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Zygomycosis: conventional laboratory diagnosis

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Abstract

A definitive diagnosis of zygomycosis caused by Mucorales can be made by histopathological examination with or without isolation of the fungus from the same site. Histopathological examination of the tissues affected typically shows characteristic broad, hyaline, ribbon-like, irregular fungal hyphae with wide-angle branching, accompanied by tissue necrosis and angioinvasion of the fungi. Tissue invasion by the fungal hyphae as seen by microscopy is essential to establish the diagnosis. Fungal elements can be stained with Gomori methenamine-silver, periodic acid-Schiff or Calcoflour white stain. All Mucorales grow rapidly on most fungal media such as Sabouraud dextrose agar incubated at 25–30 °C. Mucorales from a sterile site or repeated positive cultures of the fungi from a non-sterile site are considered significant in a high-risk patient with predisposing factors for acquisition of zygomycosis. Positive cultures from non-sterile specimens should be interpreted with caution and will require correlation between the finding and the clinical situation.

Keywords: Diagnosis, Mucorales, Zygomycetes, zygomycosis

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Introduction

Although *Aspergillus* species, particularly *Aspergillus fumigatus*, account for the largest proportion of invasive mould infections, the last decade has witnessed the emergence of new opportunistic pathogens, including non-fumigatus *Aspergillus* species, *Fusarium* species, *Scedosporium* species, the dematiaceous fungi (*Alternaria*, *Bipolaris*, *Curvularia*, *Cladosporium* and *Exserohilum* species) and the agents of zygomycosis (mucormycosis) [1–3].

The class Zygomycetes includes a variety of filamentous fungi that may cause human disease and have emerged as an important cause of morbidity and mortality among immunocompromised patients [4,5]. The medically important Zygomycetes encompass two orders of filamentous fungi with distinct morphological, epidemiological and pathogenic characteristics, the Mucorales and the Entomophthorales [5]. The majority of cases of zygomycosis in humans are caused by members of the order Mucorales. Organisms of the genus *Rhizopus* are by far the most common clinical isolates, with *Rhizopus oryzae* occcurring most frequently. Members of the genus *Mucor* are second to *Rhizopus* in order of frequency, whereas Cunninghamella, Apophysomyces, *Absidia*, *Saksenaea*, *Rhizomucor* and other genera each represent a significantly smaller percentage of clinical isolates [4–7]. Zygomycetes are ubiquitous in soil and the environment and occasionally colonize humans.

The diseases produced by these fungi are referred to by the label 'zygomycosis'. Manifestations depend on the location of involvement, but they typically concern rhinocerebral, pulmonary, cutaneous, gastrointestinal and central nervous system (CNS) diseases [4–7].

Zygomycetes are characterized in culture by broad, nonseptate or sparsely septated hyphae and by the presence of sporangiophores supporting sporangia, which contain sporangiospores [5]. During sexual reproduction in culture, zygospores may be produced. Zygomycetes are characterized in tissue by the formation of wide, ribbon-like, hyaline, aseptate or sparsely septated hyphae with wide-angle (approximately 90 °) branching. The substantial differences among these and other structures allow mycology laboratories to diagnose organisms by genus and species [5].

Laboratory Diagnosis

As infections caused by Zygomycetes, and particularly the Mucorales, in humans may be rapidly fatal, timely diagnosis is crucial to avoid treatment delay [8,9]. Although confirmation of the diagnosis and species identification of the causative organism should be pursued, treatment should be initiated as soon as the diagnosis is suspected because of the severity of

these infections. The diagnosis of zygomycosis relies on a constellation of a high index of suspicion, assessment of presenting signs and symptoms, imaging studies, cultures and direct examinations of clinical specimens, and histopathology [10,11].

Suspicion should be based on knowledge of the underlying conditions that predispose to zygomycosis and the usual presentation of the infection in each of these conditions [12,13]. A common scenario concerns the development of zygomycosis in oncological patients or transplant recipients who receive antifungal therapy for prophylaxis or treatment of other opportunistic fungal infections [9,14–18]. In such cases the antifungal agents administered are not active against Zygomycetes, such as fluconazole, voriconazole and the echinocandins. Most of the signs and symptoms associated with the clinical manifestations of zygomycosis are non-specific. However, their diagnostic significance may increase if they are interpreted in relation to the patient's underlying condition. Early diagnosis is the cornerstone of successful treatment of zygomycosis; indeed, a direct correlation has been established between early tissue diagnosis and survival [10]. However, as is the case with many fungal infections, diagnosis is often not possible until autopsy [1,10].

Clinical specimens

The reference standard for the definite diagnosis of zygomycosis concerns histopathological, cytopathological or direct microscopic examination from affected organs [1,5,19,20]. The diagnosis relies on the evidence of tissue invasion. Thus, specimens obtained should be processed for fungal stains, cultures and any other procedures (e.g. molecular-based analyses) appropriate for ruling out differential diagnoses (Fig. 1). Combining microscopy and culture will increase the diagnostic yield by 15–20% [4,10,21].

Adequate specimens are skin scrapings from cutaneous lesions, nasal discharges, scrapings and aspirates from sinuses in patients with rhinocerebral lesions, bronchoalevolar lavages and needle biopsies from pulmonary lesions, and biopsy tissue from patients with gastrointestinal and/or disseminated disease. Blood cultures are of no benefit. If CNS abnormalities are present, brain biopsy may be helpful along with analyses of the cerebrospinal fluid. Unfortunately, even in brain involvement, fungal stains and culture results are rarely positive. Overall, it is important to collect many proper clinical specimens in order to obtain a high-yield result because Mucorales are sometimes difficult to distinguish from other filamentous fungi in histopathological examination [19,20]. Zygomycetous fungi have primitive coenocytic hyphae, which become easily damaged during biopsy procedures or tissue grinding in the laboratory. Thus, they are not suitable for growing in culture despite their presence in microscopic or histopathological examinations; in fact, fungal cultures are positive in only 15–25% of cases [6, 22].

Direct microscopic examination

Aspirated material from sinuses, sputum in pulmonary disease and biopsy material should be analysed using 10% potassium hydroxide (KOH) or optical brighteners such as Fungi-Fluor™ (Calcofluor white staining solution; Polysciences, Inc., Warrington, PA, USA) or Blancophor® (Bayer AG, Leverkusen, Germany) [10, 23]. Demonstration of fungal elements from cytological preparations (i.e. sputa, inflammatory

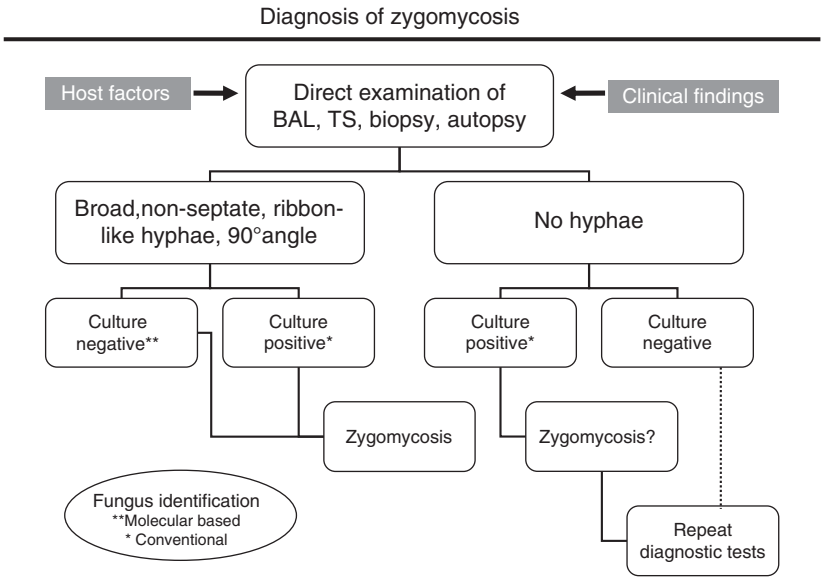

FIG. 1. Zygomycosis: diagnostic approach using microscopic and culture techniques. BAL, Broncho-alveolar lavage; TS, Tracheal secretions.

FIG. 2. Direct microscopic examination. Computed tomography-guided percutaneous lung biopsy stained with Fungi-Fluor shows typically distorted, non-septate and broad hyphae.

fluid aspirates from abscesses or sinusitis infection) may be complex as a result of the difficulty in extracting fungal elements from invaded tissues. Fungal elements may be rare in cytological specimens and, when present, are often fragmented. The key features associated with Zygomycetes are typically thick-walled, refractile hyphae 6–15 μm in diameter, swollen cells (≤50 μm) and, sometimes, distorted hyphae (Fig. 2). The width of the hyphal element varies substantially. A positive direct microscopy, especially from a sterile site, must be considered significant, even if the laboratory is unable to culture the fungus. The combined application of KOH and brighteners is possible.

Histopathology

The diagnosis of zygomycosis is made on tissue section. Acute suppurative inflammation predominates, with focal areas of granulomatous inflammation. Hyphae usually vary from 6 μm to 30 μm in diameter, and are sparsely septate and irregularly branched. The organism characteristically invades the walls of adjacent blood vessels, causing thrombosis and infarction. Stains of fixed tissues with haematoxylin and eosin (H&E) or specialized fungal stains, such as Grocott methenamine-silver (GMS) or periodic acid-Schiff (PAS) stains show broad-based, ribbon-like, non-septate hyphae with wide-angle branching (approximately 90 °) (Fig. 3) [5]. The hyphae are often not well preserved and may become crinkled or gnarled in the tissue sections. This appearance of the hyphal elements is often described as resembling 'crinkled cellophane'. Cross-sections of hyphal elements often give tissues a vacuolated appearance. These cross-sections vary in diameter and may be confused with yeast cells The

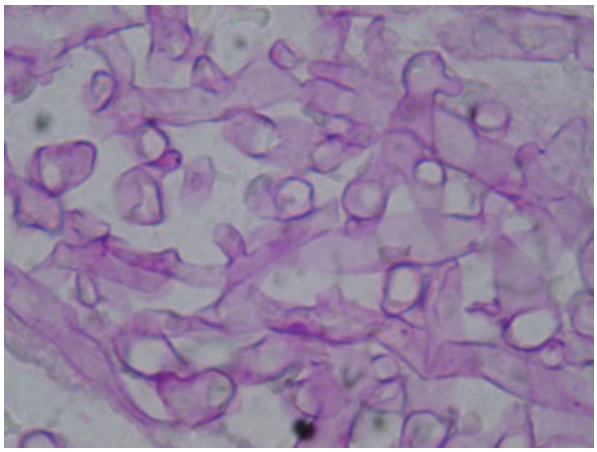

FIG. 3. Periodic acid-Schiff staining of computed tomography-guided percutaneous lung biopsy shows broad-based, ribbon-like, non-septate hyphae with right-angle branching and swollen cells.

H&E stain should always be confirmed with a more fungus-specific tissue stain such as GMS or PAS. The application of optical brighteners in such specimens is also possible.

Culture

These fungi can be very difficult to recover. It is recommended that the clinical material is inoculated onto Sabouraud dextrose agar and incubated at 30 °C (or 37 °C) for a minimum of 3–5 days [5]. The growth of the Mucorales tends to be rapid, with mycelial elements expanding to cover the entire plate in only a few (1–7) days. The sporulating surface of the colonies may demonstrate variable degrees of coloration. Depending upon the order, species or individual isolate, Zygomycetes will demonstrate surface colouration

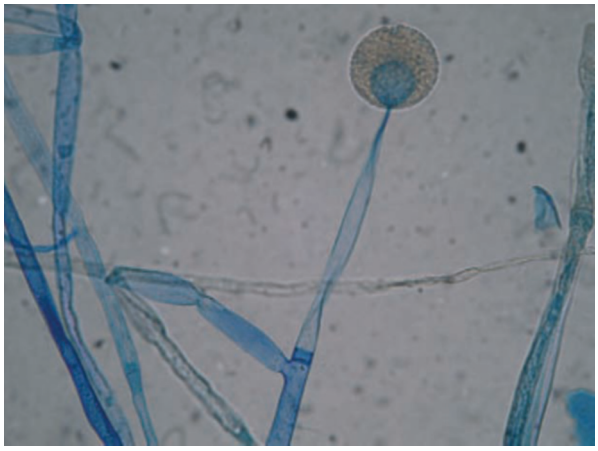

FIG. 4. Microscopic feature of *Mucor* sp. in culture. Lactophenol cotton blue staining shows a globose intact sporangium with sporangiospores and branched sporangiophores. Stolons and rhizoids are absent.

TABLE 1. Differentiating features of the sporangium-producing Mucorales

Organism	Best growth, °C	Sporangiophore	Apophysis	Columella	Sporangium	Rhizoids
Absidia sp.	37	Branched, hyaline	Conical	Dome-shaped	Pear-shaped	Primitive, rare
Apophysomyces sp.	24–42	Non-branched, brown	Bell-shaped	Rarely elongated	Pear-shaped	Tuft, originating from a foot cell
Mortierella sp.	>40	Branched, hyaline	–	–	Spherical	Primitive
Mucor sp.	<37	Branched, non-branched, hyaline	–	Varying shapes	Spherical	Absent
Rhizomucor sp.	20–60	Branched, brown	–	Spherical	Spherical	Primitive, rare
Rhizopus sp.	37	Non-branched, brown	Not prominent	Spherical	Spherical	Abundant, often well developed

Adopted from Ribes *et al.* [26].

varying from pure white to tan, brown, grey, or even black. Brain-heart-infusion (BHI) broth with penicillin is added to the normal battery when Zygomycetes are suspected. The use of broth provides optimal medium–specimen contact. The aseptic addition of sterile penicillin discs will inhibit bacteria. Malt extract agar is an effective alternative to broth media for the isolation of Zygomycetes. BHI broth may be used routinely by some ophthalmologists for corneal scrapings [5]. Sterile bread without preservatives is recommended for the recovery of Zygomycetes from clinical specimens. Bread is often superior to other media used for recovering these pathogens. A piece of bread is sterilized in a humidified Petri dish. Specimens from non-contaminated sites can be directly inoculated. Contaminated specimens should be treated with antibacterial agents before inoculation. Zygomycetes will grow rapidly, often filling the entire Petri dish within a few days [4–6].

Recovery of Zygomycetes from cultures of clinical specimens allows not only for diagnosis, but also for the identification of the causative organism to species level. Although Zygomycetes may contaminate laboratory material, their isolation from clinical specimens of susceptible hosts should not be disregarded as contamination. The isolation of Zygomycetes in patients with leukaemia had a positive predictive value of 88% [24]. Despite the ability of these organisms to invade tissues, they are rarely isolated from cultures of blood, urine, cerebrospinal fluid, faeces, sputum, paranasal sinuse secretions, bronchoalveolar lavage or swabs from infected areas. The recovery of Zygomycetes from biopsy material may be compromised if the processing of the

TABLE 2. Minimum inhibitory (MIC) and fungicidal concentrations (MFC) of various antifungals against Zygomycetes. Tests were performed according to EUCAST methodology

	MIC, mg/L			MFC, mg/L		
Species	Range	90%	Mean	Range	90%	Mean
Rhizomucor sp. (*n* = 17)						
Amphotericin B	0.5–1	1	0.68	1–4	4	1.79
Liposomal AMB	0.06–0.5	0.5	0.25	0.125–1	1	0.55
Voriconazol	>8	>8	>8	>8	>8	>8
Itraconazol	1 to >8	>8	>8	2 to >8	>8	>8
Posaconazol	1–8	4	2.7	2–8	8	5.7
Absidia sp. (*n* = 7)						
Amphotericin B	0.5–2	1	0.89	1–8	4	2.21
Liposomal AMB	0.125–4	2.5	1.25	0.25–8	4	2.54
Voriconazol	>8	>8	>8	>8	>8	>8
Itraconazol	1 to >8	>8	>8	2 to >8	>8	>8
Posaconazol	0.25–2	1	0.88	0.5–4	2	1.75
Rhizopus sp. (*n* = 12)						
Amphotericin B	1–4	2	3	2–8	8	6
Liposomal AMB	0.25–4	2	2.46	0.5–8	4	3.93
Voriconazol	>8	>8	>8	>8	>8	>8
Itraconazol	2 to >8	>8	>8	4 to >8	>8	>8
Posaconazol	1–16	4	2.86	2–8	8	5.70
Mucor sp. (*n* = 17)						
Amphotericin B	0.5–1	1	0.79	1–2	2	1.57
Liposomal AMB	0.125–0.25	0.25	0.25	0.25–1	0.5	0.54
Voriconazol	>8	>8	>8	>8	>8	>8
Itraconazol	2 to >8	>8	>8	4 to >8	>8	>8
Posaconazol	1–2	2	1.29	2–8	4	2.86
Fusarium sp. (*n* = 19)						
Amphotericin B	1–4	4	0.79	1–2	2	1.57
Liposomal AMB	0.5–4	4	0.25	0.25–1	0.5	0.54
Voriconazol	0.5–8	2	2.12	4–8	8	4.01
Itraconazol	2 to >8	>8	>8	4 to >8	>8	>8
Posaconazol	>8	>8	>8	>8	>8	>8

AMB, amphotericin B.
Data generated from Lass-Flörl *et al.* [32] and Perkhofer *et al.* [33].

specimens involves tissue grinding, a procedure that kills the non-septate hyphae of these fungi. The recovery rate is enhanced, however, if thin slices of minimally manipulated tissue are placed onto the culture medium [4–6]. Exudates and necrotic tissue contain few viable organisms; thus the inoculum from these specimens must be heavy. Zygomycetes do not survive for more than a few hours at refrigerator temperatures; therefore, if culture is delayed, storage at room temperature is recommended [6]. In any case, negative cultures do not rule out the infection.

A culture result from a non-sterile body site is not in itself diagnostic of infection because these fungi are common in the environment [24, 25]. In one study, only 16 of the samples which grew Zygomycetes (7.6%) came from infected patients [24].

Serology

Development of serological tests for diagnosis of zygomycosis by means of antigen and/or antibody detection has been attempted. Antibodies to Zygomycetes can be detected by enzyme-linked immunosorbent assays (ELISAs) and double diffusion [26]. Immunoblot analyses have also been used to detect *Rhizopus arrhizus* antigens [27]. However, these serological tests for zygomycosis cannot be recommended without further clinical evaluation and are not available for routine use at this time. Recently, Japanese researchers observed that serum IgE and Mucor IgE antibody were related to invasiveness of orbital mucormycosis [28].

Species Identification

Zygomycetes are usually fast-growing fungi characterized by primitive, mostly aseptated hyphae. Asexual spores include chlamydoconidia and sporangiospores contained in sporangia borne on simple or branched sporangiophores. Sexual reproduction is isogamous and produces a thick-walled sexual resting spore called a zygospore [5, 26].

Most isolates are heterothallic; thus zygospores are absent and identification is based primarily on sporangial morphology. This includes the arrangement and number of sporangiospores, the shape, colour, presence or absence of columellae and apophyses, as well as the arrangement of the sporangiophores and the presence or absence of rhizoids. Lactophenol cotton blue can be used to achieve better visualization (Fig. 4) [5, 26].

Growth temperature studies (25 °C, 37 °C, 45 °C) can be helpful in identifying culture characteristics. Sporulation may be stimulated by the use of nutrient-deficient media, such as cornmeal–glucose–sucrose–yeast extract agar or Czapek Dox agar. Features that help in the differentiation of these genera are summarized in Table 1.

In vitro Susceptibility

Available methods for susceptibility testing are the EUCAST-reference method for spore-forming moulds [29], the M38-A reference method for filamentous fungi [30] and the Etest® (AB Biodisk, Solna, Sweden).

With these fungi it is often difficult to achieve accurate and consistent endpoints. As the interpretive MIC breakpoints have not yet been defined for Zygomycetes and the correlations between clinical response and MIC values for a given strain are uncertain, the use of antifungal susceptibility testing in zygomycosis for routine clinical decisions is not recommended at this time [31, 32].

Zygomycetes appear to be susceptible to amphotericin B and are generally not susceptible to the triazoles and echinocandins (Table 2) [32, 33]. Among the extended-spectrum triazoles, posaconazole appears to be active against most of the Zygomycetes [32, 33].

Conclusions

Conventional, routine laboratory diagnostic techniques play an important role in the diagnosis of and treatment of zygomycosis. However, expertise is needed in isolating the agents of this disease. It is paramount to identify the causal agent so that appropriate treatment can be instigated based on our current understanding of antifungal sensitivities. Increasing effort should be put into developing new serological tests.

Transparency Declaration

CL-F serves as a consultant or speaker for Essex-Schering Plough, Gilead, MSD, Pfizer, Novartis and Basilea.

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