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Histopathologic Diagnosis of Fungal Infections in the 21st Century

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INTRODUCTION

Fungal infections are becoming more frequent because of expansion of at-risk populations and use of treatment modalities that permit longer survival of these patients (109). Some of the changes in endemic fungal infections can be attributed to climate changes, extension of human habitats, ease of travel, and shifting populations. At-risk populations for opportunistic fungal infections or disseminated endemic fungal infections include patients who have received transplants, those prescribed immunosuppressive and chemotherapeutic agents, HIV-infected patients, premature infants, the elderly, and patients undergoing major surgery. Thus, a shift in the mycoses encountered in the health care setting has occurred. Prior to the 21st century, bloodstream infections were more frequently caused by *Candida* spp., and agents of invasive pulmonary infections included primarily endemic mycoses and *Aspergillus* spp. Today, fungi previously considered nonpathogenic, including mucoraceous genera (formerly called zygomycetes) and a variety of both hyaline and dematiaceous molds, are

commonly seen in immunocompromised patients. In addition, diagnosis of infection versus colonization with these fungi is a frequent problem that has important treatment implications for these patients. Furthermore, advances in diagnostic radiology and in patient support (such as platelet transfusions, etc) have allowed greater ability to pursue specific diagnoses by collecting tissue biopsy specimens from body sites formerly not available for histopathologic examination.

The advantages of obtaining these specimens have created a series of diagnostic challenges because of the limited amount of tissue obtained and the architectural distortion produced by these new procedures. In addition, the therapeutic armamentarium now available and the presence of resistance of these fungi to different drugs have compounded the diagnostic challenges. Histopathology continues to be a rapid and cost-effective means of providing a presumptive or definitive diagnosis of an invasive fungal infection. However, the use of fungal silver impregnation stains (Grocott or Gomori methenamine silver [GMS]) cannot alone solve these challenges, and newer diagnostic techniques may be required. Microbiologists, pathologists, and clinicians need to be aware of the limitations of tissue diagnosis, the pitfalls of morphological diagnosis, and the tests that can be performed with tissue and other samples to make organism-specific diagnoses. In this review we present epidemiologic, clinical, and morphological findings and interpretation pitfalls regarding the most frequently encountered yeasts and molds, as well as alternative testing that can be performed with other samples. Table 1 summarizes the clinical presentations and host reactions produced by the mycoses discussed in this review, Fig. 1 to 3 summarize the morphologies of these fungi and the differential diagnoses for each, and Table 2 summarizes the alternate testing that can be performed with specimens that were not sent to the pathology laboratory. Additionally, we present methods that can be used for diagnosis of specific yeasts and molds in formalin-fixed, paraffin-embedded tissue submitted for histopathologic diagnosis, as well as a series of scenarios that should help guide the diagnosis and treatment of patients with mycoses.

MORPHOLOGIES OF ORGANISMS IN TISSUES IN VARIOUS DISEASES

Diseases Where Yeasts or Yeast-Like Structures Are Usually Seen in Tissues

Blastomycosis. (i) Epidemiologic and clinical situations when blastomycosis should be considered in the differential diagnosis. Blastomycosis was first described by Gilchrist at the end of the 19th century as a dermatologic infection caused by a protozoan (58). Today we know it is caused by the dimorphic fungus *Blastomyces dermatitidis*, and the majority of cases involve primarily lung disease. The organism has been isolated from moist soil rich in organic debris in the Mississippi and Ohio River valleys and around the Great Lakes and the Saint Lawrence River, which include multiple states in the United States (southeastern, south central, and upper midwestern states) and several Canadian provinces (99). Blastomycosis was so prevalent in Chicago that it has been referred to as "Chicago disease" (118); however, sporadic cases have been reported from areas where the disease is not endemic, such as Colorado, Texas, Kansas, and Nebraska in North America, and from other countries around the world (150).

Inhalation of the *B. dermatitidis* conidia is the usual route of infection, and a variety of responses can occur in the lung, including asymptomatic infections, acute and chronic pneumonia, and fatal acute respiratory distress syndrome (ARDS) (99, 118). In general, acute pneumonia is rarely identified outside outbreaks. More frequently, pulmonary blastomycosis is diagnosed in cases of chronic pneumonia or when a lung neoplasia is suspected. In 20 to 40% of cases the disease has become systemic at the time of diagnosis and there is skin, soft tissue, bone, genitourinary (GU), or central nervous system involvement (9, 87, 99, 118). In rare cases skin lesions without lung involvement have been described, suggesting direct cutaneous inoculation. The skin lesions most frequently described are painless ulcers or verrucous lesions. Blastomycosis in immunocompromised patients appears to be more severe and more frequently fatal (99). It should be noted that in patients with central nervous system involvement, diabetes mellitus is an important predisposing factor (9).

(ii) Morphological characteristics that set blastomycosis apart. *B. dermatitidis* in tissue appears as yeasts that measure 8 to 15 μm in diameter, have thick refractile cell walls, and may show a single, broad-based bud (Fig. 1). The yeasts can be observed in a variety of specimens, including sputum, bronchoalveolar lavage (BAL) fluid, fine-needle aspirates from lung, skin, or other lesions, cerebrospinal fluid (CSF), and surgical resections (9, 87). The thick refractile cell wall of this organism gives the appearance of a space between the fungal cell contents and the surrounding tissue when hematoxylin and eosin (H&E) stain is used. Inside the cell wall, the multiple nuclei of the yeast stain with hematoxylin. Occasionally, *B. dermatitidis* can show smaller yeast forms, the so-called microforms. In addition, *B. dermatitidis* can be seen with a variety of routinely used preparations and stains such as KOH and Papanicolaou stain. The contour of the yeast is best highlighted by staining the cell wall with fungal silver stains such as GMS or periodic acid-Schiff (PAS) stain (87, 118). The inflammatory reaction accompanying the yeasts is primarily granulomatous with various degrees of neutrophilic infiltrate; thus, it has been described as pyogranulomatous inflammation (118). It needs to be remembered that blastomycosis can be concomitantly present with neoplasias and tuberculosis.

(iii) Pitfalls in morphological diagnosis. Few studies have systematically compared the presence of broad-based budding yeasts in histopathologic or cytologic specimens with culture or other diagnostic methods that would confirm the diagnosis of blastomycosis. A retrospective study of 53 patients showed that *Coccidioides immitis*, *Candida albicans*, or *Aspergillus* was recovered from 4 pathologic specimens (10%) demonstrating broad-based budding yeasts in direct histopathologic examination (118). An earlier study of patients with blastomycosis commented that a high percentage of their cultures were overgrown with *Candida* (87). This suggests that not all broad based-budding yeasts in the 8- to 15-μm size range are *Blastomyces*. Since histopathologic or cytologic results can usually be provided before the culture is available, there is pressure to use these results to guide treatment, particularly because *B. dermatitidis* can take up to 3 weeks to grow or may not grow at

TABLE 1. Clinical presentation of and host reaction to the more common mycoses

Fungus(i)	Clinical presentation	Host response[a]	Comment(s)
Blastomyces dermatitidis	Asymptomatic	Tissue descriptions unavailable	Epidemiologic evidence
	Acute pneumonia	Mixed suppurative[b] inflammation	Seen in areas of endemicity
	Chronic pneumonia	Mixed suppurative and granulomatous[c] inflammation	Most frequently diagnosed
	ARDS	Diffuse alveolar damage	Can be fatal
	Disseminated	Various inflammatory responses depending on immune status	Skin, soft tissue, bone, GU, or CNS is primarily involved
	Cutaneous	Mixed suppurative and granulomatous inflammation	Rare, due to direct cutaneous inoculation
Cryptococcus spp.	Asymptomatic	Minimal reaction	Rare, epidemiologic evidence
	Pneumonia	Predominantly granulomatous inflammation, can have abundant fibrosis	More frequent in immunocompetent individuals
	Cryptococcoma	Granuloma with various degrees of necrosis and fibrosis	More frequent with *C. gattii*
	Pleural effusion	Various inflammatory responses depending on immune status	More frequent in immunosuppressed patients
	Disseminated	Various inflammatory responses depending on immune status, abundant extracellular yeasts may efface tissue architecture, necrosis may be present	Frequent in immunosuppressed patients; involves the CNS (producing meningitis or cryptococcomas), skin, bones, or other tissues
Histoplasma capsulatum	Asymptomatic	Tissue descriptions not available	Occurs when low numbers of microconidia are inhaled
	Acute pneumonia	Nodules showing vascular necrosis associated with lympho-histiocytic vasculitis and rare granulomatous inflammation	Occurs when high numbers of microconidia are inhaled
	ARDS	Diffuse alveolar damage	Can be fatal
	Mediastinitis	Granulomatous inflammation	Occurs upon initial inhalation of the microconidia
	Chronic pneumonia	Granulomas with various degrees of necrosis and calcification	Can present as a nodule or cavity
	Disseminated	Various inflammatory responses depending on immune status, abundant intracellular yeasts may efface tissue architecture, necrosis may be present	Seen upon initial infection or as reactivation of latent disease in patients with T-cell deficiencies; can involve skin, GI tract, liver, spleen, and bone marrow
Coccidioides immitis/posadasii	Asymptomatic	Tissue descriptions unavailable	Epidemiologic evidence
	Acute pneumonia	Suppurative and granulomatous inflammation	Presents as lobar infiltrates and adenopathy
	Chronic pneumonia	Mixed suppurative (including eosinophils) and granulomatous inflammation with a rim of lymphocytes, Splendore-Höeppli phenomenon likely	Can present as a nodule or cavity
	Disseminated	Various inflammatory responses depending on immune status	Occurs in certain risk groups (those with diabetes, use of steroids, and others); can involve skin, lymph nodes, bones, joints, and CNS
	Cutaneous	Mixed suppurative and granulomatous inflammation	Rare, due to direct cutaneous inoculation
Candida spp.	Superficial infections	Minimal to suppurative inflammation depending on immune status of individual	Skin and mucous membranes of GI and GU tracts in immunocompetent and immunosuppressed individuals
	Invasive disease	Various inflammatory responses depending on immune status, primarily suppurative inflammation with rare granulomas, invasion of blood vessels, necrotizing vasculitis	Occurs primarily as a health care-associated infection (patients with vascular access devices, with recent surgeries, receiving broad-spectrum antibiotics, or immunosuppressed), can involve all organs
Pneumocystis jirovecii	Asymptomatic	Minimal reaction	Has been found in the lungs of children
	Pneumonia	Minimal reaction; rarely atypical reactions such as fibrosis, granulomas, and others	Affects immunosuppressed patients
	Disseminated	Minimal reaction; rarely atypical reactions such as fibrosis, granulomas, and others	Rare, affects immunosuppressed patients
Sporothrix schenckii	Cutaneous	Mixed suppurative (including eosinophils) and granulomatous inflammation, Splendore-Höeppli phenomenon frequent, presence of asteroid bodies, epidermis with pseudoepitheliomatous hyperplasia	Associated with handling contaminated soil or animals, draining lymph nodes are frequently affected
	Disseminated	Various inflammatory responses depending on immune status	Affects bone, joints, meninges, and other organs
Penicillium marneffei	Cutaneous	Mixed suppurative and granulomatous inflammation with various degrees of necrosis	Seen mostly in Southeast Asia
	Disseminated	Various inflammatory responses depending on immune status, may consist only of necrosis and infected macrophages	Seen mostly in Southeast Asia; *P. marneffei* infection represents the most frequent AIDS-defining illness

Paracoccidioides brasiliensis	Acute pneumonia	Mixed suppurative and granulomatous inflammation	Correlates with hormonal, genetic, immunologic, and nutritional status
	Chronic pneumonia	Mixed suppurative and granulomatous inflammation surrounded by fibrosis	Correlates with hormonal, genetic, immunologic, and nutritional status; if organism is swallowed, can cause GI disease
	Disseminated	Mixed suppurative and granulomatous inflammation, bone may show osteonecrosis	Involvement of bone marrow, adrenal glands, CNS, and other tissues
	Cutaneous	Mixed suppurative and granulomatous inflammation, epidermis with epitheliomatous hyperplasia	Rare, due to direct cutaneous inoculation
Rhinosporidium seeberi[d]	Nose, nasopharynx, ocular areas	Granulomatous inflammation with fibrosis and granulation tissue	Presents as a mass or polyp
	Disseminated	Chronic and granulomatous inflammation	Rare, can involve other mucous membranes or cutaneous sites and internal organs
Aspergillus spp.	Allergic bronchopulmonary aspergillosis	Allergic mucous with eosinophils, Curshmann's spirals, Charcot-Leyden crystals; mucosa with suppurative and granulomatous inflammation, vasculitis, and fibrosis	Hypersensitivity reaction to fungi, most frequently *A. fumigatus*; seen frequently in patients with cystic fibrosis or steroid-dependent asthma
	Allergic fungal rhinosinusitis	Similar to that for allergic bronchopulmonary aspergillosis	Hypersensitivity reaction to fungi similar to that for allergic bronchopulmonary aspergillosis
	Chronic pulmonary aspergillosis	The wall surrounding the fungus ball consists of fibrosis	Occurs in immunocompetent individuals with a variety of lung conditions (tuberculosis, emphysema, and others) in which the cavity or lesion is colonized and then a "fungus ball" or aspergilloma forms
	Chronic necrotizing pulmonary aspergillosis	The wall surrounding the fungus ball consists of a layer of necrosis, granulation tissue, granulomatous inflammation, and fibrosis	Occurs in immunosuppressed individuals with chronic pulmonary aspergillosis, where the fungus is invading the tissues locally
	Invasive disease	Angioinvasion by hyphae with consequent necrosis or hemorrhage of surrounding tissue	Seen in severely immunosuppressed patients, involves the lungs, CNS, and other tissues
Mucorales genera	Cutaneous	Angioinvasion by hyphae with consequent necrosis or hemorrhage of surrounding tissue; inflammation, if present, is frequently suppurative, less commonly granulomatous, but varies depending on immune status	Necrotic (black) skin lesion in immunosuppressed patients
	Rhinocerebral	Similar to that for cutaneous disease	Particularly frequent in diabetic patients but can occur in any immunosuppressed patient
	Pulmonary	Similar to that for cutaneous disease	Multiple pulmonary nodules and pleural effusions in immunosuppressed patients
	Invasive disease	Similar to that for cutaneous disease	Risk factors include cancer chemotherapy and stem cell transplantation
Entomophthorales	Mucocutaneous	Fibrosis, granulation tissue, mixed eosinophilic and granulomatous inflammation; Splendore-Höeppli phenomenon present	Presents as a mass in immunocompetent individuals, the lesion can be in the GI tract
Hyaline septated molds (*Fusarium* spp., *Scedosporium* spp., *Trichoderma* spp., *Paecilomyces* spp., and others)	Superficial infections	Mild inflammation	Occur in skin, cornea, and nails; *Fusarium* spp. are the most common of these organisms in causing superficial infections
	Range of diseases similar to that for *Aspergillus*: allergic, chronic pulmonary, and invasive	Similar to that for *Aspergillus*	Some organisms have some peculiarities (for example, *Scedosporium* spp. are associated with pneumonia after near drowning, and *Trichoderma* spp. have been observed in patients undergoing dialysis)
Dematiaceous fungi (*Madurella* spp., *Fonsecaea* spp., *Cladophialophora* spp., *Exophiala* spp., *Curvularia* spp., *Bipolaris* spp., and others)	Superficial infections	Mild inflammation	Occur in skin, cornea, and nails
	Deep skin infections	Mixed suppurative and granulomatous inflammation with reactive epidermal changes, including pseudoepitheliomatous hyperplasia and draining sinuses	See Table 3
	Range of diseases similar to that for *Aspergillus*: allergic, chronic pulmonary, and invasive	Similar to that for *Aspergillus*	*Bipolaris* and *Curvularia* are most frequently associated with eosinophilia and allergic sinusitis or allergic bronchopulmonary mycosis, *Cladophialophora bantiana* is most frequently associated with brain abscesses

[a] Fungal morphology is presented in Fig. 1, 2, and 3.
[b] Suppurative inflammation refers to presence of congestion, edema, necrosis, and an inflammatory infiltrate with a predominance of neutrophils.
[c] Granulomatous inflammation refers to presence of epithelioid macrophages including multinucleated giant cells, lymphocytes, and necrosis.
[d] *R. seeberi* is not a fungus but is included for purposes of contrast with fungi that have similar morphology.

TABLE 2. Alternative testing that can be performed with nontissue specimens based on the morphology present in tissue and the suspected fungi

Morphology using H&E, GMS, and PAS staining	Suspect fungus(i)	Alternative test with nontissue samples	Comment(s)
Broad-based budding yeasts (10–15 μm)	*Blastomyces dermatitidis*	Antigens in urine or serum	EIA antigen assay for urine, serum, BAL fluid, or CSF is available at MiraVista Diagnostics (Indianapolis, IN) (sensitivity has been reported to be around 90%, but this test should be performed simultaneously with *Histoplasma* testing because of cross-reactivity between the two antigens); EIA can be used to follow response to treatment
		Serology	There is poor sensitivity (9 to 28%) and specificity if using complement fixation and immunodiffusion, radioimmunodiffusion and EIAs have better sensitivity (77 to 83%) and specificity (95%) but are not commercially available
		Cultures of sputum or BAL fluid	Diagnostic yields of 86 to 92%, respectively; however, delays in diagnosis are inherent to the technique
Narrow-based budding yeasts (4–10 μm) with a thick capsule	*Cryptococcus* spp.	Cryptococcal antigen	Sensitivity of >90% but may have up to 7% false-positive results; latex agglutination and EIA with serum and CSF are FDA approved, but testing in urine is not; laboratories can perform the test with urine samples if they validate for the sample type
		Cultures of blood, CSF, and other fluids	Use of canavanine-glycine-bromthymol blue medium is helpful to distinguish *Cryptococcus gattii* from *C. neoformans*
Small yeasts (2–4 μm) with narrow-based budding grouped in clusters inside macrophages	*Histoplasma capsulatum*	Antigens in urine or serum	Several commercial assays are available; sensitivity is about 90% in cases of disseminated disease and 75% during acute pulmonary disease; false-positive results occur in cases with other endemic mycoses, with blastomycosis being the most important overlap; *Aspergillus* galactomannan also cross-reacts
		Serology	Accomplished by the use of complement fixation or immunodiffusion with about 80% sensitivity, useful for immunocompetent individuals but may not be useful when the patient is immunosuppressed
		Cultures of blood and other fluids	It is important to use lysis-centrifugation to release the organisms from phagocytic cells; although specific, culture may take up to 6 weeks
Spherules with multiple endospores	*Coccidioides immitis/posadasii*	Antigens in urine or serum	An EIA for *Coccidioides* antigen in urine, serum, and CSF is commercially available (MiraVista Diagnostics, Indianapolis, IN); studies using rabbit antibodies against a *Coccidioides* galactomannan in an EIA showed that 71% of patients with coccidioidomycosis have antigenuria; however, EIA cross-reacts in 10% of patients with other endemic mycoses
		Serology	Measurement of combined IgM and IgG by EIA shows a sensitivity and specificity of >95%, immunodiffusion can also be used to measure IgM and IgG
		Cultures of any sample	*Coccidioides* is a select agent with stringent culture regulations
		DNA detection in sputum	PCR detection of ITS2 or Ag2/PRA targets has been published, showing good sensitivity, but assays are not commercially available
Small yeasts (3–5 μm) intermingled with pseudohyphae and/or hyphae	*Candida* spp.	Detection of beta-D-glucan in serum	Sensitivity will vary between 57 to 90% depending on the patient population and cutoff values used for the assay, specificity ranges from 44 to 92%
		Cultures of blood and other fluids	Blood cultures occur in 50 to 70% of cases, and when positive, they indicate invasive disease; peptide nucleic acid fluorescent *in situ* hybridization assay (Yeast "Traffic Light" PNA-FISH assay; AdvanDx Inc., Woburn, MA) of positive blood culture smears can identify the most frequent *Candida* spp. without the need for subcultures
		DNA detection in whole blood, serum, or plasma	Several PCRs have been developed and validated; they seem to have increased sensitivity compared to blood cultures, but the clinical significance of these findings is still being researched

Continued on following page

TABLE 2—*Continued*

Morphology using H&E, GMS, and PAS staining	Suspect fungus(i)	Alternative test with nontissue samples	Comment(s)
Nonpigmented (hyaline), septated hyphae with acute-angle branching	*Aspergillus* spp., *Fusarium* spp., *Scedosporium* spp., *Trichoderma* spp., and *Paecilomyces* spp.	Detection of galactomannan in serum or BAL fluid	Galactomannan antigen detection using commercially available *Aspergillus* EIA (Platelia *Aspergillus* test; Bio-Rad, Hercules, CA) has sensitivities that range from 40 to 100% and specificities from 56 to 100%, depending on the patient population; false-positive results occur in 50% of patients taking certain antibiotics or plasmalyte or having other fungal infections; other genera in this morphological category do not have specific alternative tests, although galactomannan has been shown to be positive in patients with *Paecilomyces* infections
		Detection of beta-D-glucan in serum	Measured using commercially available EIA (Fungitell kit; Associates of Cape Cod, East Falmouth, MA) with sensitivities that range from 50 to 100% and specificities of from 44 to 98%, the presence of beta-glucan is not specific for particular fungal genera
		Cultures of blood	*Fusarium* spp. may have positive blood cultures, other genera are rarely cultured from the blood, even in the presence of invasive disease
Nonpigmented (hyaline), pauciseptate ribbon-like hyphae with right-angle branching	*Mucorales* genera	Serology	Serologic tests for invasive disease have been attempted but have not been clinically useful
		Culture of blood	*Mucorales* genera are rarely recovered from blood, even with invasive disease
Pigmented irregular hyphae and yeast-like structures both with septations	Dematiaceous fungi, including *Madurella* spp., *Fonsecaea* spp., *Cladophialophora* spp., *Exophiala* spp., *Curvularia* spp., *Bipolaris* spp., and others	Serology	Not available
		Culture of blood or other fluids	These genera are rarely cultured from the blood, even in the presence of invasive disease

all from these specimens. The sensitivity of culture varies depending on the sample that was obtained and may range from 62% to 100% (87, 96). The diagnostic yield of histopathology will depend on the expertise present in the center where the patient is seen (99). Because of the possibility of histopathologic false-positive results, pathologists should describe the yeast and budding pattern that are observed in the tissue specimen and should list the yeasts that can have this morphology in the report comment field (Fig. 1). In addition, alternative tests are necessary to determine that the patient truly has blastomycosis, especially in cases from areas where the disease is not endemic or when the clinical picture is not typical.

(iv) Alternative testing. Blastomycosis antigens can be detected in the urine and serum by using an enzyme immunoassay (EIA). The sensitivity and specificity for antigen detection have been reported to be above 90%; however, cross-reactivity occurs in patients with histoplasmosis, paracoccidioidomycosis, and penicilliosis due to *Penicillium marneffei* (50). Because of the cross-reactivity, it is important that antigen tests for both blastomycosis and histoplasmosis be ordered. Detection of antibodies to *B. dermatitidis* in serum using traditional complement fixation and immunodiffusion has poor sensitivity and specificity; however, as antigens have been better purified and used in radioimmunodiffusion and EIAs, the sensitivity and specificity of serology have significantly improved (99). Cultures of the lesion should be encouraged, particularly in regions where the disease is not endemic.

Cryptococcosis. (i) Epidemiologic and clinical situations when cryptococcosis should be considered in the differential diagnosis. Human cryptococcosis is caused by several *Cryptococcus* species, including *C. neoformans* and *C. gattii*. *C. neoformans* is responsible for the majority of infections found in immunocompromised individuals, while *C. gattii* causes infections in immunocompetent and compromised hosts (72). *C. neoformans* var. *grubii* (serotype A) and *C. neoformans* var. *neoformans* (serotype D) have a worldwide distribution and are found primarily associated with pigeon guano. HIV infection is the most frequent predisposing factor for cryptococcal disease; however, other conditions are associated with cryptococcosis, including underlying lung, liver, or renal disease, immunosuppressant use, malignancies, and autoimmune diseases. *C. gattii* (serotypes B and C) has been found in eucalyptus and other tropical and subtropical trees in a more limited geographic distribution, including Australia, Papua New Guinea, parts of Africa, Mexico, and southern California. In 1999, *C. gattii* was first reported as a human pathogen in Vancouver Island, Canada, and it has now been found to cause disease in other areas in British Columbia, Canada, and in the northwest United States (Washington and Oregon) (53).

Independent of the species, humans inhale cryptococcal

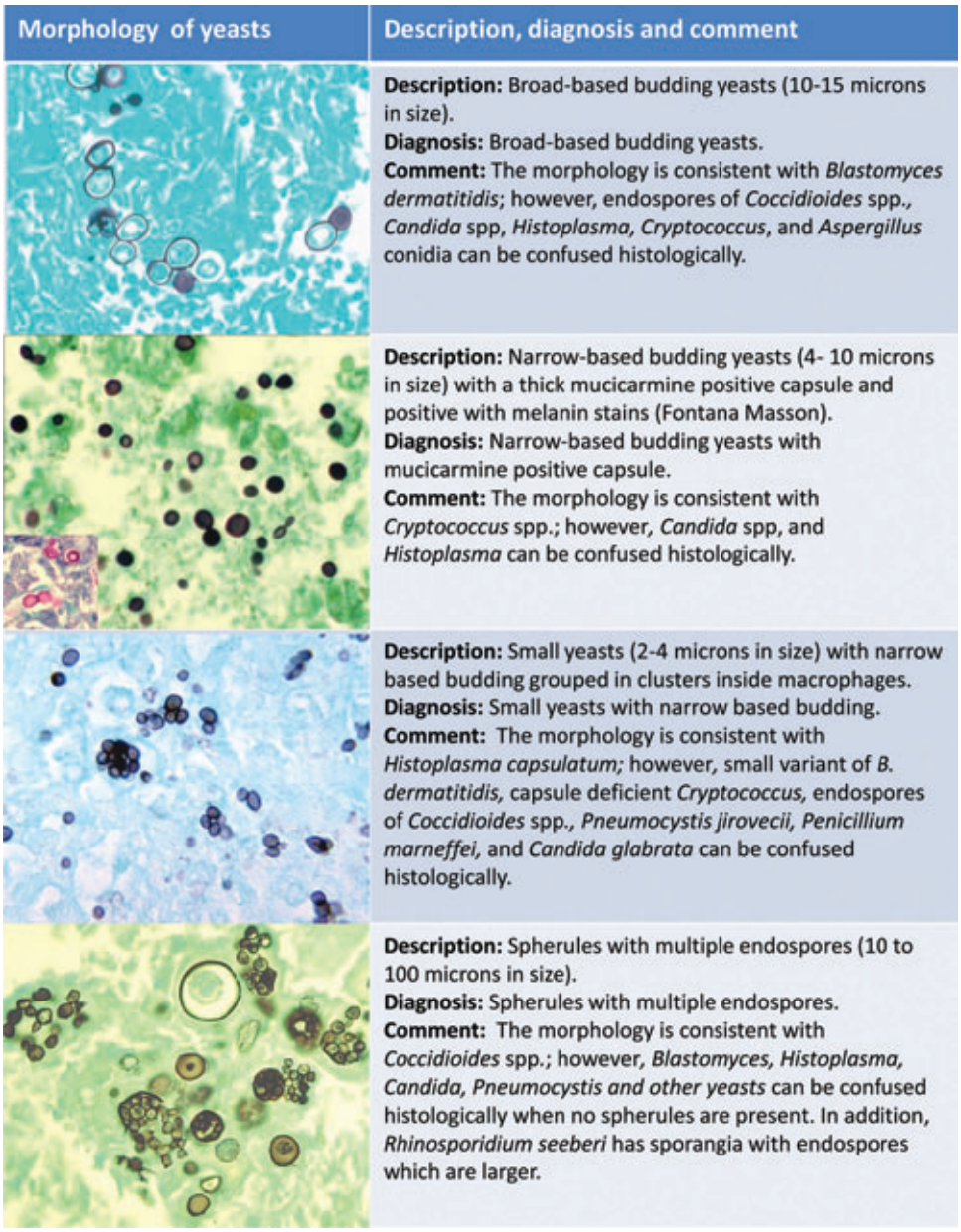

FIG. 1. Morphology, description, diagnosis, and comment for endemic fungal infections that present as yeasts in tissues. All photographs are of Grocott methenamine silver (GMS)-stained specimens except for the inset in the second row, which is a mucicarmine stain. For each type of infection, alternative testing and correlation with culture, epidemiologic, and clinical features are necessary. (The photographs of *Cryptococcus* spp., *Histoplasma capsulatum*, and *Coccidioides* spp. are reprinted from the CDC Public Health Image Library collection.)

yeasts or basidiospores, and thus the lung is the primary infection site (53, 72, 90). Few exposed individuals remain asymptomatic, while the majority display pneumonia, cryptococcomas, or pleural effusions. From the lung, cryptococci can disseminate to the central nervous system (producing meningitis or cryptococcomas), skin, bones, or other tissues. *C. gattii* is associated with a higher incidence of solid lesions in the lungs and brain than *C. neoformans.* The frequency of disseminated disease is dependent on the immune status of the patient: in immunocompetent individuals the most frequent presentation is pulmonary, while immunosuppressed patients commonly present with central nervous system involvement.

(ii) Morphological characteristics that set cryptococcosis apart. Cryptococci are encapsulated, spherical to oval yeast that measure 5 to 10 μm in diameter and have narrow-based budding (Fig. 1) (31, 56). A thick polysaccharide capsule gives these organisms the characteristic appearance of having a clear space around them that can be seen in tissue sections with H&E stains. When testing CSF, India ink can be used as a negative stain to highlight the capsule. Because of the capsule, the buds appear separate from the mother cells. The polysac-

charide capsule stains with Alcian blue and Mayer's or Southgate's mucicarmine stain. As with all other yeasts, the wall of the organism stains with GMS and PAS stains. In addition, cryptococci stain with Fontana-Masson stain because they contain melanin.

The inflammatory reaction against cryptococci seen in histopathology varies from well-formed granulomas where the organisms are found inside macrophages and giant cells to minimal inflammation with abundant extracellular organisms that efface the tissue architecture (31, 56). The granulomatous inflammatory reactions also show a spectrum from abundant necrosis to fibrous granulomas. In some instances the fibrosis is intense, with the fibroblast having plump spindle cells in a storiform pattern accompanied by a background of lymphocytes and plasma cells, giving the appearance of an inflammatory pseudotumor (151). Some authors have correlated the inflammatory reaction to the immune status of the patient and the presence or absence of capsule.

(iii) Pitfalls in morphological diagnosis. In some patients with cryptococcosis, the yeast may produce lesser amount of the characteristic polysaccharide capsule; thus, these organisms may resemble other yeasts of similar size, such as *Candida* spp. or *Histoplasma*. Staining these specimens with Fontana-Masson stain may prove that the yeast produces melanin, which is characteristic of cryptococci. The use of cryptococcal antigen tests with serum and CSF may not be helpful for patients with poorly encapsulated cryptococci, because most of the serologic tests detect antigens present in the capsule (56).

(iv) Alternative testing. Cryptococcal antigen testing using latex agglutination or EIA can be performed with serum and CSF. These techniques show a sensitivity and specificity of above 90%; however, false-negative results can occur due to low fungal burden or a prozone phenomenon, while false-positive results have been seen in patients infected with *Trichosporon* spp. or *Klebsiella pneumoniae*, in those with positive rheumatoid factor, or if the reagent was incubated with the specimen beyond the recommended time. The presence of antigens or yeast forms in the various fluids and tissues is important for diagnosis and for determining the amount of cryptococci present, but continued infection can be determined only by cultures (72). Cultures, particularly using canavanine-glycine-bromthymol blue medium, which turns blue in the presence of *C. gattii*, are helpful to determine the species of *Cryptococcus* and are indispensable for determining antifungal susceptibility when indicated. However, it should be noted that breakpoints for antifungal drugs have not been established for *Cryptococcus* spp.

Histoplasmosis. (i) Epidemiologic and clinical situations when histoplasmosis should be considered in the differential diagnosis. *Histoplasma capsulatum* is found worldwide in old buildings, caves, and soil rich in bird and bat droppings; nevertheless, there are areas of endemicity, including the Ohio and Mississippi River valleys in the United States, Central and South America, southern Europe, parts of Africa, and southeastern Asia (78). In most areas of the world human histoplasmosis is caused by *H. capsulatum* var. *capsulatum*; however, in western and central regions of sub-Saharan Africa the African clade of *Histoplasma capsulatum*, formerly named *H. capsulatum* var. *duboisii*, can be found (93). Although histoplasmosis can occur in outbreaks when old buildings are renovated/demolished or groups of tourists visit caves, most cases are sporadic.

Histoplasmosis is acquired by inhaling microconidia, and depending on the amount of fungus inhaled and immune status, the host may show no symptoms or may have acute or chronic pulmonary disease or disseminated infection (22, 78). Once inhaled, the conidia are ingested by lung alveolar macrophages, where the organisms convert into the yeast phase. The phagocytized organisms survive inside macrophages for weeks and can disseminate as the macrophages travel in the lymphatic system. *H. capsulatum* is an intracellular pathogen that can remain viable inside macrophages until specific cell-mediated immunity kills the organisms. Disseminated disease occurs upon initial infection or as reactivation of latent disease in individuals who have T-cell immunodeficiencies such as AIDS, hematologic malignancies, or solid organ transplants or who use corticosteroids or tumor necrosis factor antagonists (63).

Patients with high exposure loads or those who are immunosuppressed can present with acute pneumonia or ARDS (32, 78). As macrophages travel to mediastinal lymph nodes, patients may present with mediastinitis, and as macrophages disseminate through the body, the organisms spread to other organs, forming nodules. Usual sites of dissemination include the skin, gastrointestinal (GI) tract, liver, spleen, and bone marrow. Although rare, central nervous system infection can occur. Chronic cavitary histoplasmosis that may be clinically indistinguishable from tuberculosis is a frequent presentation of older patients with emphysema or immunocompetent individuals exposed to lower fungal loads.

(ii) Morphological characteristics that set histoplasmosis apart. *H. capsulatum* var. *capsulatum* in tissue is an oval 2- to 4-μm yeast that may show narrow-based buds (Fig. 1) (32). With H&E stain, the basophilic yeast cytoplasm is separated from the surrounding tissue by a clear zone corresponding to the cell wall. The cell wall is highlighted with GMS and PAS stains. Because the yeasts are initially ingested by macrophages, they appear to be clustered, and some authors have suggested that this is an important diagnostic feature. This clustering within histiocytes and occasionally within neutrophils is the presentation of *Histoplasma* in fluids stained with Papanicolaou stain or blood smears stained with Giemsa stain (60). African histoplasmosis shows similar clustering inside phagocytic cells (particularly large multinucleated giant cells), but the yeasts are larger (8 to 15 μm in diameter) than with *H. capsulatum* and may be pigmented.

Few lung tissue samples from patients with acute pulmonary histoplasmosis have been studied, and these have shown nodular areas of parenchymal and vascular necrosis associated with lympho-histiocytic vasculitis (105). The histopathologic picture resembles that for lymphomatoid granulomatosis, but scattered small granulomas with small yeasts in the parenchyma should suggest the diagnosis of histoplasmosis. Chronic lung infections that radiographically appear as coin lesions show typical granulomatous inflammation with central necrosis and calcified material (32). Yeasts are usually found in this necrotic calcified material, which can be lost during processing and cutting of the tissue. In immunosuppressed patients, sheets of macrophages filled with yeasts characterize disseminated disease. The collections of macrophages distort the organ architecture and produce necrotic areas. Because the morphol-

ogy of *H. capsulatum* is not specific, it is important to perform clinico-epidemiologic correlation.

(iii) Pitfalls in morphological diagnosis. Several fungi can be confused with *H. capsulatum* var. *capsulatum* when studying tissue sections (22, 60): the small variant of *B. dermatitidis* (in these cases the presence of broad-based budding and seeking larger forms can be helpful in making the diagnosis of blastomycosis), capsule-deficient cryptococci (in these cases size variation and looking for weakly positive mucicarmine-staining yeasts may suggest the diagnosis of cryptococcosis), endospores of *Coccidioides* spp. (looking for remnants of a ruptured spherule or an intact spherule is paramount for diagnosis of coccidioidomycosis), *Pneumocystis jirovecii* (this organism lacks budding and has an intracystic focus), *Penicillium marneffei* (this organism shows formation of a transverse septum rather than budding), and *Candida glabrata* (this organism may show more size variability than in histoplasmosis, and the inflammation is primarily neutrophilic).

In addition, several protozoa can also show intracellular organisms of similar size, including the agents of leishmaniasis, toxoplasmosis, and Chagas' disease, which should be differentiated from histoplasmosis (32, 60). The histopathologic difference between these organisms and *Histoplasma* is that H&E stains the entire protozoan and none shows the halo produced by the fungal cell wall. Kinetoplasts (a distinct hematoxylin-stained bar to the side of the nucleus) should be observed if the patient has leishmaniasis or Chagas' disease. Infected cells in toxoplasmosis and Chagas' disease are somatic (cardiomyocytes or neurons) rather than histiocytes.

In summary, definitive diagnosis of histoplasmosis can be difficult with tissue sections, and if a portion of the tissue specimen was not sent for cultures, alternative testing should be considered.

(iv) Alternative testing. Cultures for *Histoplasma* using blood samples can aid in the diagnosis of disseminated disease. However, since these are primarily intracellular organisms, lysis-centrifugation methods should be used to release the yeasts from histiocytes. Furthermore, the organism grows slowly, so cultures require incubation for 4 to 6 weeks before they are called negative (77). Although testing for antibodies can be performed using complement fixation or immunodiffusion, production of antibodies may not occur in immunodeficient patients (22, 78). False-positive serology can occur in patients with lymphoma, tuberculosis, and other fungal infections, particularly blastomycosis. Detection of antigen in urine and serum can be performed using EIA with various results. Antigen is concentrated in the urine, making *Histoplasma* antigen detection in this specimen more reliable. Similarly to antibody testing, there are false-positive results with antigen testing. The cross-reactivity with blastomycosis is particularly problematic because histoplasmosis and blastomycosis have overlapping endemicity and histopathologically these yeasts can sometimes be confused. Nonetheless, in patients with nondisseminated histoplasmosis the antigen burden is lower, and thus sensitivity is lower. Combining the results of detection of antigen in urine and serum may increase the sensitivity in patients with pulmonary histoplasmosis (157). Diagnosis of exposure to *Histoplasma* has been performed using intradermal reaction to histoplasmin, but this reagent is not available in the United States (55).

Coccidioidomycosis. (i) Epidemiologic and clinical situations when coccidioidomycosis should be considered in the differential diagnosis. *Coccidioides immitis* is endemic in California desertic areas, in particular the San Joaquin valley (1, 117). *Coccidioides posadasii* is present in desertic regions of northwest Mexico, Arizona, Utah, New Mexico, and West Texas and in desertic areas in Argentina, Paraguay, and parts of Central America (5). However, very little difference in morphology or clinical presentation has been found between the two species. There is a clear correlation between the incidence of disease and environmental factors: coccidioidomycosis increases when there are rainy summers followed by dry winters, following earthquakes, or when humans settle and develop the previously mentioned desertic areas. In any of these instances, *Coccidioides* arthrospores are released in higher numbers than the usual baseline (117).

Humans inhale the arthroconidia, which in the lung are transformed into multinucleated spherical structures that contain hundreds of endospores (117). It is estimated that up to 60% of individuals exposed have no symptoms, while the remainder may present with what appears to be acute community-acquired pneumonia (1, 5). In those patients with acute pneumonia, the chest X rays show lobar infiltrates and adenopathy. Several erythematous rashes (multiforme, nodosum, or toxic) are reflections of immune response to the acute infection. The majority of acute pulmonary infections known as "valley fever" resolve; however, in a minority of patients the infection may become chronic progressive, showing either a cavity or nodule. Extrapulmonary disease can occur in members of certain risk groups, including African Americans, Asians, pregnant women, patients with diabetes, or patients receiving corticosteroids for a variety of conditions. The most common sites of dissemination include skin, lymph nodes, bones, and joints; nonetheless, the most feared complication is extension to the central nervous system. Besides acquisition of infection through the respiratory route, there are rare reports of direct inoculation of skin, giving rise to primary cutaneous lesions, or acquisition through transplanted organs (21, 102, 116).

(ii) Morphological characteristics that set coccidioidomycosis apart. Spherules of various sizes (10 to 100 μm) with multiple endospores (2 to 5 μm) are characteristic of coccidioidomycosis and can be seen with routine H&E staining (116, 142). The walls of some of the spherules may appear to be ruptured, and the endospores spill into surrounding tissues. Active lesions contain multiple organisms, while resolving or residual lesions usually show lower number of organisms. GMS highlights spherule walls and endospores (Fig. 1). In contrast, reactions with PAS stain vary with age of the structures: young endospores and spherules stain strongly, while staining fades as the organisms mature. Occasionally, mycelia can be observed in cavitary lung or skin lesions (142). The sensitivity for histopathologic detection of *Coccidioides* is 84%, and that for cytology is 75% (1).

The inflammatory reaction to endospores is predominantly neutrophilic, while reaction to spherules is granulomatous. Thus, early in the infection the lesions tend to show pyogranulomas because the concentration of spherules and endospores is high. Lymphocytic clusters of B and T cells around well-formed granulomas with necrosis have been described and appear to be an important response to coccidioidomycosis

(88). Eosinophils can be abundant, releasing eosinophilic major basic protein, and can create the Splendore-Höeppli phenomenon (an intense rim of eosinophilic material around the fungal elements) (127).

(iii) Pitfalls in morphological diagnosis. *Rhinosporidium seeberi*, a mesomycetozoan parasite that causes palate and nasopharyngeal polyps, produces large sporangia (some can be seen with the naked eye) with multiple internal endospores. *R. seeberi* has very similar morphology, but its sporangia and endospores are larger than *Coccidioides* spherules, and its inner sporangial wall stains with mucicarmine. When coccidioidomycosis is suspected, it is important to look for spherules; endospores outside spherules or young spherules without endospores can be confused with *Blastomyces*, *Histoplasma*, *Emmonsia*, *Candida*, *Pneumocystis*, and other yeasts (142). It also needs to be remembered that in immunosuppressed patients, more than one infection may coexist; thus, in areas of endemicity, *Pneumocystis* and *Coccidioides* could be found in the same specimen.

(iv) Alternative testing. In the United States, *Coccidioides* spp. are select agents that are governed by specific rules related to their possession, use, and transfer. These fungi grow easily in the laboratory (93% sensitivity), and the arthroconidia can be easily aerosolized; thus, it is mandatory that all work with mycology cultures is performed using a class II biological safety cabinet (1, 142). Because of the culture constraints, laboratories have studied genomic assays targeting either internal transcribed spacer 2 (ITS2) or proline-rich antigen (Ag2/PRA) for diagnosis of coccidioidomycosis with up to 98% sensitivity (5).

Detection of antibodies to *Coccidioides* can be an important diagnostic tool. Today, IgM and IgG are generally measured using EIA and/or immunodiffusion; however, some laboratories continue to use tube precipitin to measure IgM and complement fixation to measure IgG antibodies (142). False-negative serology has been seen in up to 38% of patients with hematogenous infection and 46% of fatal cases (1). Detection of antigens in the urine using EIA has been shown in 71% of patients with coccidioidomycosis but shows cross-reaction in 10% of patients with other endemic mycoses (49).

Candidiasis. (i) Epidemiologic and clinical situations when candidiasis should be considered in the differential diagnosis. *Candida albicans* colonizes the human oropharynx and vagina, and a small number of viable organisms can be cultured from these surfaces (38, 153). Superficial infections in the gastrointestinal or genitourinary tract occur when there are microbial imbalances caused by fluctuations in reproductive hormones, antibiotic use, and immunosuppression that may have causes ranging from HIV infection to diabetes (38, 52). Invasive candidiasis occurs primarily as a health care-associated infection, and patients at risk include those receiving broad-spectrum antibiotics, immunosuppressants, or total parenteral nutrition and those with vascular access devices, recent surgeries, malignancies, and neutropenia (40). *C. albicans* is the most frequent species isolated from the blood, accounting for one-third to two-thirds of bloodstream isolates (38). Certain species are associated with particular risk factors; for example, *Candida parapsilosis* is seen in patients with hyperalimentation or indwelling devices or in neonates, while *Candida glabrata* is seen in patients who are receiving azoles. Once *Candida* organisms gain access to the blood, secondary seeding into all organs can occur. Common localizations include the liver and spleen in neutropenic hemato-oncologic patients, the endocardium in those with prosthetic heart valves or other intravascular prosthetic devices, and the eye in those with longstanding candidemia (160). *Candida* organisms are capable of forming biofilms in catheters and devices, permitting their growth even when achievable doses of appropriate antifungal agents are given (40, 160).

(ii) Morphological characteristics that set candidiasis apart. In tissues *Candida* organisms appear as mats of yeasts measuring 3 to 5 μm in diameter intermingled with pseudohyphae (also referred to as filaments) (Fig. 2) (94). The filaments may show periodic constrictions. The organisms can be seen with H&E, GMS, and PAS stains. The predominant *Candida* species that does not produce filaments is *C. glabrata*. Histopathologic examination of specimens is very important to define invasion of tissues and vessels, since growth from skin, lung, and the gastrointestinal or genitourinary tract is only indicative of colonization (40).

The usual host reaction, whether in superficial or invasive candidiasis, consists primarily of neutrophilic inflammation with some lymphocytes and macrophages, fibrin, and coagulative necrosis (94). Giant cells and granulomas can be seen but are sparse. As *Candida* organisms invade blood vessels, they can cause mycotic aneurysms or thrombophlebitis. Necrotizing vasculitis has been described in candidemia, but organisms are not observed in the necrotic vessels, supporting the concept that *Candida* soluble fractions cause the necrotizing lesions (141). In patients with neutropenia the necrosis is usually accompanied by hemorrhage, and few lymphocytes and macrophages can be observed. In patients with endocarditis and vegetations, platelets are an important component (20). In gynecologic Pap smears, superficial *Candida* infections are associated with enlarged, hyperchromatic nuclei with perinuclear halos; these changes can be confused with low-grade, squamous intraepithelial lesions (104).

(iii) Pitfalls in morphological diagnosis. *Candida* spp. are yeasts that can produce pseudohyphae, and they thus require differentiation from other yeasts and molds that produce true hyphae in tissue. The most frequent differential diagnosis is with *Aspergillus* spp. and *Trichosporon* spp. (94). Elongated *Candida* pseudohyphae can appear to be branching but are differentiated because pseudohyphae are slender and do not have septations. Germinating *Candida* blastospores can also appear to be branching but can be distinguished by the absence of a constriction between the base of the blastospore and the germ tube. *Histoplasma* is the differential diagnosis for *C. glabrata*, since pseudohyphae will not be produced by these species.

(iv) Alternative testing. Blood cultures are indispensable for diagnosis in invasive disease, although positive blood cultures are estimated to occur in 50 to 70% of cases. The peptide nucleic acid fluorescent *in situ* hybridization assay can be used to identify the most common species of *Candida* in smears made from positive blood culture bottles. Multiplex-tandem PCR detection of *Candida* spp. in whole blood, serum, or plasma has yielded better and faster results than blood cultures; however, this method is still for research use only and will need to be validated if used for diagnostic purposes (85). Detection of β-D-glucan in serum has been used, but the sensitivity and specificity will vary with the type of patient and the cutoff value used for the test (167). Sensitivity is

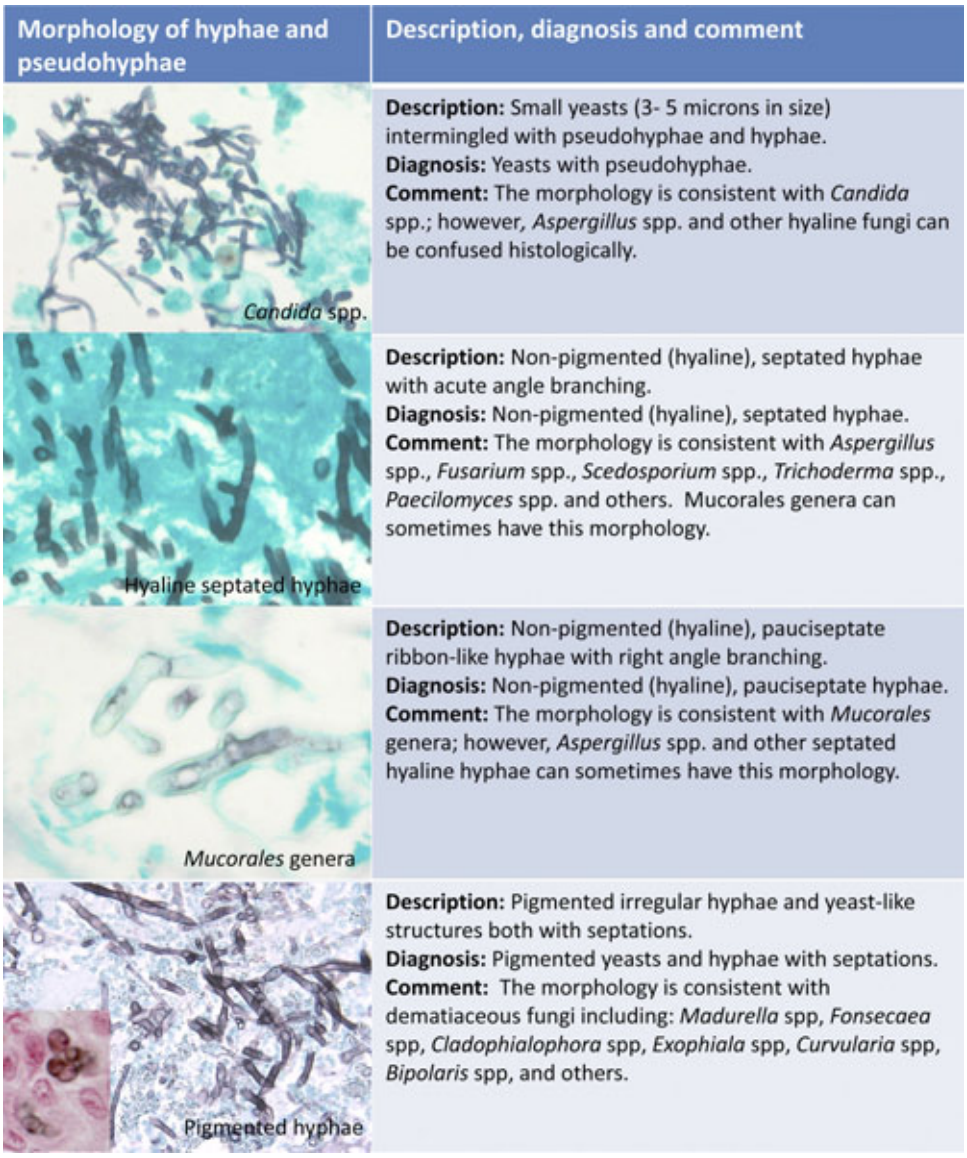

FIG. 2. Morphology, description, diagnosis, and comment for fungal infections that present with hyphae or pseudohyphae in tissues. All photographs are of Grocott methenamine silver (GMS)-stained specimens except for the inset in the fourth row, which is a Fontana-Masson stain. The brown color observed in the fungal element is melanin. For each type of infection, alternative testing and correlation with culture, epidemiologic, and clinical features are necessary. (The photograph in the fourth row is reprinted from the CDC Public Health Image Library collection.)

lowest (57%) when using single specimens from patients with candidemia in the intensive care unit or monitoring once weekly with liver transplant patients, while it is highest (97%) when using a low cutoff value (60 pg/ml). Specificity ranges from 44 to 92% with single specimens from patients with candidemia using high cutoff values (80 pg/ml).

Diseases caused by other fungi and organisms resembling fungi that display yeasts or yeast-like organisms in tissue. (i) Epidemiologic and clinical situations when yeasts or yeast-like organisms should be considered in the differential diagnosis. *Pneumocystis* pneumonia was an unusual infection until 1982, when AIDS appeared in the western world (28). Until the advent and widespread use of highly active antiretroviral treatment (HAART), *Pneumocystis* pneumonia was frequently diagnosed in patients with AIDS and was associated with high mortality. Although the prevalence of *Pneumocystis* pneumonia in the United States has dropped, it continues to be a problem in patients with AIDS who have suboptimal access to HIV testing and health care, patients receiving immunosuppressant medications chronically, or those with altered immune status (79, 83). *Pneumocystis* is an interesting organism that has protozoan and fungal characteristics and has been classified as both at different times in history (112). In addition, the species that is pathogenic to humans (previously named *P. carinii*) has recently been renamed *P. jirovecii*. Although the mode of transmission of *Pneumocystis* is not known, an airborne mechanism is suspected since the majority of infected patients present with pneumonia. Extrapulmonary disease has

been documented but is very rare (112).

Sporothrix schenckii is the cause of lymphocutaneous and fixed cutaneous lesions traditionally associated with injuries that occur during handling of contaminated soil, plants, and wood. Transmission in persons who handle sick animals but do not recall having had a skin injury has also been documented, particularly in cases involving children and cats (10). Most of the lesions occur in exposed areas, predominantly in hands, arms, and face, with rare instances of systemic disease affecting bone, joints, meninges, and other internal organs. Erythema nodosum and arthralgias (without infection of the joints) have been described in patients with cutaneous and lymphocutaneous disease. Although *S. schenckii* is found worldwide, infections are more common in tropical and temperate climates. Outbreaks of this disease in humans and animals in South Africa and Brazil have been described (144).

Of all the *Penicillium* species, *P. marneffei* is the most frequent cause of pathology in humans (98). Disseminated disease with this organism is seen primarily in immunosuppressed patients, particularly those with AIDS, who have resided in or visited Southeast Asia (Thailand, Vietnam, and the southern part of China) (48). In some areas it is the third most common opportunistic infection in patients with AIDS, after tuberculosis and cryptococcosis. The disease presents with fever that may be accompanied with chills, weight loss, anemia, cutaneous or subcutaneous lesions, lymphadenopathy, hepatosplenomegaly, respiratory symptoms, and osteoarticular lesions.

Paracoccidioides brasiliensis is the agent of paracoccidioidomycosis, which occurs mostly in Brazil, Colombia, Venezuela, Ecuador, and Argentina, with occasional cases in countries in Central and South America (126). Rare cases have been documented in other countries decades after the patient resided in a country where the disease is endemic, suggesting reactivation of paracoccidioidomycosis that had been acquired at an earlier age. Exposure to *P. brasiliensis* does not guarantee the development of active disease. Hormonal, genetic, immunologic, and nutritional statuses appear to determine if the patient will present with acute or chronic or with localized or disseminated disease. In adults the disease predominates in males, which has been supported by *in vitro* studies showing that 17β-estradiol blocks or delays the transition from the mycelial or conidial forms into the pathogenic yeast forms (139). The majority of patients present with lung disease, which can disseminate hematogenously to bone marrow, adrenal glands, brain, and other tissues, or the organisms are expectorated and lesions can form in the oropharyngeal tract. In the lung the disease can present as interstitial infiltrates or cavities. Less frequently, *P. brasiliensis* is inoculated directly into the skin or oral mucosa, causing lesions in these areas. If there is lymphadenopathy, the necrotic contents may drain into the skin.

R. seeberi (described above) is another organism that cannot be cultured and for which animal models do not exist. It has been placed phylogenetically at the divergence between fungal and animal organisms (6). The organism causes rhinosporidiosis, a disease characterized by polyploid tumors that occur primarily in the nose, nasopharynx, and ocular areas (27). Rarely the disease can disseminate to other mucous membranes or cutaneous sites as well as viscera. Rhinosporidiosis occurs in hot tropical climates in all continents, and cases have been reported from Uganda, Sri Lanka, India, Brazil, Argentina, Texas, and other locations.

Another unusual fungus that can be present in histopathologic specimens is *Emmonsia crescens*. This dimorphic fungus is inhaled or inoculated into the skin when humans are in close contact with rodent burrows and aerosolized conidia. The amount of conidia inoculated will determine the presentation: small inocula are asymptomatic and present as walled-off granulomas, while larger inocula can result in acute severe pulmonary disease. The disease is known as adiaspiromycosis or haplomycosis, due to the presence in tissue of adiaspores, which are large, thick-walled structures formed by transformation of hyphae. *Emmonsia pasteuriana* has also been reported in a patient with AIDS (47); this species does not produce adiaspores in tissue.

(ii) Morphological characteristics. In tissue sections stained with H&E, *Pneumocystis* pneumonia presents as foamy intraalveolar eosinophilic exudates with minimal inflammatory infiltrate. In Papanicolaou-stained respiratory cytology specimens, the organisms blend into the mucous blue-green background. GMS staining demonstrates that the foamy material in tissue sections or cytologic specimens corresponds to multiple organisms which are thin-walled spheres of 2 to 5 μm that have an intracystic focus (capsular dot) (Fig. 3) (64). Collapsed organisms are usually found intermingled with intact organisms. Atypical inflammatory reactions to *Pneumocystis* have been documented, including interstitial pulmonary fibrosis, granulomas, hyaline membranes, and interstitial lung infiltrates (64). In these instances, it is difficult to suspect that *Pneumocystis* is present in the lesion.

S. schenckii in tissues appears as round, oval, or cigar-shaped yeasts of 2 to 6 μm or larger in diameter that may show narrow-based or tube-like budding (34). *S. schenckii* yeasts are not easy to identify with H&E stains, and thus GMS and PAS stains should be used to highlight their contour. In cases of sporotrichosis, star-like, eosinophilic material (Splendore-Höeppli phenomenon) surrounding yeasts can be observed in 40 to 92% of cases (24, 138). These structures have been called asteroid bodies, and *Sporothrix* has been demonstrated in the centers of these structures using immunohistochemistry (138). The infection is usually present in a background of granulomatous inflammation with neutrophils and microabscesses and various degrees of fibrosis. The epidermis in cutaneous lesions shows pseudoepitheliomatous hyperplasia and microabscesses. Yeasts are most abundant in the microabscesses. In epidermal microabscesses, the yeasts may be mixed with hyphae. *S. schenckii* has also been identified using fine-needle aspiration of lesions (57).

P. marneffei yeasts are small (2 to 5 μm in diameter) and do not bud but divide by fission, thus resembling a sausage with a transverse septum (Fig. 3) (98, 168). These transverse septa will appear to be thicker than the wall of the yeast when using the GMS stain. *P. marneffei* yeasts are usually found inside macrophages and thus appear in clusters with occasional single extracellular organisms. Immunosuppressed patients show an anergic necrotizing host response with abundant infected macrophages accompanied by various degrees of necrosis; however, in patients where immunity is less compromised, granulomatous and abscess formation have been described. *P. marneffei* has also been identified using fine-

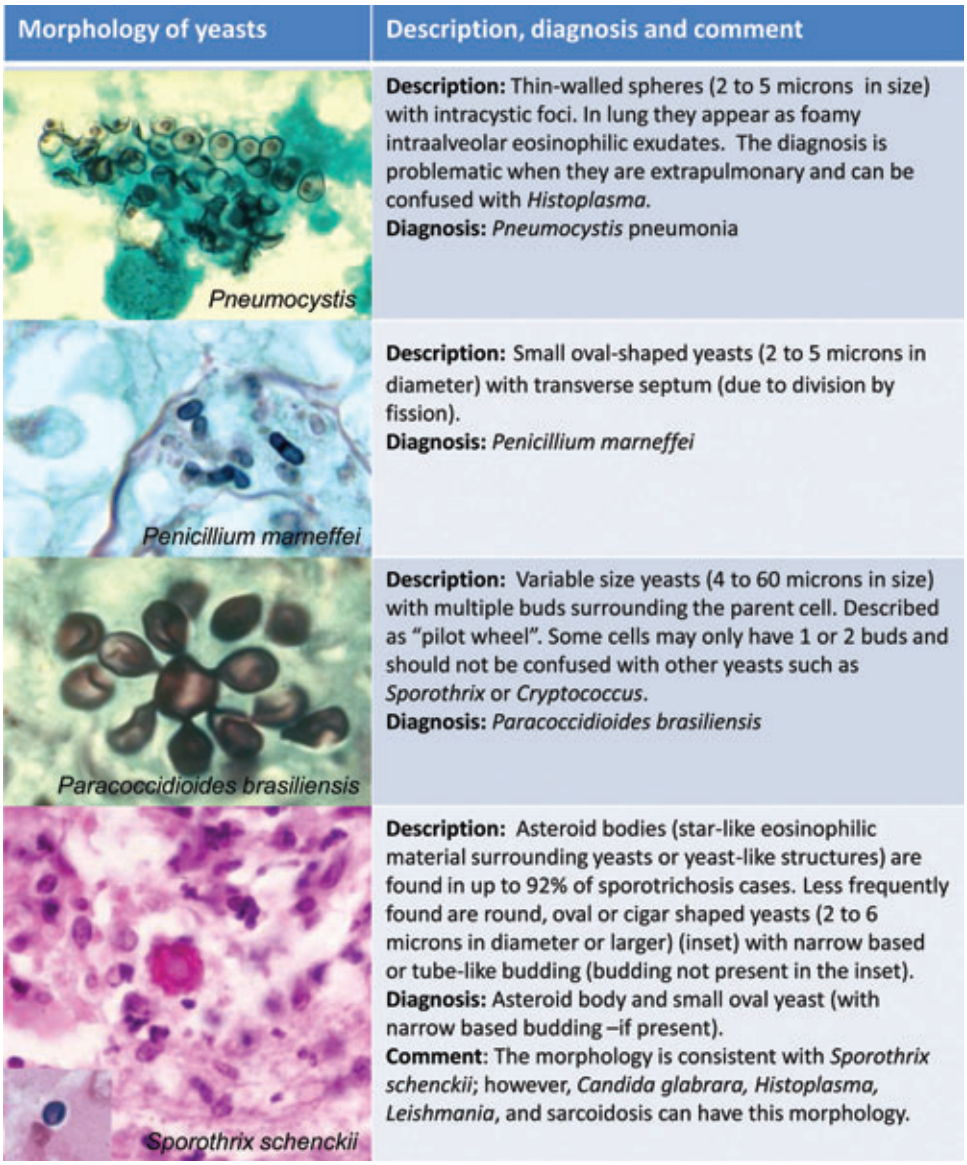

FIG. 3. Morphology, description, diagnosis, and comment for fungal infections that show characteristic yeast morphology in tissues. Except for the last row, which shows an H&E-stained asteroid body, all photographs are of Grocott methenamine silver (GMS)-stained specimens (including the inset of *S. schenckii*, which is counterstained with H&E). For each type of infection, alternative testing and correlation with culture, epidemiologic, and clinical features are necessary. (The photographs of *Pneumocystis*, *Penicillium marneffei*, and *Paracoccidioides brasiliensis* are reprinted from the CDC Public Health Image Library collection.)

needle aspiration of a lymph node (89).

P. brasiliensis in tissue sections stained with H&E are spherical yeasts that vary in size from 4 to 60 μm and have an optically clear space between the fungus and surrounding tissue (92). The pathognomonic "pilot wheel" created by multiple buds surrounding the parent cell is highlighted with GMS but may be difficult to observe with H&E stains (Fig. 3). Not all yeasts show buds around the entire circumference. The yeasts are usually found inside multinucleated giant cells. The host reaction ranges from mixed granulomatous and neutrophilic inflammation to necrotic granulomas surrounded by fibrosis. The epidermis in skin lesions shows pseudoepitheliomatous hyperplasia. In the bone marrow there may be coagulative necrosis with osteonecrosis (129).

Diagnosis of rhinosporidiosis can be performed only using histopathology. *R. seeberi* presents as large (50- to 100-mm) round structures that can be seen with the naked eye as yellowish pinhead-sized spots in the polyp (27). Microscopically, these structures vary in size, corresponding to different stages in the development of the organism, and have a densely eosinophilic wall that either encloses smaller round structures or can be empty and containing amorphous eosinophilic material. Microscopic features of this organism are enhanced by using GMS, PAS, and mucicarmine stains (164). The host response is predominantly granulomatous inflammation admixed with

fibrosis and granulation tissue. The epidermis and mucosal epithelium show hyperplastic features, and *R. seeberi* can be seen in these cellular layers.

On H&E staining, adiaspores are the primary feature of pulmonary adiaspiromycosis. Adiaspores are thick, double-walled spherules that measure 20 to 400 μm or more and are empty or contain eosinophilic hyaline globules (162). When stained with GMS, the entire wall thickness stains and shows fenestrations. Adiaspores usually elicit a granulomatous inflammatory reaction.

(iii) Pitfalls in morphological diagnosis. The differential diagnosis of *Pneumocystis* includes *Histoplasma.* In the usual clinico-pathologic setting, the intracystic body of *Pneumocystis* is the key to differentiate this organism from *Histoplasma*. However, *Pneumocystis* may be difficult to differentiate from *Histoplasma* when present inside granulomas or when there is extrapulmonary disease.

S. schenckii should be differentiated from *Candida* (the presence of pseudohyphae may be useful unless it is *C. glabrata*), *Histoplasma* (finding clusters of organisms may indicate histoplasmosis), and *Leishmania* (by finding kinetoplasts). In addition, Hamazaki-Wesenberg bodies (pigmented elliptical structures seen in sarcoidosis) have been confused with *S. schenckii* (34). The presence of the Splendore-Höeppli phenomenon is an important distinctive feature of sporotrichosis.

P. marneffei should also be differentiated from *Candida*, *Histoplasma*, *Toxoplasma gondii*, and *Leishmania*, and this can be achieved by finding the characteristic *P. marneffei* transverse septum that occurs during fission of the organism.

Paracoccidioidomycosis may not show the characteristic multiple budding but may show only one or two buds. Since *C. neoformans*, *S. schenckii* and *Lacazia loboi* may have two and three buds, these fungi should be considered in the differential diagnosis. *L. loboi* is the cause of a nodular skin infection that histopathologically forms chains of cells connected to one another by a tubular structure in a granulomatous background (121). The diagnosis of paracoccidioidomycosis should be considered only when yeasts are variable in size and multiple teardrop and tubular buds are seen coming from a parent cell. Because of the variability in size, many yeasts can be confused with *P. brasiliensis* if organisms are present in low numbers and the characteristic "pilot wheel" is not seen.

Morphologically, coccidioidomycosis and rhinosporidiosis show either a spherule (*Coccidioides*) or a sporangium (*Rhinosporidium*) containing distinct endospore-like structures; however, *R. seeberi* sporangia are larger and mucicarmine positive, and the clinical setting is different from that of coccidioidomycosis (27).

The differential diagnosis for adiaspiromycosis in tissues has usually been helminthic infections, since there are no other fungi with these histologic characteristics (42). Helminths will show musculature and internal organs that are not present in adiaspiromycosis. Adiaspores may collapse and form various shapes that resemble other fungi, helminths, or pollen grains. Adiaspores are distinguished from *Coccidioides* spherules in that spherules contain endospores while adiaspores are empty.

(iv) Alternative testing. *Pneumocystis* cannot be recovered in culture, and thus alternative testing is usually performed using direct immunofluorescence. Although β-D-glucan in serum cross-reacts with *Candida* and *Aspergillus* organisms, it has been shown to have good sensitivity for detection of *Pneumocystis* (83). In addition to cultures for detection of *S. schenckii*, a skin test (sporothricin) has been used in areas of endemicity outside the United States. *P. marneffei* can be cultured from blood, skin lesions, or aspirates from lymph nodes. Serologic testing specific for *P. marneffei* has been studied in Southeast Asia (48). Serologic tests detecting IgG antibodies against *P. brasiliensis* are available in countries where it is endemic and are used for diagnosis and to follow up treatment(123). Skin tests using paracoccidioidin also have been used for epidemiologic studies (75). Alternative testing for rhinosporidiosis does not exist. Molecular diagnosis with fresh tissues has been used for several of these infections, using either specific primers for these fungi or panfungal primers (23, 45, 83).

Diseases Where Hyphae Are Usually Seen in Tissue

Aspergillosis. (i) Epidemiologic and clinical situations when aspergillosis should be considered in the differential diagnosis. The genus *Aspergillus* encompasses a large number of molds that reproduce asexually by producing unbranched chains of conidia from a bulbous structure called a vesicle (13). *Aspergillus* spp. are ubiquitous in the environment and have been used for centuries to ferment rice to produce sake or soybeans to produce soy sauce. In industry, *Aspergillus niger* is used to produce citric acid and numerous commercial enzymes. The genus has evolved to survive in a variety of habitats from damp basements and decaying vegetation to colonization of mammals. *Aspergillus fumigatus* is the species most frequently associated with human disease, but other species, including those that are important for industry (*A. niger*), can cause disease in immunosuppressed hosts. Besides the host immune status, genetic predisposition to disease with *Aspergillus* spp. is an important component.

Aspergillosis encompasses three entities: allergic bronchopulmonary aspergillosis (ABPA), chronic pulmonary aspergillosis/aspergilloma, and invasive or systemic aspergillosis (13, 136). ABPA is an exaggerated hypersensitivity reaction to a variety of fungi, most frequently *A. fumigatus*, in cystic fibrosis and steroid-dependent asthma patients. These patients may be asymptomatic with infiltrates on chest X rays or have symptoms of bronchiectasis with copious production of sputum that contains brown specks or hemoptysis in addition to wheezing, fatigue, weight loss, and chest pain. Diagnostic criteria for the different stages have been established for the disease (2, 59, 119). However, all have in common the recognition of specific immunity toward *Aspergillus* antigens, which are not standardized tests (136). Once the patient has reached the fibrotic stage, the disease is not reversible; thus, diagnosis at earlier stages is important. Related to ABPA is allergic fungal rhinosinusitis, a hypersensitivity reaction to noninvasive fungal elements, particularly of the *Aspergillus* spp. (29, 145). Both ABPA and allergic fungal rhinosinusitis show allergic mucin with noninvasive hyphae, respiratory atopy, positive skin tests to the etiologic fungal organism, elevated total IgE, peripheral eosinophilia, association with major histocompatibility complex (MHC) class II antigens, and a favorable response to steroids. In addition, ABPA shows elevated fungus-specific IgE and IgG antibodies.

Chronic pulmonary aspergillosis usually occurs in nonimmu-

nocompromised individuals with a variety of predisposing conditions (152). Lung cavities caused by tuberculosis are the most frequently described predisposing condition; however, emphysema, sarcoidosis, bronchiectasis, ankylosing spondylitis, and other infections have been described. As the lung tissue is destroyed by the fungus, a cavity forms or enlarges and a fungus ball or aspergilloma can be produced. From the pathologic perspective, two different entities with similar signs and symptoms have been described: thin-walled aspergillomas and chronic cavitary/necrotizing pulmonary aspergillosis (136). In patients with chronic necrotizing pulmonary aspergillosis, further immunosuppressive conditions can be encountered, such as diabetes, HIV infection, advanced age, chronic steroid use, malnutrition, or alcohol abuse (155). These entities evolve slowly over several months to years. Hemoptysis is the most common sign and the one associated with life-threatening events. Some aspergillomas are asymptomatic and found only when an X ray is performed for other reasons, or the patient may have cough, fever, weight loss, and malaise. Patients with chronic pulmonary aspergillosis have serologic or microbiologic evidence that an *Aspergillus* sp. is involved in the process.

Invasive pulmonary aspergillosis is a disease that occurs in severely immunocompromised patients, including patients with prolonged neutropenia, hematopoietic stem cell and solid organ transplant recipients, patients with AIDS, premature newborns, and patients with chronic granulomatous disease (65, 148). The disease most frequently involves the respiratory tract, and the signs and symptoms include fever, cough, dyspnea, and hemoptysis. Pleuritic chest pain may be present if there is lung infarction. In a computed tomography (CT) scan, there is a lung nodule with the halo sign (ground glass opacity surrounding the nodule) or with the air crescent sign that occurs when there is fungal vascular invasion and hemorrhage. Neurological signs of stroke or seizures indicate that the fungus has reached the central nervous system. An international consensus defining proven, probable, and possible invasive fungal infections for research purposes was published in 2002 and revised in 2008 (7, 43). It is important to notice that the highest level of certainty for proven invasive fungal infections includes demonstration of fungal elements in diseased tissue obtained by either a biopsy sample or fine-needle aspiration of the lesion; however, the procedures to obtain these specimens may not be possible because of the host's underlying condition. In those cases, clinicians will have to rely on alternative testing.

(ii) Morphological characteristics that set aspergillosis apart. *Aspergillus* spp. are usually described as thin (3- to 12-μm), septate, acute-angle (45°) or dichotomous branching hyphae (Fig. 2). Vesicles with conidia can be observed when the fungi are present in cavitary lesions or sinuses. However, it needs to be remembered that multiple *Aspergillus* species may cause aspergillosis, and this description may not reflect the characteristics of all species or changes that may occur after antifungal treatment. For example, with *A. niger* infection, calcium oxalate crystals may be found in respiratory specimens. *A. terreus* is the only *Aspergillus* species known to produce round or pear-shaped aleurioconidia directly along the lateral hyphal walls.

In ABPA, the lung appears to have thick, tenacious mucous material in normal, fibrotic, or bronchioectatic airways. Microscopically, the mucus contains inflammatory cells (mostly eosinophils, lymphocytes, and macrophages), Curshmann's spirals (desquamated epithelium with eosinophils), Charcot-Leyden crystals, and scant hyphae. The bronchial wall may show a spectrum of changes that include inflammation with eosinophils, neutrophils, and macrophages, granulomas, vasculitis, interstitial fibrosis, and microabscesses (136). The presence of microabscesses with hyphae could represent an early stage of invasive aspergillosis. The diagnosis of allergic fungal rhinosinusitis is one of exclusion and is based on histopathologic demonstration in mucosa obtained during surgery of scattered hyphae in the inspissated allergic mucus (mucus showing laminated concretions of pyknotic and degranulated eosinophils and Charcot-Leyden crystals) with no fungal invasion or necrosis (145). Some authors describe scattered, mild granulomatous inflammation in the mucosa of the sinuses (41).

The fungus ball in chronic pulmonary aspergillosis consists of hyphae enmeshed in necrotic material. In thin-walled aspergillomas the reaction surrounding the fungus ball consists of fibrosis, while in chronic cavitary/necrotizing pulmonary aspergillosis there is a necrotic tissue layer with abundant hyphae surrounded by granulation tissue and an outer layer of fibrosis (155). Additional reactions in the cavity wall may include granulomas, eosinophils with formation of the Splendore-Höeppli phenomenon around hyphae, calcium oxalate crystalloids, and hemorrhage or hemosiderin-laden macrophages (170). Differentiation of cases with chronic cavitary/necrotizing pulmonary aspergillosis from those with invasive aspergillosis may be difficult, since vessels in the wall of the cavity may show hyphal invasion and various degrees of thrombosis. Aspergillomas are usually parenchymal lesions, but if the aspergilloma arises in a bronchiectasis, it will be bronchocentric.

In tissue from neutropenic patients with invasive pulmonary aspergillosis, angioinvasion is demonstrated by the presence of septate, right-angle-branching hyphae in the vessel wall and hemorrhage (154). The lesions result in either wedge-shaped pulmonary infarctions or a well-circumscribed spherical nodule with a vessel in the center (65). Because of the patient's neutropenia, these lesions show little inflammatory reaction.

(iii) Pitfalls in morphological diagnosis. A study of 122 specimens showed concordance in 83% of cases with septated, acute-angle-branching hyphae in histology and the presence of *Aspergillus* spp. in culture, while *Scedosporium* spp., *Fusarium* spp., *Pseudallescheria* spp., *Phialophora verrucosa*, and *Trichophyton* spp. were recovered in culture from discordant cases (86). Cultures of the allergic inspissated mucus of patients with allergic fungal rhinosinusitis have yielded multiple species of *Aspergillus*, *Bipolaris spicifera*, and *Curvularia lunata* as well as *Staphylococcus aureus*. For these patients nasal swabs are not useful for diagnosis (145). Fontana-Masson stains may be useful to demonstrate pigments in dematiaceous organisms (*Bipolaris* and *Curvularia*).

Cases with dual infection with *Aspergillus* spp. and *Candida* or mucoraceous genera have been described and pose important diagnostic dilemmas. To be able to identify dual infections, it is crucial to have alternative diagnostic testing of tissues such as immunohistochemistry, *in situ* hybridization, or PCR, which at this time are available only for research use (70, 147). In addition, in invasive pulmonary aspergillosis, cultures are positive in only 50% of bronchoalveolar lavage fluid specimens (148), and organisms recovered from BAL fluid samples may reflect colonization rather than the actual pathogen.

Lastly, detection of *Aspergillus* spp. in blood cultures in cases with invasive disease is approximately 5%.

(iv) Alternative testing. Galactomannan and (1→3)-β-D-glucan are components of the *Aspergillus* cell wall and can be measured using commercially available EIAs. Galactomannan can be measured in serum and bronchoalveolar lavage fluid specimens using the Platelia *Aspergillus* test (Bio-Rad, Hercules, CA). Different studies of this test have shown sensitivities that range from 40 to 100% and specificities of from 56 to 100%, depending on the population of patients tested and the cutoff values used (62, 97, 167). The most important problem with galactomannan testing is that false-positive results occur in approximately 50% of patients taking antibiotics (piperacillin, amoxicillin, or ticarcillin), 100% of patients receiving substances that contain products of *A. niger* fermentation (plasmalyte), and various percentages of patients with infections with other fungi, including *Penicillium*, *Paecilomyces*, *Alternaria*, and *Histoplasma.* Serial galactomannan testing of sera from patients at risk is one strategy to define whether the patient is colonized, has invasive disease, or is responding appropriately to treatment.

The (1→3)-β-D-glucan is a characteristic fungal cell wall constituent common to a broad range of fungal pathogens. The commercially available assay is the Fungitell kit (Associates of Cape Cod, East Falmouth, MA) (114). Circulating (1→3)-β-D-glucan was detected in patients with systemic fungal infections, including invasive aspergillosis, candidemia, and *Pneumocystis* pneumonia (3, 120). False-positive reactions are known to occur in some patients who are receiving piperacillin/tazobactam (101).

Mucormycosis (zygomycosis). (i) Epidemiologic and clinical situations when mucormycosis should be considered in the differential diagnosis. Even though diseases caused by ribbon-like, pauciseptate, hyaline molds were originally described in the 1800s, the nomenclature of these molds has not been completely settled (132). The names that have been given to these molds in the medical literature include *Zygomycota* (currently regarded as invalid) and *Mucorales*. It is currently accepted that the broader subphylum *Mucoromycotina* has two orders, the *Mucorales* and the *Entomophthorales.* Fungi classified as *Entomophthorales* were originally identified as parasites or pathogens of insects that occasionally cause mucocutaneous disease in immunocompetent human hosts. Conversely, those fungi classified as *Mucorales* cause a spectrum of predominantly angioinvasive disease in immunosuppressed patients (109). Of the *Mucorales*, *Rhizopus* is the genus most frequently causing human disease, while *Mucor* spp. cause disease in fewer than 20% of cases.

Mucorales genera are ubiquitous in the environment and can be found in soil and decomposing matter (132). Their spores are easily airborne, which can cause contamination of laboratory media; thus, finding these molds in clinical cultures has to be correlated with the patient's history and clinical findings to define whether the cultured mold should be considered the cause of disease. Inhaled spores cause disease in the upper and lower respiratory tracts of immunosuppressed persons. In immunocompetent or immunosuppressed hosts, spores can be inoculated into the skin and subcutaneous tissues by trauma, needle exposure, or insect bites, and they can be ingested, causing gastrointestinal disease. In immunosuppressed patients, cutaneous lesions have been linked to adhesive hospital products such as the tape used for maintaining intravenous devices or tubing in place.

Host defense against the *Mucorales* genera is primarily through macrophages that inhibit germination of spores and neutrophils that use the oxidative burst to kill proliferating hyphal elements; thus, patients who have diseases affecting the function of these two cell types will be at risk for infection (132). Diabetic ketoacidosis causes dysfunction of macrophages and is the most frequent risk factor for sinusitis and rhinocerebral infection. As expected, cancer chemotherapy and stem cell transplantation have emerged in the past 2 decades as major risk factors for invasive pulmonary mucormycoses. In addition, these fungi thrive when iron is present in the host, and those patients receiving iron-chelating agents such as deferoxamine to reduce iron overload are also at risk. Other risk factors include prematurity and injection drug use. If host defenses are poor, the spores germinate in the original inoculation site and invade tissues, including blood vessels. During the initial phases of the infection there is edema, but as the hyphae invade blood vessels the tissue undergoes necrosis and has a characteristic black color. In contrast, immunocompetent individuals infected with *Entomophthorales* produce an intense inflammatory response and present with a mass in the skin, respiratory sinuses, or gastrointestinal tract.

The three most frequent primary clinical manifestations of mucormycosis are rhinocerebral, pulmonary, and cutaneous infections. Any of these primary manifestations can give rise to disseminated disease. Patients with rhinocerebral mucormycosis have fever and nasal discharge. Those with pulmonary mucormycosis have fever, multiple pulmonary nodules, and pleural effusions. Mortality due to disseminated disease is extremely high but varies depending on the associated risk factor and clinical presentation (137). Early detection at the primary site is imperative to institute surgical and antifungal treatment. For diagnosis, tissue should be obtained for both culture and histopathology.

(ii) Morphological characteristics that set mucormycosis apart. *Mucorales* genera can be seen in biopsy and cytologic (needle aspirate and bronchoalveolar lavage fluid) specimens. Tissue identification of these molds is a very important diagnostic tool, since it distinguishes the presence of the fungus as a pathogen in the specimen from a culture contaminant and is indispensable to define whether there is blood vessel invasion. *Mucorales* genera produce nonpigmented, wide (5- to 20-μm), thin-walled, ribbon-like hyphae with few septations (pauciseptate) and right-angle branching (132). The hyphae may vary in width, appear folded or crinkled, and be sparse or fragmented. In lesions exposed to air, thick-walled spherical structures can form at the ends of the hyphae (35). Routine H&E stains may show only the cell wall with no structure inside. In cytologic specimens the hyphae will be highlighted with Papanicolaou and calcofluor white stains. On occasion the hyphae are very degenerate, and many of the characteristics may not be appreciated in the specimen; however, the pathologist should mention that degenerate hyphal elements are observed in the specimen, since this identifies the source where the fungus is found and will rule out contamination if there are questions about contamination in the culture. Stains that can help highlight the fungal wall include GMS and PAS stains, although fragmen-

tation and necrosis of the fungal elements may cause these stains, in particular GMS, to be either faintly positive or negative (Fig. 2).

In immunosuppressed hosts, the hyphal elements will be found with abundant necrosis, hemorrhage, and blood vessel thrombosis (12). Important diagnostic features include identification of fungal elements invading the blood vessel wall or inside their lumen. Sparse neutrophilic inflammation can be found in the periphery of the lesion. *Entomophthorales* infections in immunocompetent hosts are accompanied by intense granulomatous inflammation with abundant neutrophils and eosinophils, fibrosis, and granulation tissue (111). The Splendore-Höeppli phenomenon in these cases can be prominent. Sporulation can occasionally be observed.

(iii) Pitfalls in morphological diagnosis. The major morphological differentiation between *Mucorales* genera and other molds is with other fungi that produce nonpigmented hyphae in tissue, including *Aspergillus* spp., other hyaline septated molds (such as *Fusarium* and *Scedosporium*), and *Candida* spp. (132). The presence of abundant septation and acute-angle branching should suggest the diagnosis of *Aspergillus* spp. or another hyaline septate mold, while yeasts with pseudohyphae should suggest *Candida* spp. Poor staining of hyphae with GMS should suggest mucormycosis. To be able to specifically identify *Mucorales* in tissues or to detect dual infections by *Mucorales* genera and other fungi, it is important to use immunohistochemistry, *in situ* hybridization, or PCR (70, 147).

(iv) Alternative testing. Culture of the tissue specimen is indispensable for organism-specific diagnosis. Attention to using gentle processing, is important since aggressive grinding of the tissue may render the fragile fungal elements nonviable (132). *Mucorales* genera are fast-growing fungi, but unfortunately, the yield of cultures is low. Although serologic tests have been attempted, they are not recommended.

Diseases caused by *Fusarium*, *Scedosporium*, and other hyaline septated molds. (i) Epidemiologic and clinical situations when hyaline septated molds should be considered in the differential diagnosis. Infections caused by colorless septate fungi are referred to as hyalohyphomycoses (109). The major organisms included in this category are *Fusarium* spp., *Scedosporium* spp., *Trichoderma* spp., *Paecilomyces* spp., *Scopulariopsis* spp., *Acremonium* spp., *Schizophyllum* spp., *Phaeoacremonium* spp., and *Trichosporon* spp. These organisms are ubiquitous in nature, leading to frequent mucocutaneous and inhalational exposure, and the distinction between colonization and infection may be difficult to assess in immunosuppressed patients with positive cultures. Although histopathology has limitations, it remains crucial because it provides information regarding tissue invasion. Neutropenia is an important risk factor for invasive disease caused by all of the hyaline molds (39, 113).

Fusarium spp. can cause superficial infections such as keratitis, onychomycosis, sinusitis, and cutaneous infections in normal hosts (113). It has been recovered frequently from wounds and skin of burned patients and has been described in association with contaminated water supplies. Invasive infection, particularly in the lung, has been seen in patients with hematologic malignancies, prolonged neutropenia, and graft-versus-host disease and is second in frequency to infections by *Aspergillus* spp. in these patients. Skin lesions that result from disseminated disease are painful, pruritic nodules that evolve to have central necrosis, giving a characteristic "bull's eye" appearance, and eventually ulcerate to produce ecthyma gangrenosum-like lesions. Dissemination can occur to all organs, including the heart (26). *Fusarium* spp. are frequently recovered from blood cultures in patients with disseminated disease, and *Fusarium solani* is the most common species encountered. In addition, *Fusarium* has the ability to produce toxins, and mycotoxicosis can occur in animals and humans when they ingest food contaminated with a toxin-producing species (113).

Scedosporium spp., and for some species their sexual state *Pseudallescheria* spp., are also ubiquitous in soil and water and can cause disease through inhalation of conidia or direct inoculation of mucous membranes and skin (39). These organisms can cause a range of diseases similar to those observed with *Aspergillus*, from allergic responses in sinuses and lungs and colonization of lung cavities with formation of fungus balls to invasive pulmonary and disseminated organ involvement. Disseminated disease caused by *Scedosporium* spp. is seen primarily in patients with AIDS, primary immunodeficiencies, and hematologic malignancies and patients who have received transplants and/or corticosteroids. However, pneumonia or brain abscesses can be observed in normal hosts and should be particularly considered after near drowning in polluted water. In addition, individuals who participate in agricultural activities may present with white-grain mycetomas caused by *Scedosporium* spp. that involve skin, soft tissues, and bone.

Lastly, *Trichoderma* spp. have been observed in patients undergoing dialysis in addition to those with the typical causes of immunosuppression (109). *Paecilomyces* spp. are a cause of keratitis and endophthalmitis (109). Organisms of both of these genera as well as *Scopulariopsis* spp., *Acremonium* spp., *Schizophyllum* spp., *Phaeoacremonium* spp., and *Trichosporon* spp. can cause invasive diseases with clinical presentations indistinguishable from those caused by *Aspergillus*.

(ii) Morphological characteristics that set hyaline molds apart. In tissue the features of hyaline septated molds are similar to those seen with aspergillosis (113). The fungi invade vessels and cause thrombosis and necrosis of the surrounding tissues. Microscopically the hyphae are septated, show acute-angle branching, and are not pigmented (Fig. 2). The contours of the hyphae and presence of septa are highlighted by using PAS and GMS stains. Occasionally, the hyphae of hyaline fungi swell and appear globose.

Fusarium and *Scedosporium* may show prominently constricted hyphae with varicosities and intercalated chlamydoconidia (163). *Fusarium* spp. can sporulate in tissues, and thus there may be a combination of yeast-like structures and hyphae in histopathologic preparations (113). *Scedosporium* spp. rarely sporulate in tissues but can do so in patients with fungus ball cavities; in this case the ovoid conidia are pigmented, correlating with the melanin-like pigment of the colonies (39).

(iii) Pitfalls in morphological diagnosis. These organisms cannot be distinguished from each other or from *Aspergillus* spp. Thus, the presence of nonpigmented, septate hyphae with acute-angle branching in tissue should not be signed out as "compatible or suggestive of *Aspergillus*," since there are important treatment implications (67). These organisms should be signed out descriptively, and a comment stating that the fungal elements could correspond to *Aspergillus* spp., *Fusarium*

spp., *Scedosporium* spp., *Trichoderma* spp., *Paecilomyces* spp., *Scopulariopsis* spp., *Acremonium* spp., *Schizophyllum* spp., *Phaeoacremonium* spp., or *Trichosporon* spp. should be added (Fig. 2). When the hyphae of these fungi appear globose, the differential diagnosis is with fungi in the *Mucorales* genera. In these instances, the width of the hyphae can be assessed at the areas of septation, since these areas are less affected by the swelling.

(iv) Alternative testing. Blood cultures for *Fusarium* have a high yield compared to those for *Aspergillus* because of adventitious sporulation in tissue. However, caution has to be exercised when these fungi are cultured, since these molds are ubiquitous in the environment and frequently cause contamination or colonization. The (1→3)-β-D-glucan test is positive in these infections and cannot help distinguish them from other invasive fungal infections, including candidiasis (113). If culture is not available, galactomannan testing should be performed to try to differentiate hyaline molds from *Aspergillus* spp. It is important to make organism-specific diagnoses of these molds because some *Fusarium* species and *Scedosporium* frequently demonstrate resistance to azoles and echinocandins, thus requiring combination therapy (36, 113).

Diseases caused by *Bipolaris*/*Curvularia* and other dematiaceous fungi. (i) Epidemiologic and clinical situations when dematiaceous fungi should be considered in the differential diagnosis. Dematiaceous fungi are naturally pigmented molds whose hyphae and conidia contain melanin. Dematiaceous fungi are ubiquitous in the environment and can be found in soil, plants, and organic debris worldwide. More than 60 genera have been implicated in human disease (130). Melanin is considered an important virulence factor, since disruption of pigment production by *Exophiala dermatitidis* leads to reduced virulence in animal models (44).

Dematiaceous molds primarily cause skin and soft tissue infections that are preceded by trauma or environmental exposure, and they can give rise to three clinical entities: eumycetoma, chromoblastomycosis, and phaeohyphomycosis (54, 109). The name "chromo" comes from the Greek root meaning color, while "phaeo" also comes from the Greek and means dusky, with both names indicating the color of the lesion or the pigment production by the causative organism. Table 3 presents the differences among these three clinical entities. In addition, dematiaceous fungi can cause a variety of other clinical syndromes, including onychomycosis, keratitis, allergic disease, pneumonia, brain abscesses, and disseminated disease. In patients with respiratory syndromes, the infection is acquired by inhalation of conidia. The dematiaceous fungi most frequently associated with eosinophilia and allergic sinusitis or allergic bronchopulmonary mycosis are *Bipolaris* and *Curvularia* (130). The clinico-pathologic characteristics of allergic diseases are similar to those described for *Aspergillus*. Pneumonia, brain abscesses, and disseminated disease are frequently seen in immunocompromised patients such as HIV-infected patients, those who have received a transplant or are receiving corticosteroids, and patients with cancer, diabetes, or chronic diseases (11). However, brain abscesses have also been described in immunocompetent hosts (131). It is postulated that brain abscess and disseminated disease are secondary to a primary pneumonic focus rather than skin disease. *Cladophialophora bantiana* is the dematiaceous fungus most frequently

TABLE 3. Clinical presentation of dematiaceous molds

Condition	Clinical presentation	Tissues involved	General agents that can cause the entity	Molds most frequently associated with clinical presentation	Other molds associated
Eumycetoma	Painless mass in extremities with multiple sinuses that drain pus and grains	Skin, soft tissues, fascia, bone	Bacteria, hyaline and pigmented molds	*Madurella* spp.	*Scedosporium*, *Acremonium*, *Exophiala*, *Aspergillus*, and others
Chromoblastomycosis	Tumor-like lesions of skin (nodules, verruca, scar, plaques)	Skin, underlying soft tissues	Pigmented molds	*Fonsecaea pedrosoi*, *Cladophialophora carrionii*	*Phialophora verrucosa*, *Rhinocladiella* spp., *Exophiala* spp., and others
Phaeohyphomycosis	Immunocompetent individuals present with single lesion (cyst, plaque) in the area of inoculation; in immunosuppressed patients the inoculation site may be a nodule, eschar, ulcer, or other and may disseminate to other organs	Skin, soft tissue, sinuses, lungs, brain	Pigmented molds	*Exophiala jeanselmei*, *Exophiala dermatitidis*, *Bipolaris* spp.	*Alternaria*, *Curvularia*, *Exserohilum*

associated with brain abscesses (130). Mortality is low for patients with superficial diseases, but once the disease becomes disseminated, mortality increases to approximately 80%.

(ii) Morphological characteristics that set dematiaceous fungi apart. Diagnosis of these entities requires histopathologic examination of the tissues and culture. Although biopsy specimens are best for histology and culture, scrapings of the lesions can also be used. The biopsy specimens should be obtained from areas with pigment, as these are sites where fungi can be found. All dematiaceous fungi show pigmented hyphae; however, the degree of pigmentation will vary. The pigmented round cells characteristic of chromoblastomycosis, which are also called "copper penny lesions" or sclerotic bodies, may show internal septations. In cases where pigmentation is not evident with H&E stains, Fontana-Masson staining may be necessary to demonstrate the melanin pigment (54). The hyphae tend to be thin (2 to 6 μm wide) but are irregularly swollen (also referred to as toruloid or moniliform) with prominent septa that show constrictions (Fig. 2) (33, 135). The frequency of branching will depend on the fungus. The hyphae may show terminal or intercalated vesicular swellings with thick walls resembling chlamydoconidia. Pigmented yeast-like cells can also be seen and may show septation and budding. In addition, GMS and PAS stains can be used to highlight the fungal wall. The fungal elements are usually seen in multinucleated giant cells or in the extracellular necrotic material. Specific morphological features have been described for the three distinct skin and soft tissue entities.

Mycetomas are characterized by the production of grains. Dematiaceous fungi produce black grains while other fungi (usually *Scedosporium* and *Acremonium*) or bacteria produce white grains. Histologically, grains are interwoven mycelial aggregates that are lined with intense eosinophilic material (Splendore-Höeppli phenomenon) (30). The grains are localized in dermal abscesses and sinus tracts that connect the abscess to the skin surface. The sinus tracts are surrounded by granulation tissue and granulomatous inflammation.

Chromoblastomycosis forms characteristic sclerotic bodies in tissue or muriform cells. These are darkly pigmented, thick-walled, round to polyhedral fungal elements that have undergone septation in multiple planes (124). They are usually found in the dermis or epidermis, including the keratin layer, and are surrounded by connective tissue and neutrophilic inflammation. Fungal elements are found singly or in clusters. In chromoblastomycosis, the epidermis usually shows an intense reaction with pseudoepitheliomatous hyperplasia and hyperkeratosis. The inflammatory infiltrate is primarily granulomatous with microabscesses.

The phaeohyphomycotic cyst presents histopathologically as a single dermal lesion with minimal changes in the epidermis (33). The cyst wall consists of dense collagenous tissue and granulomatous inflammation with abundant giant cells. In the center of the cyst there are foci of geographic necrosis and foreign bodies, presumed splinters that carried the infection. Fungal elements (yeast-like structures and septated hyphae) can be found throughout the lesion. When phaeohyphomycosis becomes disseminated in patients with cancer, fungal elements can be observed in 83% of specimens (11). The inflammatory reaction in disseminated disease is usually nonspecific (neutrophils and mononuclear cells), with only one-fourth of the specimens showing necrosis and even less commonly granulomas.

(iii) Pitfalls in morphological diagnosis. The different dematiaceous fungi cannot be distinguished from one another by histology. Some dematiaceous fungi may show very little melanin and may appear as hyaline hyphae; thus, a Fontana-Masson stain is needed to highlight the pigment. However, caution should be exercised when interpreting Fontana-Masson staining, since many *Aspergillus* spp., some *Mucorales* genera, and *Trichosporon* can also show positive staining (80).

(iv) Alternative testing. Cultures should be obtained to have an organism-specific diagnosis. Because dematiaceous fungi are ubiquitous in the environment, contamination of cultures occurs frequently. A study in a cancer center showed that only 11% of 348 specimens from which dematiaceous fungi were cultured could be associated with cases of proven or probable invasive fungal disease (11). Thus, finding the fungal elements by histopathology is indispensable for diagnosis. Serodiagnostic tests are not available for these infections.

Dermatophyte disease. (i) Epidemiologic and clinical situations when dermatophytes should be considered in the differential diagnosis. Superficial mycoses are believed to affect 20 to 25% of the world population (4). Dermatophytes are the predominant cause, with three main anamorphic (asexual or imperfect) genera infecting humans: *Epidermophyton*, *Trichophyton*, and *Microsporum* (165). Dermatophytes are found worldwide, usually associated with keratinous material that serves as the source for human and animal infections. Some species have more restricted geographic locations; for example, *Trichophyton soudanense* is primarily found in central Africa, while *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Microsporum canis*, and *Epidermophyton floccosum* are distributed worldwide. Migration of populations has resulted in changes in the distribution of the different dermatophytes. In addition, there has been a decline of these infections with improvements in living conditions and the use of antifungal agents.

Dermatophytes are acquired through contact of an individual with conidia present in soil, animals, or objects (e.g., combs, shoes, or clothing) (100). Certain conditions that allow longer contact between conidia and skin (spaces between nails) and have the appropriate humidity and temperature (interdigital areas or hair) are more prone to harbor these infections. These infections are designated tinea or ringworm, followed by the affected body site. Examples include tinea unguium or onychomycosis (in nails), tinea cruris (in the groin), tinea corporis, tinea circinata, or tinea glabrosa (in the extremities), tinea pedis (athlete's foot), tinea capitis or tonsurans (in the scalp) and tinea barbae (in the beard). All tineas have a similar clinical pattern, showing a ring of scaling inflammatory skin that is accompanied by burning and itching. If there is an intense reaction in hair follicles, the disease is known as Majocchi's granuloma.

Dermatophytes are fungi that invade the keratin of immunocompetent and immunosuppressed hosts by virtue of their keratinolytic proteases (161). These fungi express carbohydrate-specific adhesins that allow attachment to epithelial cells, and they produce multiple serine and metallo-endoproteases that allow digestion of the keratin network. Dermatophytes cause chronic skin disease since they adapt and are not rapidly

eliminated by the host immune response. *Trichophyton* cell wall mannans are capable of inhibiting lymphoproliferative responses *in vitro* and may also be responsible for inhibiting turnover of the stratum corneum. The host response is primarily delayed-type hypersensitivity and varies in degree depending on the host immune status and the infecting dermatophyte. In addition, there is an association between dermatophyte infections and allergy, particularly asthma.

Besides dermatophytes, other fungi can cause superficial skin and hair infections (8). The clinico-pathologic presentations and the responsible fungi are presented in Table 4. The two most important groups are *Malassezia* spp. and *Candida* spp. *Malassezia* infections cause tinea versicolor and are particularly associated with application of oils and lotions, use of corticosteroids, exposure to sunlight, hydrosis, and possibly seborrheic dermatitis (100). In patients with AIDS, superficial *Candida* infections are common and include cheilitis (painful fissures in the lip commissures) and a variety of mucocutaneous lesions, including atrophic plaques, pseudomembranous lesions, or leukoplasia (125).

(ii) Morphological characteristics that set dermatomycosis apart. To detect dermatophytes in the keratin skin layer, it is necessary to use GMS or PAS stain since these are hyaline fungi that are difficult to observe in the keratin layer using H&E (66). Hyphae and aleurioconidia can be visualized and are particularly prominent in hair follicles. The host reaction to the fungus is very variable. In the keratin layer there may be mild hyperkeratosis with focal parakeratosis. In acute lesions the epidermis shows spongiosis and neutrophilic microabscesses. Lastly, the dermis shows various degrees of perivascular lymphocytes and plasma cells, and in some cases there may be prominent papillary dermal edema (71). When a dermatophyte causes severe inflammation of hair follicles and shafts, it is called kerion if the infiltrate is primarily neutrophilic or Majocchi's granuloma if chronic with prominent mononuclear inflammation. In both instances giant cells can be present and frequently contain fragments of hyphal elements. On rare occasions dermatophytes can invade the epidermis and dermis, producing nodular lesions that resemble mycetomas.

(iii) Pitfalls in morphological diagnosis. Superficial fungal infections caused by *Candida* spp. and *Malassezia* spp. should be considered in the differential diagnosis of dermatophytes. In comparison with dermatophytes, these organisms tend to stain basophilic with H&E. In the case of *Candida* spp., it is indispensable to make sure that the infection is superficial and no vascular invasion is present. If dermal or vascular invasion is observed, it is important to rule out skin manifestations of systemic fungemias.

(iv) Alternative testing. Lesion scrapings can be clarified with potassium hydroxide (KOH), and then either viewed under a light microscope or stained with calcofluor white and viewed under a fluorescence microscope. This will identify the presence of fungal elements but will not determine which fungus is present. Cultures are important to differentiate between dermatophytic disease and superficial skin infections caused by other fungi or yeasts. Dermatophyte test medium (DTM) changes color with production of alkaline metabolites produced by dermatophytes, potentially allowing faster identification than when using traditional fungal culture media. The diagnosis should be established before starting treatment because of the length, cost, and potential side effects of the drugs used. In addition, knowing the dermatophyte species may help establish preventive measures with pets or other possible sources of reinfection. PCR testing of skin scrapings and other specimens has been used to identify dermatophytes and other fungi that can cause onychomycosis and superficial skin infections; however, PCR testing has not been approved in the United States for diagnostic purposes (76).

Diseases caused by other molds (coelomycetes). (i) Epidemiologic and clinical situations when coelomycetes should be considered in the differential diagnosis. Fungi that in the asexual stage produce conidia inside a fruiting body-containing cavity lined by either host or fungal tissue are known as coelomycetes. The fruiting body can have a variety of shapes (spherical or disc-like), and the cavity opens to the environment through an ostiole or pore (156). In general these fungi infect plants and thus are found ubiquitously in the environment. Humans acquire these organisms during contact with infected plants or soil through cuts or punctures in the skin. Coelomycetes cause superficial skin infections, onychomycosis, and keratitis/endophthalmitis and can occasionally become systemic in immunocompromised hosts (51). All these fungi are pigmented and cause cutaneous and subcutaneous phaeohyphomycosis and black-grain mycetomas. A number of fungi are considered to be in this group; *Phoma* spp., *Colletotrichum* spp., and *Nattrassia mangiferae* are some examples.

(ii) Morphological characteristics that set coelomycetes apart. It is important to perform histopathologic studies in these cases, since coelomycetes are ubiquitous and culture contamination may occur. The fungal elements are quite pleomorphic and can be moniliform, bead-like yeasts to short branched or unbranched hyphae. Some tissues can display yeast-like elements, depending on the plane in which the tissue was cut. The fungal elements show pigment.

(iii) Alternative testing. These fungi can be difficult to identify because they do not produce reproductive structures in tissue although they show moderate to rapid growth. Thus, panfungal PCR amplification with sequencing of the product has been used to identify cultured isolates (51).

TECHNIQUES THAT ARE USED FOR IDENTIFICATION IN HISTOPATHOLOGIC MATERIAL

Histochemical Staining

Methods. Histopathologic examination of tissues to detect fungi is and will remain an important tool to define the diagnostic significance of positive culture isolates, including fungal invasion of tissues and vessels as well as the host reaction to the fungus. Histopathology can also provide rapid presumptive diagnosis of the fungus while waiting for fungal culture results, or it may provide the only available material when no culture growth occurs or cultures were not ordered. Histopathologic examination of biopsy specimens, surgical resection specimens, and autopsy material should always start with H&E staining of the tissue. GMS and PAS staining should be performed if a fungus is suspected after review of tissue sections because of presence of an inflammatory tissue response or when there is high clinical suspicion even if the H&E stain is unrevealing. In addition, mucin (mucicarmine) or melanin (Fontana-Masson)

TABLE 4. Clinical presentation of dermatophytes

Name of superficial infection	Clinical presentation	Extension to hair follicle	Fungus(i)	Systemic disease	KOH preparations	Morphology in tissue sections
Tinea or ringworm, followed by the location in the body	Round lesions with scaly border, accompanied by pruritus and burning	Yes; when suppurative known as kerion, when chronic known as Majocchi's granuloma	Dermatophytes (*Epidermophyton* spp., *Trichophytum* spp., *Microsporum* spp.)	Very rare but can invade the dermis and soft tissues, causing mycetomas	Hyphae with or without septations	Hyphae cannot be visualized in the keratin with H&E, special stains are needed
Tinea versicolor	Hypo and hyperpigmentation in patients with oily and sweaty skin, fine scales when scratching	Yes, known as *Pityrosporum* folliculits	*Malassezia* spp.	Systemic infections may occur in premature neonates receiving parenteral nutrition and in other immunosuppressed hosts	Yeasts and hyphae ("spaghetti and meat balls")	Faintly basophilic hyphae in the stratum corneum
Tinea nigra	Brown to black macule, usually in palms, with some scaling	No	*Phaeoannellomyces werneckii*	Not described	Darkly pigmented, septated, and branching hyphae	Pigmented hyphae in the stratum corneum
White piedra	Creamy-white, small, soft nodules in hair shafts	No	*Trichosporon* spp.	Immunosuppressed patients may have lung infiltrates, renal involvement, and fungemia	Septate hyphae perpendicular to hair shaft	Not used for diagnosis
Black piedra	Hard dark nodules in hair shafts	No	*Piedraia hortae*	Not described	Collections of crescent ascospores surrounded by pigmented hyphae	Not used for diagnosis
Superficial candidiasis	Intertrigo, chronic paronychia, onychodystrophy, cheilitis	Yes	*Candida* spp.	Yes, particularly in patients with AIDS and depending on the level of immunosuppression	Yeasts, pseudohyphae may be observed	Fungal elements may be seen through the biopsy, vascular invasion must be determined

TABLE 5. Stains and alternative methods that can be used with tissue sections

Stain(s)	Application(s)	Color and fungi stained
Hematoxylin and eosin (H&E)	Used routinely in pathology to demonstrate tissue morphology; in the case of fungal infections, this stain helps identify the inflammatory host reaction, such as multinucleated giant cells, necrotic material, hemorrhage, and the Splendore-Höeppli phenomenon; most fungi can be observed with this stain, particularly the nuclei of yeast-like cells or if the fungus is naturally pigmented (however, the fungal elements may be difficult to distinguish from the background)	All fungi show pink cytoplasm, blue nuclei, no color for the wall
Fungal silver stains (Grocott and Gomori methenamine silver [GMS])	They highlight the wall of the fungus and thus are useful for screening the tissue sample, can be combined with H&E in such a way that the fungus and the host reaction can be clearly observed	The fungal cell wall appears black or dark brown for all fungi, the surrounding tissue is usually green, the *Mucorales* may stain very pale
Periodic acid-Schiff (PAS)	Detects glycogen in tissues, fungal walls contain large amounts of glycogen and thus PAS can be used for screening for fungal organisms	The fungal cell wall appears pink to red purple; depending on the counterstain used, the nuclei can be blue
Gridley fungus	Stains the walls of most fungi	The fungal cell wall appears purplish red, the background is usually yellow
Mucine stains (Mayer's or Southgate mucicarmine, Alcian blue)	Stains mucopolysaccharides, including the capsules of a variety of organisms; also stains mucus, which can be present in a variety of human cells	Useful to highlight the capsules of cryptococci, which appear red or blue depending on the stain used
Melanin stains (Fontana-Masson)	Stains melanin present in some fungi; also stains melanin present in human tissues, such as in the skin epidermis	*Cryptococcus* and the dematiaceous fungi will take a black to dark brown color
Bacterial stains (tissue Gram stains or acid-fast stains)	Many fungi take bacterial stains; additionally, some filamentous bacteria (actinomycetes and *Nocardia*) have to be differentiated from fungi	*Candida* spp. stain purple/blue (Gram positive) with the Gram stain; *Blastomyces* and *Histoplasma* can be acid-fast staining (take a red color)
Immunohistochemistry (IHC)	Uses antibodies against the different fungi, antibodies can be monoclonal or polyclonal, assays are not FDA approved and require validation by each laboratory	Assays for *Blastomyces*, *Cryptococcus*, *Histoplasma*, *Coccidioides*, *Pneumocystis*, *Sporothrix*, *Paracoccidioides*, *Penicillium*, *Candida*, *Aspergillus*, and *Mucorales* have been published in the literature; depending on the colorimetric developer, the fungi can stain dark brown or red; the counterstain is usually hematoxylin; cross-reactivity of the antibodies is a concern
In situ hybridization (ISH)	Uses molecular probes for different fungi, probes are generally ribosomal since there are multiple copies of ribosomal genes in each fungal cell, assays are not FDA approved and require validation by each laboratory	Probes published in the literature include those for *Blastomyces*, *Cryptococcus*, *Histoplasma*, *Coccidioides*, *Pneumocystis*, *Sporothrix*, *Candida*, *Aspergillus*, *Mucorales*, *Fusarium*, and *Pseudallescheria*; depending on the colorimetric developer, the fungi can stain dark brown or red; sensitivity and specificity of the probes vary

stains can be extremely useful for identification of *Cryptococcus* (mucin and melanin) and dematiaceous fungi that may not produce abundant pigment (melanin). Table 5 presents the different stains and alternative methods for fungi that can be used in tissue sections. It is important to remember that some fungal infections, particularly during the acute phases, give rise to neutrophilic or suppurative inflammation, so epidemiologic circumstances and clinical history may also prompt the need to use these special stains. In immunosuppressed patients there may be no inflammation but necrosis may be present because of fungal invasion of blood vessels and associated thrombosis. Fungal invasion of blood vessels weakens the wall and can result in hemorrhage. Communication between clinicians and pathologists is invaluable to define the cases in which GMS, PAS, Fontana-Masson, or mucicarmine stains may be useful to highlight fungi. As the use of less invasive procedures has become more prevalent in medicine, cytologic specimens have become common samples. Bronchoalveolar lavage fluid is a frequent specimen used to diagnose pulmonary infections (81). Other cytologic specimens include sputum, cerebrospinal fluid, and fine-needle aspirates of lesions. Most fungi can be visualized with the routine stains used for cytologic preparations,

including Papanicolaou and Giemsa stains; however, the wall of the fungi is not highlighted with either stain. In order to stain the fungal wall, GMS and PAS stains can be used in cytologic specimens. Calcofluor white, an alternative stain that highlights the fungal elements, can be used with fresh cytologic specimens if a fluorescence microscope is available; alternatively, potassium hydroxide wet preparations can be studied using bright-field or phase-contrast microscopy. Bronchoalveolar lavage and cerebrospinal fluid specimens can also be used for detection of fungal antigens.

Retrospective studies that correlate culture results with histopathology and cytology showed that the overall accuracy for microscopic morphological techniques can vary from 20 to 80% (140, 143, 158). The lowest correlation has been reported for invasive septate molds (158). Even though GMS and PAS stains were used more frequently in the cases correctly diagnosed than those misclassified, special stains did not significantly improve pathologists' diagnostic capabilities (140). Misclassification of cases occurs when the pathologist has a false sense of his or her ability to categorize fungal organisms by genus based on microscopic morphology alone, when inappropriate terminology is used such that other potential molds within a particular category are not included in the differential diagnosis, or when there is a lack of knowledge of morphological mimics of yeasts and hyphal forms. The misclassifications with greater potential for adverse consequences occurred when there were few, folded, fragmented, and/or necrotic fungal elements in the specimen and the structures could not be adequately categorized as septate versus pauciseptate hyaline molds. Therefore, clinicians need to be aware that misclassifications in histopathologic examination occur in at least 20% of cases, and pathologists need to give as much information as possible without overextending their diagnostic capabilities (140, 143).

In order to avoid misclassifications, pathologists should describe the fungal elements observed in the sample and refrain from trying to offer a specific diagnosis. Pathologists need to remember that there are very few instances where morphological characteristics are specific. Some groups have suggested the use of templates or synoptic reporting for the diagnosis of fungal infections. In Fig. 1, 2, and 3 we have suggested templates for reporting on histopathologic specimens according to the morphological characteristics found. It is important to recognize that the diagnosis is primarily descriptive of the fungus and should include whether or not there is invasion of the tissues and vessels, the amount of fungal elements observed, and the host reaction to the infection (inflammation, necrosis, or hemorrhage). The comment section of the report should clearly state the fungi most frequently associated with that morphology as well as other possible organisms (fungi and parasites) that should be considered in the differential diagnosis. All pathology reports should also include a statement in the comment section regarding the importance of correlating clinico-epidemiologic features and results of cultures and other laboratory tests.

Advantages. Histopathology is indispensable in some instances to define whether an organism recovered in culture represents contamination, colonization or true infection. Tissue and vascular invasion and necrosis are important histopathologic features that can help make the distinction.

Fungal cultures may not always grow; thus, histopathologic presumptive diagnosis may be the only evidence of a fungal infection. For example, *Mucorales* genera are molds that usually grow within 24 to 48 h; however, if the original specimen is ground too aggressively, the hyphal elements may be destroyed and the culture may not grow (132). In immunocompetent individuals with a chronic solitary nodule caused by an endemic mycosis, yeasts may be observed in tissue sections but the culture may not grow because the yeasts are nonviable (166). Poor recovery of *Aspergillus* spp. and other septate hyaline molds in cultures has been attributed to previous treatment, use of prophylactic antifungal medications, or possible differences in the physiologic states of the mold *in vivo* versus *in vitro* (158). Another situation that can result in loss of ability to recover an organism in culture even when a viable organism was initially present in the clinical material is when a small quantity of biopsy material is sent to the microbiology laboratory with a request for multiple cultures, such as for aerobic bacteria, anaerobic bacteria, acid-fast bacilli (AFB), and fungi, all of which require inoculation of multiple media and slide preparations. Sometimes this small amount of material must be even further split between microbiology and histopathology. Lastly, certain fungi, such as *Pneumocystis*, do not grow using current microbiology practices and require detection using histopathologic or cytologic techniques (140).

In some instances, fungal cultures may take weeks to be completed, and thus a preliminary histopathologic diagnosis of a fungal infection may be ready before the culture and may provide sufficient information for clinicians to start treatment. For example, *Scedosporium*, *Sporothrix*, *Blastomyces*, and *Coccidioides* may take up to 3 to 4 days to grow and *Histoplasma* and *Paracoccidioides* may take more than 2 weeks; thus, the histopathologic diagnosis could be available earlier than culture results (140).

Disadvantages. In the previous sections we have discussed the pitfalls for each fungus in the comparison of its morphology with those of other fungi or parasites. In addition to these pitfalls, pathologists must differentiate possible fungal structures from stained normal human tissue structures. Particularly when using GMS stains, normal tissue structures that can be confused with yeasts include neurosecretory granules and melanin, while hyphae require differentiation from collagen fibers, basement membranes, and other silver-staining filamentous structures. Figure 4 shows neurosecretory granules, which compared to any of the yeasts tend to be irregular, smaller, and located inside neurosecretory cells, while basement membranes and collagen fibers tend to show lower staining intensity and less sharpness than hyphae. To aid in the recognition of these different tissue structures and fungi, it is important to colocalize the GMS-stained structures in tissue sections (usually from a consecutive level) stained with H&E or PAS stain. The combination of GMS counterstained with H&E, instead of Light Green, is a way to achieve the colocalization in one slide. In addition, pathologists should assess the presence or absence of internal structures that can be observed in fungi (nuclei and cytoplasm) which stain with H&E but not with GMS.

Another disadvantageous occurrence that can happen during interpretation of special stains is when the sample shows some transversally cut hyphae, which then appear as yeasts that

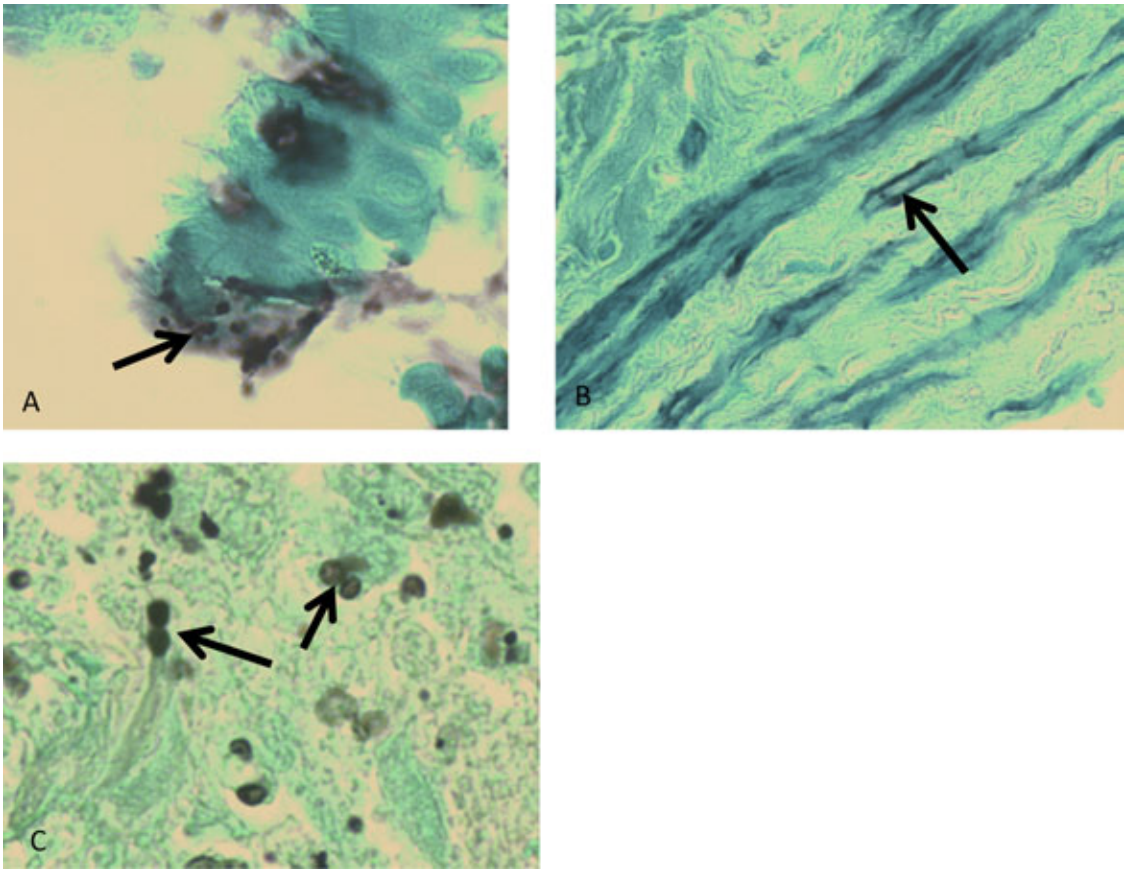

FIG. 4. Diagnostic pitfalls. When using GMS stains, normal tissue structures can appear as yeasts or hyphae. (A) Neurosecretory granules (arrow). (B) Collagen fibers, with one even showing a "pseudoseptum" (arrow). (C) In specimens with few organisms, hyphae cut transversally can appear as yeasts that may have "pseudobudding" (arrows).

may even appear to be budding (Fig. 4). In these cases it is important to go deeper into the block to see if more fungal elements cut longitudinally are present in the sample.

Histopathology usually cannot provide the fungal genus and species, which are very important for treatment. For example, a case with hyaline septate hyphae includes in the differential *Aspergillus* spp., *Fusarium*, *Scedosporium*, and others. Thus, treatment should be with voriconazole, which is effective for all these fungi; however, treating with or adding amphotericin B should be done only for *A. fumigatus* and *F. solani*. Itraconazole or echinocandins could be used for *A. fumigatus* but would not be effective for *F. solani* or *Scedosporium* (140). Detection of yeasts with pseudohyphae suggests *Candida* spp. with a differential diagnosis that includes septate hyaline molds; thus, fluconazole could be used to cover most *Candida* spp. but would lack activity against *C. krusei*, *C. glabrata*, or the other septated hyaline molds.

Infections with more than one fungus have been reported in burn and immunosuppressed patients (70, 143). Even though histopathology might be expected to be an adequate method to identify these double infections, the morphological diversity may be subtle and not appreciated. Thus, other tests should be used to determine if more than one organism is present.

In immunosuppressed patients, isolation of different fungi during short periods of time may raise the question of whether there are either different fungal infections in different locations, subsequent infections by different fungi, or an infection with two different fungi in which one of the organisms had intrinsic resistance to the treatment given. In Fig. 5 we present an example of a neutropenic patient with chronic lymphocytic leukemia who was diagnosed first as having an invasive mold (by culture *A. fumigatus*) in the lung and then 3 days later with invasive fungal rhinosinusitis (by culture *Fusarium* spp.) and died 22 days later with disseminated fungal infection (suspected mucormycosis by immunohistochemistry). The sequence of photographs stained with GMS shows the usual difficulty in distinguishing hyaline septated molds from those that are pauciseptated by using histopathology, particularly in the second and third samples, where morphological characteristics could have been altered because of previous treatment of this patient with amphotericin B and voriconazole.

Immunohistochemistry

Methods. Immunohistochemistry refers to the use of antibodies to detect targets (fungal antigens) in tissue sections so that the morphology of the target as well as the surrounding tissues can be seen. For these assays it is necessary to cut a very thin section of the tissue (similar to that used for histopathology) and place it on a glass slide. The tissue can be fresh frozen

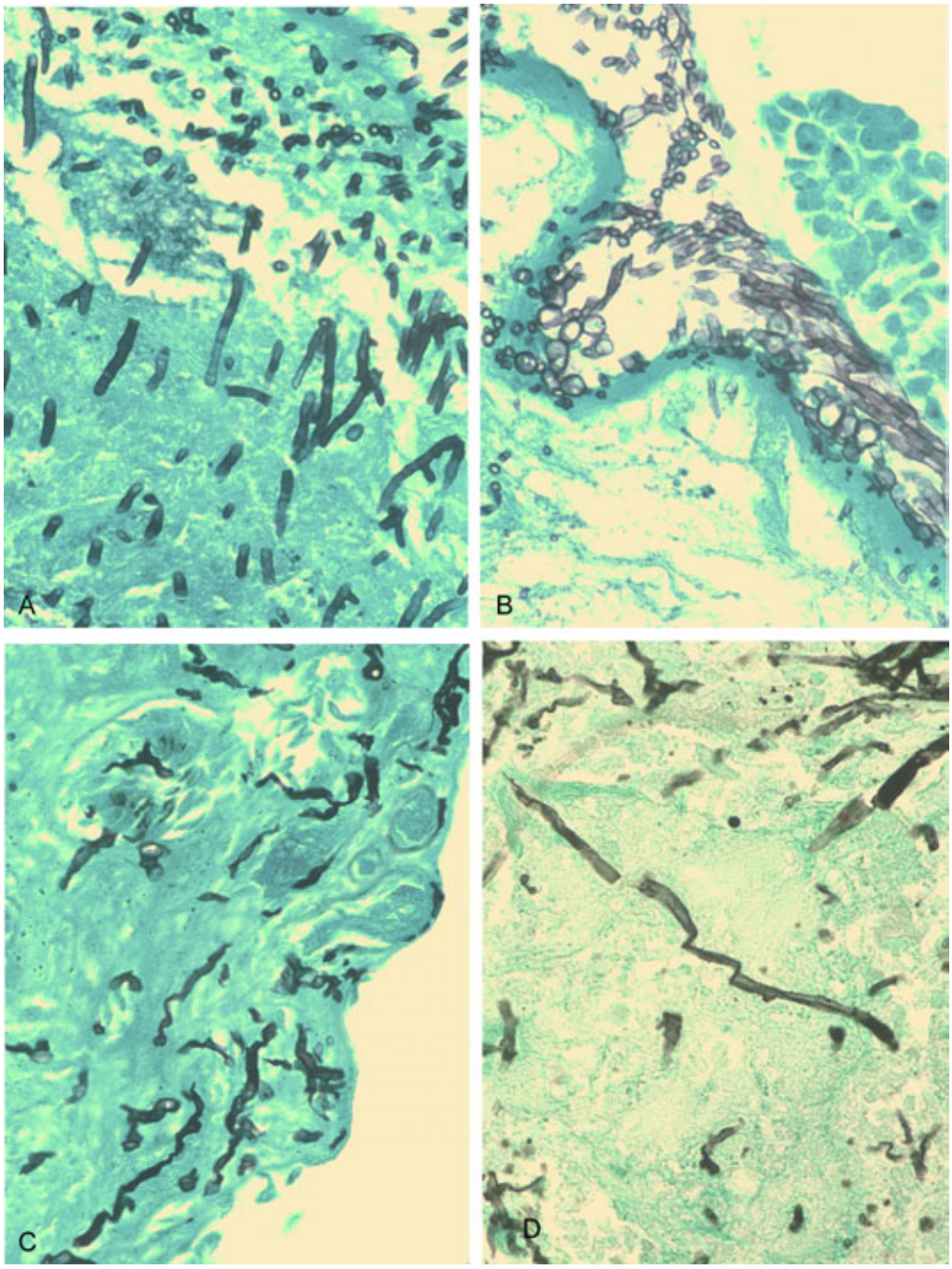

FIG. 5. Sequential specimens stained with GMS (magnification, ×120) showing mold infections in a neutropenic patient with chronic lymphocytic leukemia. (A and B) Hyaline septated hyphae in the lung. The culture was positive for *Aspergillus fumigatus*. (C) Hyphae (by culture a *Fusarium* sp.) in a nasal debridement sample obtained in the same patient 3 days after the lung biopsy. The morphology of fungal elements could be confused with mucormycosis, since there are few septations and the hyphae twist and turn. (D) Hyaline pauciseptated hyphae in a lung specimen obtained at autopsy 22 days after the nasal debridement. The specimen stained positive using immunohistochemistry for mucormycosis. The morphology of the hyphae is distorted, probably due to previous antifungal treatment.

or paraffin embedded. Tissue that has been embedded is usually formalin fixed, which can cause distortion of antigens, particularly if fixation has been lengthy. In general, pathologists prefer to use formalin-fixed, paraffin-embedded (FFPE) tissues since this is the routine histopathologic procedure that renders the material noninfectious and easily stored at room temperature. For performing the assay, paraffin needs to be removed from the tissue, which is usually done with reagents that dehydrate the tissue. The tissue needs to be rehydrated and either treated with enzymes or subjected to other antigen

retrieval techniques that will make fungal antigens available for the antibodies, and then the antifungal antibodies are applied. In some methods the primary antibody has been enzyme or fluorescence labeled, while other procedures call for a secondary labeled antibody whose target is usually the Fc portion of the primary antibody. Once the labeled antibodies have been incubated for an appropriate amount of time, the tissue sections are washed to remove unbound antibodies. If fluorescent labels are used, the tissue section can be visualized with a fluorescence microscope. Although fluorescent antibodies have been used for fungi in specialized centers, this labeling method does not permit visualization of surrounding tissues and the preparations are not permanent. In the case of enzyme labels such as peroxidase, the color needs to be developed with the appropriate substrates and the tissue then counterstained, enabling visualization of the fungus and the surrounding tissues.

The choice of the primary antibody is very important to define the best targets. Antibodies used in other methods such as immunodiffusion or complement fixation have been tested in immunohistochemical assays, since there are few commercially available antibodies specifically validated for immunohistochemistry in FFPE tissues (128). Immunohistochemical reagents that detect *Aspergillus* spp. and mucormycetes in tissue are commercially available (AbD Serotec). It is very important to validate these assays, starting with extensive assessment of cross-reactivities of the primary antibodies with cultures and tissues that have been treated in a manner similar to that for the unknown specimens that will be tested (73). The widespread presence of common antigens in fungi has resulted in very few clinically useful specific primary antibodies. For example, several studies using a variety of monoclonal and polyclonal anti-*Aspergillus* antibodies have shown a broad range of cross-reactivities with other hyaline septated molds, mucormycetes, and some yeasts (122, 128, 146).

Studies of immunohistochemical assays for fungi have reported uniformly distributed staining of the fungal organism (128), although we have noted that nonviable hyphae may not show staining when these assays are used, particularly in tissues with mucormycosis. In addition, antigen staining has been noted outside the fungal organisms, similar to what has been well documented in immunohistochemical assays for bacterial infections (149). To be able to adequately interpret immunohistochemical assays, it is imperative to use appropriate antibody controls (an irrelevant antibody of the same type [monoclonal versus polyclonal]) in a sequential patient tissue slide so as to define the amount of nonspecific staining present for each case (46).

Advantages. In theory, immunohistochemistry for fungi has many advantages, including the combination of morphology (the fungal element itself, its localization in the tissue, and the inflammatory reaction) with specific detection of the organism using specimens that are routinely processed in pathology laboratories to render them noninfectious. Multiple automation platforms are commercially available for immunohistochemical assays, reducing the cost and turnaround time. Lastly, enzyme-labeled antibodies result in a permanent record of the reaction. As new non-cross-reactive antibodies are developed and tested using this technique, immunohistochemistry may provide an inexpensive and rapid alternative to more costly assays that do not combine morphology with detection of the specific fungus. In addition, double-staining immunohistochemical assays will permit the simultaneous detection of more than one fungus in tissue sections (70, 73, 143).

Disadvantages. At this time, many of the available antibodies cross-react with multiple fungi and cannot be used for detection of specific organisms. Evaluation, verification, and validation of the antibodies and immunohistochemical assays must be performed in the laboratory before results can be used for patient care purposes. Since antibodies are analyte-specific reagents (ASR), verification and validation in the United States must follow federal regulations (25).

In Situ Hybridization

Methods. *In situ* hybridization refers to the use of probes to detect the presence of specific fungal nucleic acids while preserving the tissue morphology so that the morphology of the fungus and the tissue reaction to the organism can be visualized. For these assays, a thin tissue section is placed on a slide and the hybridization is performed directly on the slide. Similarly to immunohistochemistry, the tissue can be either frozen or FFPE. If the tissue is paraffin embedded, preparation of the tissue sections before hybridization includes deparaffinization and rehydration. After rehydration, proteins bound to nucleic acids should be digested using enzymes (pepsin or proteinase K). Tissues are then prehybridized with a solution that contains formamide, salmon sperm DNA, and yeast RNA to decrease the amount of nonspecific binding of the probe to DNA and RNA in the tissues. The tissue DNA is then denatured and hybridized with the probe of choice. After the hybridization, excess or unbound probe is washed using different concentrations of standard saline citrate. The probe is then detected in a variety of ways, depending on how the probe was labeled. Lastly, the tissues are counterstained.

Most probes used to detect fungi using *in situ* hybridization assays have been unique, organism-specific rRNA (18S, 28S, or 5.8S) targets for various molds and yeasts (67–69, 82, 103). rRNA is distributed through the organism in large amounts, providing ample opportunity for hybridization. rRNA probes have shown strong signals when hybridized to their homologous target. For *Aspergillus*, another target that has also been used is alkaline proteinase (108). Choosing the size of the probe is important for penetration of tissues and can have a significant impact on signal intensity (61). Probes of between 400 and 750 bp seem to have the best penetration. Most frequently probes have been labeled with digoxigenin, and an immunohistochemical assay using antidigoxigenin is used for detection. Probes with over 200 bp have more digoxigenin labels, and thus their signal is stronger. Probes have also been designed with locked nucleic acids, which are modified nucleotides linked through a methylene unit, or peptide nucleic acid-labeled probes that provide uncharged, neutral backbones have been used (103, 110). Both the locked and peptide nucleic acid probes improve hybridization and help reduce the time of detection, making *in situ* hybridization assays much more rapid to complete (3- to 4-h assays). Various systems, such as catalyzed reported deposition, have been used to enhance detection (68, 69).

The probes that have been used are for the most part genus specific. Probes for *Blastomyces*, *Coccidioides*, *Cryptococcus*,

Sporothrix, *Pneumocystis*, *Candida*, *Fusarium*, and *Pseudallescheria* have shown strong signals with good analytic specificity (67–69, 82). On the other hand, probes for *Histoplasma* showed very low sensitivity when the organism was demonstrated in an area of necrosis (69). Also, some *Aspergillus* probes have shown cross-reactivity with other septate hyaline molds, particularly *Fusarium* (67). In some assays, probes for *Mucorales* genera have been difficult to interpret because of high background and low signal intensity, in addition to uneven distribution of the signal in the hyphal elements (68).

In most published reports, *in situ* hybridization is performed in cases where fungal elements can be first demonstrated with routine histopathologic stains, since GMS and PAS stains have shown better sensitivity than hybridization assays as a screening method for detection of fungal elements (67–69). *In situ* hybridization is used to confirm and identify the specific organism present in the tissue. Thus, each case usually requires *in situ* hybridization with an array of probes designed to identify the fungi in the differential diagnosis. However, hybridization may be uneven, and nonstaining hyphal elements may represent either infection by another fungus for which there is no probe or nonviable portions of the organism. The use of multiple probes in one case can potentially demonstrate the presence of dual infections.

Advantages. *In situ* hybridization offers the highest degree of specificity compared to histochemical stains and immunohistochemistry (the other morphology-based techniques). As with histochemistry and immunohistochemistry, *in situ* hybridization assays performed with FFPE tissues have the advantage of using noninfectious specimens that are routinely processed in pathology laboratories. Automated platforms for *in situ* hybridization already exist; however, costs are higher and the turnaround time is usually longer than for immunohistochemical assays. At this time, differentiation of *Fusarium* from other septate hyaline molds is very important clinically and has been demonstrated using *in situ* hybridization (67, 103). Although probes for all fungi are not yet commercially available, probes that are used in other settings, such as fluorescent *in situ* hybridization for *Cryptococcus* and *Candida* spp. in blood cultures and CSF, could potentially be validated for *in situ* hybridization platforms for FFPE tissue (91, 95).

Disadvantages. *In situ* hybridization is not a screening method, since GMS and PAS stains are more sensitive for detection of fungi (67–69). Once a yeast or mold has been detected in tissue using histochemistry, a panel of probes should be used to define the genus present. The panels should be constructed based on the fungi in the differential diagnosis for that tissue and that patient population. Although more expensive, the use of panels will allow for detection of single and dual infections. At this time, probes for *in situ* hybridization assays in tissues are not commercially available. Laboratory-developed assays should be evaluated, verified, and validated in the laboratory before results are used for clinical diagnosis and patient care as indicated by federal regulations (25).

PCR-Based Methods

Methods. The PCR has been used to detect fungal DNA in FFPE tissues. FFPE tissue is not the sample of choice. Fresh nonembedded tissues have shown a sensitivity for PCR detection of fungi of 97%, while the sensitivity of paraffin-embedded material is only 68% (84). The fungal DNA extracted from FFPE specimens can be degraded and in low concentration, and it often contains substances that inhibit protein digestion or DNA amplification. However, when fungal elements are detected in FFPE tissue sections and fungus culture is not available, PCR can in some cases determine the organism that is causing the infection. Depending on the DNA extraction method, the nucleic acid quality, determined as the percentage of samples in which a human housekeeping gene control is recovered, can vary from 60 to 90%, and consequently the PCR efficiency is between 57 and 93% (106). Each tissue sample needs to be tested with a human control DNA to determine the quality of the nucleic acid extracted.

Either of two approaches can be used for analyzing extracted DNA preparations: one is to target sequences specific to a particular organism; the second is to amplify a gene that is present in all fungi (i.e., panfungal) and then sequence the fungal DNA. Genus-specific PCR probes that have been used with FFPE samples include those for *Aspergillus*, *Rhizopus*, *B. dermatitidis*, *Coccidioides*, *H. capsulatum*, and *P. brasiliensis* (14–16, 18, 19, 23). The majority of the published assays target specific rRNA genes (18S, 28S, and 5.8S), the intervening internal transcribed spacer (ITS1 and ITS2), or particular portions of the fungal genome and use nested or seminested PCR methods. The second approach of amplifying a panfungal gene and then sequencing the product is very appealing because this approach is expected to detect a large variety of fungi that can infect humans (84, 133). For this approach, primers should include sequences present in multiple copies within the fungal genome but contain highly variable regions that allow species identification. Primers for the ITS1 and ITS 2 regions have been used for the amplification reaction (106). The PCR product(s) obtained is then visualized, purified, and sequenced. The identification is made by comparing the sequence to those in a sequence database such as GenBank (37).

There is no side-by-side comparison of culture with PCR from FFPE tissues. A prospective study comparing culture to PCR from fresh frozen tissue showed that cultures were positive in 63% of cases while PCR was positive in 96% (134). However, results from retrospective studies have shown that once tissues are formalin fixed, PCR positivity can be as low as 60% (106). Studies that compare all detection methods for fungi, including histopathology, PCR, *in situ* hybridization, and immunohistochemistry performed with FFPE tissues, are not available. Histopathology still appears to be the best screening tool to define the presence of fungal elements and verify that the fungus is causing disease in the tissue (19); however, to define the specific fungus or fungi present, nucleic acid detection (PCR or *in situ* hybridization) is probably more specific than immunohistochemistry at this time.

Advantages. Although morphology is not preserved, the great advantage of PCR in FFPE tissue samples is determination of the specific agent that has been observed by histopathology. With the use of PCR it has become evident that infections with multiple agents are more frequent than previously suspected (70, 84, 106, 133). To detect double infections, it is necessary to use panels of primers for more than one organism.

Disadvantages. Nucleic acids obtained from FFPE material are frequently damaged (cross-linked) or may contain PCR

inhibitors and thus may be unable to generate an adequate PCR product, may not display homology to the primer used, or may generate a product that cannot be sequenced (84). Selection of the DNA extraction method is crucial to obtain the best yield from this material (106). In addition, when fungal elements are scant in tissues, the amount of DNA obtained may be insufficient to perform a PCR assay.

The choice of PCR primer is important. There is insufficient variation in the ITS1 region to differentiate certain species, including *C. neoformans*, some *Candida* spp., and *Fusarium* spp.; thus, analysis of other regions should be considered (37, 84). In addition, false-positive results with specific *H. capsulatum* primers and difficulties in identifying *Coccidioides* in formalin-fixed tissues have been reported (15, 17). A Mayo Clinic study of 147 FFPE samples showed that histology found more coccidioidomycosis cases than PCR (19). Lastly, it is estimated that 10 to 20% of the sequences in GenBank are misidentified (106).

PCR assays continue to be labor-intensive and costly. The turnaround time for paraffin material is still approximately 4 to 5 days (for deparaffinization, DNA extraction, PCR, and sequencing). At this time, PCR assays for tissues are not commercially available, so laboratory-developed assays should be evaluated, verified, and validated by the laboratory before results are used for clinical diagnosis and patient care as indicated by federal regulations (25).

Laser Microdissection

Methods. Laser microdissection combines microscopy with laser technology to enable the study of specific cell types. Once the cells of interest are isolated, a variety of studies/tests can be performed using these cells specifically, and the results are not masked or diluted by surrounding cells or tissue constituents within the tissue sample. Two microdissection technologies are available: laser capture microdissection and laser cutting (107).

Tissue sections (usually thicker than the ones used for histopathology) are placed in special slides that allow for easy separation from the rest of the specimen. The tissue can be fresh frozen or FFPE and can be stained with H&E or by other methods that permit visualization of the desired cells in a bright-field microscope. A narrow-beam laser is used to focus on (for laser capture) or cut around (for laser cutting) the cells of interest. Capture microdissection uses an infrared laser, while cutting microdissection uses a UV laser. The cells that have been targeted or cut out are collected in a plastic cap or tube.

An important aspect of microdissection is that the tissue preparation before the tissue is placed on the slides must be tailored to the secondary test. Preparation includes fixation (or lack thereof) and staining. Exposing the tissues to the smallest amounts of chemicals ensures the least alterations to the sample for subsequent tests. Possible secondary tests include PCR for nucleic acids, electron microscopy for cellular organelles, and mass spectrometry or two-dimensional polyacrylamide gel electrophoresis for proteins (115, 159). If prepared correctly, the microdissected cells can even be cultured.

Until now laser microdissection has been used primarily for diagnosis and research on neoplastic diseases, but several researchers have reported using this technique to study infectious diseases (74, 169). Use of this tool in the ecology of dual fungal infections would enable us to better understand the pathophysiology in these cases. Laser microdissection has been used for identification of a single hypha of *A. fumigatus* from fresh frozen bird tissues (115). Although DNA sequences were obtained from 60% of the animals, a PCR product was not obtained from the remainder, suggesting that the amount of DNA will depend on the number of hyphae and the number of intact nuclei selected.

Advantages. The greatest advantage of laser microdissection is that the elements to be tested can be specifically selected. Thus, dual infections and the local environment in which this occurs can be studied in detail. Another advantage is that the material obtained will not be contaminated with nonfungal tissues.

Disadvantages. Laser capture microdissection instruments are expensive, and many laboratories may not have one or be able to afford them. From the technical perspective, there is the possibility that certain components may not be preserved because the heat in the laser may destroy the elements selected for secondary testing (107). Contamination may occur if a microdissection system requires using particular devices to select the cells, compared to placing the cells in the tube where the second test will take place. The disadvantages that occur for the secondary testing will be the same as those observed in these tests, with the added disadvantage of potentially obtaining very small amounts of material. Another disadvantage that has been reported is that intact fungal nuclei must be obtained for DNA extraction. Particularly with mucormycete organisms that are pauciseptate, hyphal material may be successfully collected but may not contain nuclei.

INTERPRETATION IN DIFFERENT SITUATIONS

Fungal Elements in Tissue but All of the Specimen in Formalin

The situation where fungal elements are in tissue but all of the specimen is in formalin is relatively frequent for several reasons: (i) cancer is the most frequent diagnosis of lesions that are resected or biopsied, and the routine procedure for tissues obtained in operating and endoscopy rooms is to place them in formalin so that morphology for cancer diagnosis is preserved; (ii) not all lesions are studied using frozen sections (iii) when a frozen section is obtained and shows fungal elements, the pathologist may not think it necessary to remind the surgeon that a sterile portion of the lesion should be sent to the microbiology laboratory for cultures; and (iv) communication between infectious disease physicians and surgeons is not always established before the tissue is obtained. Training and better communication among surgeons, interventional radiologists, pathologists, and infectious disease physicians needs to take place so that this situation decreases to a minimum.

However, when fungal elements are seen in tissue but the entire specimen is in formalin, the pathologist should describe the fungal elements, define as part of the diagnosis line whether tissue invasion by the fungi is occurring, and provide a comment that enumerates the fungi that can display the morphological features described (see "Histochemical Staining" above) (Fig. 1, 2, and 3). Identifying the pathology caused by the fungal elements (inflammatory reaction, invasion of vessels, necrosis, or hemorrhage)

tells the clinician that the fungi are not contaminating or colonizing the tissue. Descriptive diagnoses of the fungal elements together with a comment listing the fungi with consistent morphology are important to guide treatment (140). For example, the presence of septated, nonpigmented hyphae will result in treatment with voriconazole plus amphotericin B since *Fusarium*, *Aspergillus*, and *Scedosporium* could have that morphology, while a patient whose tissue displays pauciseptate hyphae will receive amphotericin B or posaconazole since this morphology most probably corresponds to the *Mucorales* genera. If the pathologist observes more than one type of fungal element in the tissue (for example, pauciseptate hyphae and yeasts with pseudohyphae), this should be noted, particularly in view of the increasing numbers of dual infections that are being reported.

In addition, alternative testing that is relevant to the clinicoepidemiologic presentation of the patient and can be performed with nontissue specimens (serology, antigen detection, skin testing, and blood cultures) can be suggested to clinicians by the microbiology laboratory and infectious disease specialist (as presented for each fungal infection in this review). Lastly, immunohistochemistry, *in situ* hybridization, and PCR with FFPE samples have already been validated for research purposes in some centers, enabling detection of specific fungi when the entire specimen is placed in formalin.

Positive Cultures but Tissue without Fungal Elements

The situation where there are positive cultures but tissue without fungal elements can occur in four instances: (i) the fungus present in the cultures is a colonizer in the patient; (ii) the fungus present in the culture is a contaminant in the laboratory; (iii) tissue is sampled from two different areas, with one sample sent to microbiology and the other to pathology; or (iv) the pathologic specimen has not been extensively studied with adequate special stains. In this situation it is important to know the pathology that was observed in the specimen and the location and number of colonies found in the plates. When only one or two colonies are observed at the area of inoculation of the plate and there is minimal pathology in the tissue sections, it is reasonable to think that the fungus present in the culture may be a colonizer. When only one or two colonies are observed away from the area of primary inoculation of the plate and there is minimal pathology in the tissue sections, it is reasonable to think that there was contamination of the fungal culture. It is also possible that the surgeon sampled two different areas of the tissue and that the one containing viable fungi was sent to microbiology while the second sample, not containing the fungal elements, was sent to pathology. Thus, there will be positive cultures, but the tissue will not contain fungal elements. Lastly, the specimen sent to pathology may not have shown the pathology usually associated with fungal infections and thus the pathologist may not have asked for special stains, or the fungal elements may be so sparse that they could have been missed. In any of these instances it is indispensable to correlate the laboratory findings (pathology and microbiology) with the clinical presentation of the patient and determine if alternative testing is required. For example, if a *Fusarium* colony was found in the middle of the plate and there was necrosis or hemorrhage in the tissue sample from a severely neutropenic patient, obtaining blood cultures may be a reasonable alternative test since invasive infections with *Fusarium* can be found in the blood. However, in the same scenario, if the organism cultured had been an *Aspergillus* sp. or a member of the *Mucorales* genera, obtaining blood cultures would not be helpful.

In instances where the tissue sections show pathology (inflammation, necrosis, or hemorrhage), it is important that the pathologist, clinician, and microbiology laboratory communicate to define the need to examine deeper in the tissue block and verify that slides were stained with GMS and PAS stains. Any fungus in low abundance may be difficult to identify, and deeper sections stained with GMS and PAS stains may be necessary. In addition, hyphae in patients infected with *Mucorales* genera may be quite distorted and negative or faintly GMS positive; thus, using PAS stains is indispensable. If the pathology present in the tissues is consistent with allergic fungal disease, finding the fungal elements may be difficult. Appropriate alternative testing may be required, depending on the culture findings, the pathology encountered, and the clinical presentation.

Fungal Elements in Tissue but Cultures without Growth

The situation where there are fungal elements in tissue but cultures without growth can occur in three instances: (i) when the tissue in the microbiology laboratory is ground too aggressively and the fungal cells are destroyed; (ii) when the fungus in the tissue is not viable; or (iii) when tissue is sampled from two different areas, with one sample sent to microbiology and the other to pathology. *Mucorales* genera are particularly prone to be destroyed with aggressive processing of the tissues. Thus, it has been recommended that the sample not be ground or homogenized but instead that direct plating of larger tissue pieces on fungal media should be routinely performed. If the pathology specimen shows hyphae with few septations compatible with *Mucorales* genera, positive growth should occur within 3 days, and the microbiology laboratory can then give the clinician a presumptive diagnosis. If growth has not occurred in the expected time, it is possible that there will be no growth. However, because *Histoplasma* and *Paracoccidioides* grow slowly *in vitro*, fungal cultures are usually incubated for at least 4 weeks in most laboratories.

Nonviable fungi in tissue are frequent in chronic, walled-off infections with endemic yeasts, such as in cryptococcosis, histoplasmosis, or coccidioidomycosis. This could also happen if the patient has received antifungal medications. It is also possible that the surgeon sampled two different areas of the tissue and that the one containing fungi was sent to pathology while the second sample, not containing the fungal elements or containing nonviable fungi, was sent to microbiology. Thus, there will be negative cultures but the tissue will contain fungal elements. Regardless of the reason for the lack of growth, fungal elements in tissue causing pathology should be treated as described in the first situation, i.e., where there are fungal elements in tissue but all of the specimen is in formalin.

Discrepancy between Culture Results and Histopathologic Findings

A discrepancy between culture results and histopathologic findings can occur because either (i) the characteristic morphology of the fungus has been altered due to use of antifungal medications or host responses or (ii) there is a dual infection and only one fungus is growing in culture. With more cases being studied using

PCR, it has become evident that dual infections are more frequent than previously realized. Although the selection of cases from the CDC study may be biased (due to difficult cases being sent for diagnosis to a reference facility), dual infections may occur in up to 20% of cases (106). In these situations, the pathologic description may refer only to the most abundant fungus present in the specimen, and the second fungus may not have been mentioned. If a second fungus is not mentioned but grows in the culture, the results may appear to be discrepant. Reasons for the recovery of only one fungus in instances of dual infections include too-aggressive processing where larger fungal cells are destroyed but smaller ones survive, the growth of one fungus inhibiting the growth of the second, or inappropriate culture conditions such that only one fungus can be recovered.

The true frequency of dual fungal infections is not well established, and the technology that exists can now permit studies that address this important question. Dual-color immunohistochemistry and *in situ* hybridization as well as laser microdissection followed by panfungal or specific PCR could be used to address this issue.

Discrepant results can appear problematic but can be solved by reviewing the patient's clinical history to see if the patient has received antifungals or how long the infection has been present and by reviewing with the pathologist the morphological characteristics of the fungal elements present in the slide. Providing descriptions of the fungal elements in the tissue sections for the diagnosis with a comment listing possible organisms that can show that particular morphology should help decrease the number of discrepancies between culture results and histopathology.

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