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Zygospores: the Last Word in Identification of Rare or Atypical Zygomycetes Isolated from Clinical Specimens

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Three zygomycetes (order Mucorales), isolated from clinical specimens, whose generic or species identification were uncertain, were definitively identified on the basis of the production of zygospores resulting from mating studies. These three isolates were identified as *Mucor circinelloides* f. *circinelloides*, *Rhizomucor pusillus*, and *Absidia corymbifera*. The production of true zygospores, the "last word" in zygomycete taxonomy, should be considered as a diagnostic tool for the definitive identification of rare, unusual, or atypical heterothallic zygomycetes. Practical considerations, however, limit this method to reference laboratories.

Identification of zygomycetes (agents of zygomycosis [mucormycosis]), especially members of the order Mucorales, is both time-consuming and labor-intensive, requiring special media, some not commercially available (5, 8), extensive microscopic observation and measurements (e.g., of sporangia, sporangiophores, sporangiospores), and a variety of biochemical and physiological tests (carbon and nitrogen assimilation, fermentation, requirement for thiamine, maximum temperature of growth, etc. [8]). Therefore, most microbiology laboratories do not identify beyond the genus level, and the species designation is often omitted in the medical literature. Laboratories that attempt species identification may find this task especially difficult if their observations do not match precisely the descriptions or illustrations in monographs or texts or if all the laborious tests required for identification have not been performed (8).

Three such troublesome zygomycetes, isolated from clinical specimens, were received by the senior author for identification or confirmation. We present in this note the method used for the confirmatory identification of these fungi, along with a brief clinical history of each patient.

The first clinical isolate (from patient 1) was cultured postmortem from the spleen of a 10-month-old baby born with Down's syndrome from a human immunodeficiency virus-positive mother. The baby had been treated with broad-spectrum antibiotics and fluconazole for a high fever. The pathology laboratory observed hyphae suggestive of *Mucor* spp. in tissue. Similar-appearing zygomycetes were isolated from cultures of the liver and spleen. Macroscopic and microscopic observations; a positive nitrate assimilation reaction using Salkin's nitrate agar (BBL Microbiology Systems, Cockeysville, Md.), a convenient but not conventional nitrate assimilation medium for zygomycetes (8); and good growth at 36°C but not at higher temperatures suggested *Mucor circinelloides* (8). However, the characteristic short circinate branches depicted in the literature were not observed (6, 8); branches were long, erect, and sympodial.

The isolate from patient 2 was cultured from a postmortem lung of a 55-year-old male with a history of chronic lymphocytic leukemia who had received fludarabine and prednisone for autoimmune hemolytic anemia. The culture was considered a possible *Mucor* sp. because rhizoids were not initially observed and it did not grow at 55°C (maximum temperature of growth of *Rhizomucor* spp. is 54 to 58°C [8]), although good

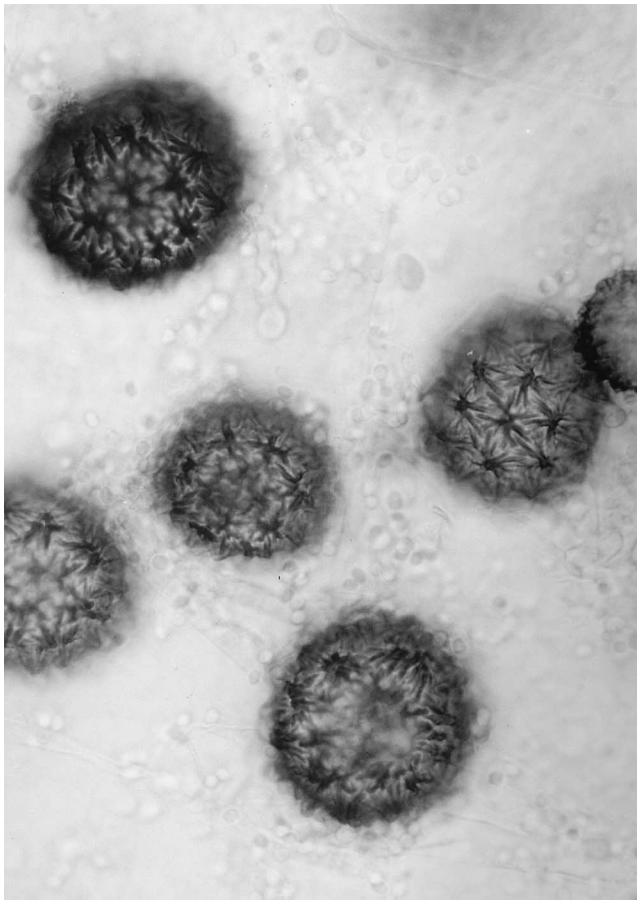

FIG. 1. Globose zygospores with stellate spines characteristic of *M. circinelloides* f. *circinelloides* on PDA (lactophenol; magnification, ×440).

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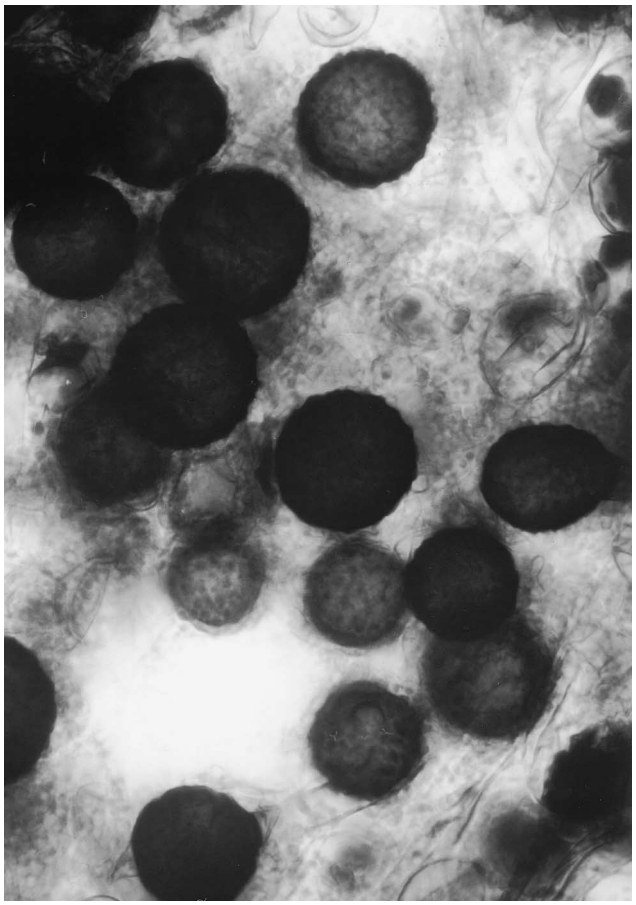

FIG. 2. Globose zygospores of *R. pusillus* with blunt spines (stellate warts) on PDA (lactophenol blue; magnification, ×440).

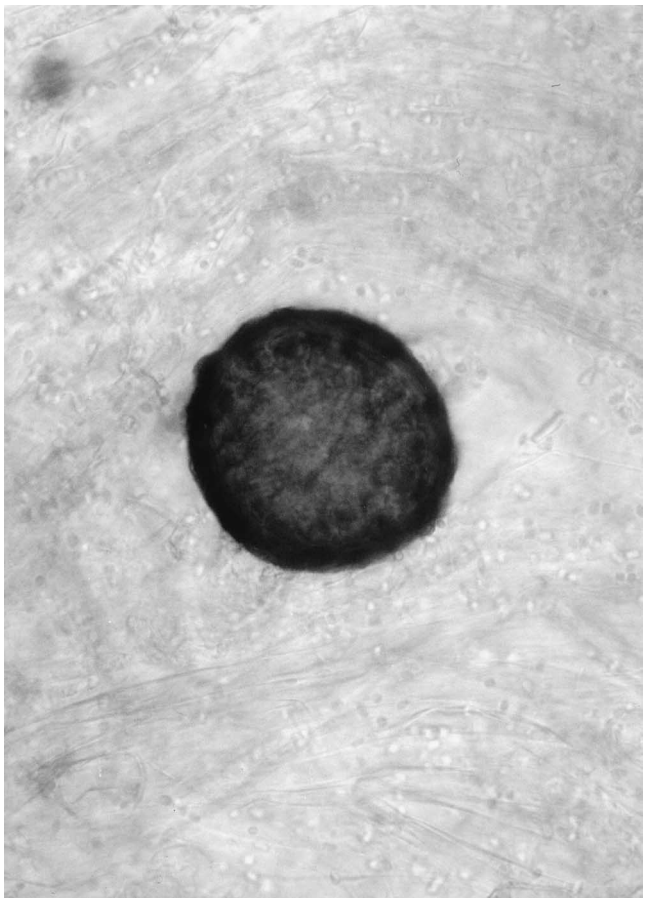

FIG. 3. Slightly roughened zygospore with four equatorial grooves of *A. corymbifera* on yeast extract agar (lactophenol; magnification, ×440).

growth and sporulation were obtained up to 49°C on potato dextrose agar (PDA) (Remel, Lenexa, Kans.).

The zygomycete from patient 3 was cultured from a bronchial wash from a 25-year-old male with cystic fibrosis. The isolate was suggestive of *Absidia corymbifera*, but its maximum temperature of growth was 45°C, not 48 to 52°C (8).

In order to definitively identify each isolate it was decided to attempt to obtain the teleomorphic stage (perfect or sexual), i.e., zygospores, since the formation of true zygospores in matings of self-incompatible similar strains is proof of the same species (8). The isolates from patients 1 and 3 were suggestive enough to warrant mating with tester (+) and (−) strains of *M. circinelloides* f. *circinelloides* and *A. corymbifera*, respectively. We also included pairing with a few other species in the collection, e.g., *Mucor indicus* and *Mucor ramosissimus*, the isolate from patient 1 and the isolate from patient 3 with several strains identified as *A. ramosa*, now generally considered synonymous with *A. corymbifera* (4, 8). More careful scrutiny of the suspected *Mucor* isolates from patient 2 revealed few simple or weakly branched rhizoids, stolons, and other features suggestive of *Rhizomucor pusillus* (7, 8); therefore, this culture was paired with strains of *R. pusillus*. The mating procedure was carried out as indicated below.

A turbid suspension was prepared from 5- to 7-day-old cultures grown on PDA by overlaying each slant culture with sterile distilled water and then gently scraping the surface to release sporangiospores. One to two drops of the suspension from each culture were delivered, via a capillary pipette, onto the surface of PDA and/or on homemade yeast extract agar (2) in petri dishes approximately 10 to 20 mm apart from the (+) or the (−) tester strain and incubated at 30°C in the dark (3) for 2 weeks. Each petri dish culture was placed in a gas-permeable plastic bag when fully grown to avoid cross-contamination. Positive and negative controls were included with each series. The positive controls consisted of matings of (+) and (−) mating types of each species where available. The negative controls comprised each isolate paired with itself as described above to ascertain the purity of each culture and rule out homothallism.

The *Mucor* isolate from patient 1 produced zygospores when paired with *M. circinelloides* f. *circinelloides* NRRL 3614(+) on PDA, confirming its identification (Fig. 1). Zygospores are typically globose, up to 100 μm in diameter, reddish brown to dark brown, with stellate spines up to 7 μm in length (6, 8). The suspected *Mucor* isolate from patient 2 produced zygospores on PDA with *R. pusillus* NRRL 3470 (no mating type is listed for this culture), establishing its identification (Fig. 2). These zygospores are up to 70 μm in diameter, globose, dark brown to black, with blunt spines also described as stellate warts (7, 8). Crosses involving the presumptive *A. corymbifera* from patient 3 and the positive controls did not result in any zygospores on PDA. However, on yeast extract agar, the medium of choice for this species (1), the controls produced a moderate number of zygospores and the clinical isolates produced a few zygospores when paired with *A. corymbifera* NRRL 2982(+) (Fig. 3). Zygospores are typically 50 to 80 μm in diameter,

globose to slightly flattened, dark brown, and slightly roughened with one to five equatorial ridges (1). Several other (+) and (−) crosses were nonfertile, indicating the presence of incompatibility factors and the necessity of a library of tester strains for this species. None of the pairings between the negative controls or the interspecific crosses yielded zygospores.

In conclusion, species identification of certain zygomycetes may be determined by the formation of zygospores in pairings with compatible strains belonging to the same species. This method, rarely used to confirm the identification of unusual clinical isolates, is presented to alert microbiologists and clinicians to the importance of this diagnostic tool in confirming the identification of zygomycetes. However, the last word in zygomycete taxonomy is limited to reference laboratories by the need for a library of tester strains and the inability of certain species to form zygospores (8).

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