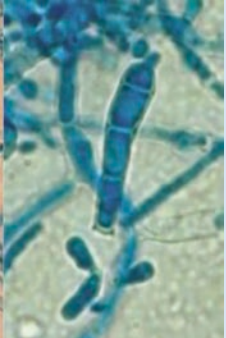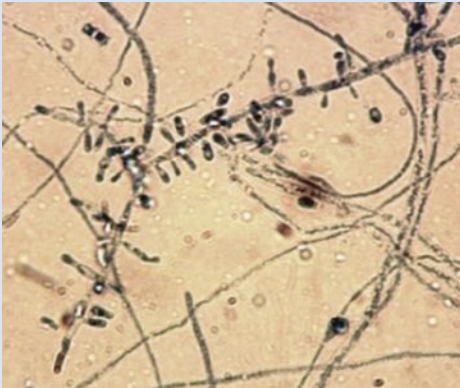| Dermatophyte	Culture morphology	Accessory structures and fruiting bodies
Microsporum canis (zoophilic fungus)	Colonies grow at moderate rates and are cottony or wooly with whitish center and lemon-yellow pigment at the periphery; radial grooves may also be present. The reverse is lemon-yellow, becoming yellow-brown with age	Few pyriform microconidia; numerous large macroconidia with thick rough walls, spiky, boat-like septate (navicular) with more than 6 cells (multilocular)
Microsporum gypseum (geophilic fungus)	Colonies grow moderately rapidly with dusty granular to sugar texture, whitish surface darkening to cinnamon brown, especially on the reverse	Macroconidia are more numerous than *M. canis*, but ellipse-like shaped, less spiky with rounded tips, and fewer cells
Microsporum audouinii (anthropophilic fungus)	Colonies grow at moderate rates and are gray-white, flat, or velvety, with a characteristic light pink to brown reverse	Usually no microconidia are present, but the presence of pectinate hyphae and terminal chlamydospores suggests the identification. Macroconidia are fusiform, beaked, and septated, with irregular intervals
Trichophyton mentagrophytes (zoophilic fungus)	Colony grow slowly, with variable appearance, usually white-creamy with a shielded center and dusty periphery; the reverse may be tan-brown, yellow, or red similar to *T. rubrum*	Numerous round or pyriform microconidia arranged in grape-like clusters, presence of spiral hyphae. Macroconidia are cylindrical and septate

Table 123.6 (continued)
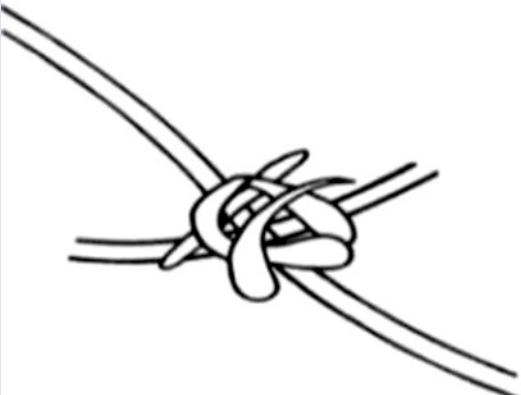
Dermatophyte	Culture morphology	Accessory structures and fruiting bodies
Trichophyton rubrum. (anthropophilic fungus)	Colonies grow slowly; with a convex central surface (like a shield boss or umbo) and dusty periphery; the colonies are white pigmented with a characteristic wine red reverse	Numerous grape-like microconidia and aleuriospores arranged along thick unbranched hyphae; macroconidia are rare, cylindrical or clavate, and multiseptate
Trichophyton schoenleinii (anthropophilic fungus)	White shielded surface, with yellow pigment on the reverse	There are no micro- or macroconidia; divergent branching and swellings at the hyphae tips producing chandelier or favus accessory structures are useful for the identification
Trichophyton verrucosum (zoophilic fungus)	Colonies with a velvety convoluted surface, creamy to ochre in tan; the reverse may be colorless or yellow	Hyphae with swollen tips, and symmetrical arthrospores arranged like a string of beads. True pyriform microconidia and rattail macroconidia are rare
Trichophyton tonsurans (anthropophilic fungus)	Colonies mature slowly and have variable color, texture, and shape; usually yellow-brown tan	Clavate to elongate microconidia; some are usually swollen and attached to conidiophores by short stalks. Macroconidia are rare
Epidermophyton floccosum (anthropophilic fungus)	Colonies are yellow-khaki or olive green with flat aerial surface conveying a soft leather appearance (suede)	Microconidia are absent; very numerous bunches of clavate or club-shaped macroconidia (banana-like) with 2–6 cells and smooth walls

Table 123.7 Specialized accessory features help to further distinguish several dermatophyte species

Accessories structure	Morphology	Dermatophyte species
Spiral hyphae: subtle filaments coiled on their axis spirally		*Trichophyton mentagrophytes*
Pectinate body: short, unilateral projections from the hyphae that resemble a broken comb		*Microsporum audouinii*
Favic chandelier: hyphal tips grouped to resemble a chandelier or the antlers of the deer (antler hyphae)		*Trichophyton schoenleinii and Trichophyton violaceum*
Nodular organ: enlargement in the mycelium that consists of closely twisted hyphae		*Trichophyton mentagrophytes and Microsporum canis*
Racquet hyphae: regular enlargement of one end of each segment with the opposing end remaining thin		*Epidermophyton floccosum, Trichophyton mentagrophytes*

may be helpful in distinguishing *T. rubrum* from *T. mentagrophytes*: milk agar medium favors *T. mentagrophytes* growth, while *T. rubrum* is restricted; *T. mentagrophytes* is urease positive while *T. rubrum* is urease negative.

Culture Pitfalls

A negative culture may arise because:
- The condition is not due to fungal infection.
- The specimen was not collected properly, especially as regards sterile procedure and eventual bacterial contamination.
- Antifungal treatment had been used prior to collection of the specimen.
- There was a delay before the specimen reached the laboratory.
- The laboratory procedures were incorrect.
- The organism grows very slowly.

It is advantageous to collect several samples from the patient, to eventually repeat the cultural examination when a fungal infection appears likely, preferably prior to treatment.

Another major problem is false-positive results due to contamination, especially *molds development* in nail specimens. Molds are ubiquitous and their etiologic role in onychomycosis is very controversial. Onychomycosis due to opportunistic fungi, especially *Scopulariopsis brevicaulis*, have no specific clinical features, and simple isolation from the nails is not sufficient to assess infection. As a general rule, the suspected pathogen fungus must be detected at least in three different samples of the pathologic material, obtained from several different sites, plated out on at least three different media, including Sabouraud's agar + actidione.

Other Complementary Examinations in Mycology

Wood's Lamp Examination

This very simple examination introduced in medical mycology in 1925 by Margarot and Devèz is no more very popular in routine use, requiring a source of ultraviolet rays filtered with a 9–10 % nickel salt sheet of glass (366 nm wavelengths allowed to pass). The examination is performed in a dark room and natural luminescence phenomenon is limited to *microsporum species (*green fluorescence*)*, *pityriasis versicolor* (yellow green), and erythrasma (reddish fluorescence). It is otherwise useful to rapidly distinguish the malassezia infection from other skin coloring changes such as vitiligo and to detect tinea capitis during epidemics, especially in schools.

Histological Examination

Biopsy and histological examination does not replace native preparation and usually is unnecessary in superficial fungal infections, but it is sometimes performed to exclude major skin diseases and superinfections, especially if dealing with yeasts. Histological examination allows for primary candidiasis to be distinguished from superinfection, by the presence of a parakeratotic horny layer, stuffed with PAS-positive yeast cells and pseudohyphae.

It is otherwise mandatory to confirm the clinical suspect of rare deep-seated or disseminated fungal diseases, such as actinomycosis, cryptococcosis, histoplasmosis, and other infections. It is also effective to confirm a pathologic role of opportunistic fungi and their relation with the infected tissue. Histology is superior to the other methods in its negative predictive value. The combination of direct microscopy plus histology was 97.8 % sensitive with 98 % negative predictive value and remains the most sensitive diagnostic approach for onychomycosis.

The specific request must be clearly indicated to the pathologist, because simple hematoxylin-eosin stains give more information on the tissue reaction pattern than on the fungal presence and require a very careful examination. It rarely enables a more precise diagnosis than simple mycosis and the fungus cannot be identified as a rule. The most used stains are Hotchkiss-McManus stain (Periodic Acid Schiff reagent, PAS) in all conditions, May-Grunwald-Giemsa and Gram stain to visualize very fine Gram-positive filaments (actinomycosis, nocardiosis), and methenamine silver nitrate staining by Gomori's method, which is particularly useful to fungal membrane detection.

Electron Microscopy

The extensive use of both transmission and scanning electron microscopy is devoted to research on the morphology and physiology of the various pathogenic fungi. It does not play a role in diagnostic procedures.

Future Perspectives

Mycology is sometimes considered an obsolete assessment, because clinical diagnosis is usually simple and antifungal treatment highly efficacious. Laboratory exam has relied on direct examination for such a long time that interest on research and implementation has been limited for decades. The sensibility of the traditional mycological examinations is not very high and conditioned from the investigator's expertise. The diagnostic error accounts for about 30 % of the cases. Therefore, last decade's research has devoted a certain interest to faster, more sensitive, and culture-independent techniques. The molecular biology techniques, especially the *polymerase chain reaction (PCR)* and *in situ hybridization* for fungal identification, are highly sensitive but expensive and require complex post-amplification procedures to differentiate relevant fungal types. At the moment, research is limited to deep-seated and disseminated fungal infections (blood infections, drug-resistant *Candida* and *Aspergillus* species) or to severe special site infections (fungal keratitis). Preliminary data using real-time polymerase chain reaction high-resolution melting analysis (PCR-HRM) suggest the capacity to detect and differentiate yeasts from filamentous fungi and to discriminate among relevant species of yeasts.

Current limitation of the molecular assays is the lack of methodological standardization and validation, with continuous new method proposals whose results interpretation are not always clear and reproducible.

New impulse to collect samples and make correct fungal identification arises from the *assay mass spectrophotometry*, which seems to be a cost-effective rapid tool and will probably be largely adopted as routine laboratory determination in an immediate future.

Wide consensus and validation from large prospective studies are necessary to allow widespread adoption of these assays into the clinical setting, but the basic approach with direct microscopy remains the clue to the diagnosis of superficial skin fungal infections.

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