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Molecular Identification of *Rhizomucor pusillus* as a Cause of Sinus-Orbital Zygomycosis in a Patient with Acute Myelogenous Leukemia

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Sinus-orbital zygomycosis caused by *Rhizomucor pusillus* in a patient with acute myelogenous leukemia is described. Identification was achieved by sequencing of the internal transcribed spacer (ITS) regions of the rRNA gene and by expression of zygospores in mating. This report highlights the value of ITS sequencing as a diagnostic tool for the identification of *R. pusillus* and expands the understanding of infection types caused by this zygomycete.

CASE REPORT

A 62-year-old male Native American rancher from Nebraska was diagnosed with acute myelogenous leukemia. He was treated with standard induction and consolidation chemotherapy followed by a low-intensity related allogeneic peripheral stem cell transplant. The patient received fluconazole prophylaxis and was placed on granulocyte-macrophage colony-stimulating factor (GM-CSF) on the day of transplant. On day 8 posttransplant (PT) the patient had an absolute neutrophil count of 300 cells per mm^3 and complained of nasal congestion. A nasal washing was positive for parainfluenza virus, but cultures for bacteria and fungi were negative. A computed tomography scan of the sinuses showed pansinusitis. On day 9 PT, the absolute neutrophil count was 1,380 cells per mm^3, at which time GM-CSF was discontinued. Over the next 4 days, the sinusitis symptoms continued with the development of right-sided periorbital edema. On day 13 PT a magnetic resonance image confirmed pansinusitis with diffuse mucosal thickening and bilateral proptosis. Broad-spectrum antibiotics were initiated. The patient was subsequently taken to the operating room for a bilateral total ethmoidectomy and sphenoidectomy; culture and histopathological exam of tissue were negative for pathogens. Amphotericin B (AMB) lipid complex (Abelcet; Enzon Pharmaceuticals, Bridgewater, NJ) was started at 5 mg per kg for presumed fungal sinusitis. Over the next 3 days, the patient had fever spikes to 39.5°C, and on day 16 PT he developed complete loss of vision in the right eye with orbital fixation. On day 18 PT, the patient underwent a bilateral total ethmoidectomy, bilateral maxillary and sphenoid debridement, and a right orbitotomy. The pathological exam of the sphenoid and ethmoid tissues reported "fibrovascular changes and acute inflammation with necrosis containing broad and irregularly shaped non-septate hyphae." Based on diagnosis of an invasive mold infection, the AMB lipid complex was increased to 7.5 mg per kg, AMB nasal irrigation was also initiated, and GM-CSF was restarted. Fungal cultures from the sinus biopsy material plated onto Sabouraud dextrose agar (Remel, Lenexa, Kansas) incubated at 30°C grew a mold within 4 days that was morphologically identified as a *Mucor* species. Sinus tissue extract and a subculture of the isolate were subsequently submitted to the Molecular Mycology Laboratory at the University of Nebraska Medical Center (UNMC) for further evaluation. DNA extracted from both the sinus tissue and the culture isolate was tested using a molecular assay targeting the internal transcribed spacer (ITS) regions of the rRNA gene complex followed by sequencing. Sequence comparison analysis using the GenBank database (National Center for Biotechnology Information, Washington, DC) identified the molecular products as *Rhizomucor pusillus* in both cases. The isolate was also sent to the University of Alberta Microfungus Collection and Herbarium (UAMH) for micromorphological and phenotypic characterization. Studies at UAMH also identified the isolate as *R. pusillus*. Additional imaging studies along with orbital and sinus debridement were performed on day 24 PT. Fungal and bacterial culturing of the tissue and imaging showed no evidence of fungus. The patient was discharged from the hospital 33 days after the transplant in stable condition with AMB nasal irrigation administered three times daily. Five days after discharge, the patient developed mental status changes and progressive respiratory deterioration. Laboratory evidence showed recurrent leukemia. Due to his aggressive recurrent disease, no further measures were taken to halt disease progression. The patient died on day 55 PT with the cause of death recorded as organ failure secondary to acute myelogenous leukemia. No postmortem exam was done.

Mycological studies. The isolate cultured from a biopsy of sphenoid tissue was deposited under accession number UAMH 10076. The isolate was grown on potato dextrose agar (PDA; Difco Laboratories, Detroit, Mich.) to obtain sporulation, and on Sabouraud dextrose agar at 25°C, 30°C, 37°C, and

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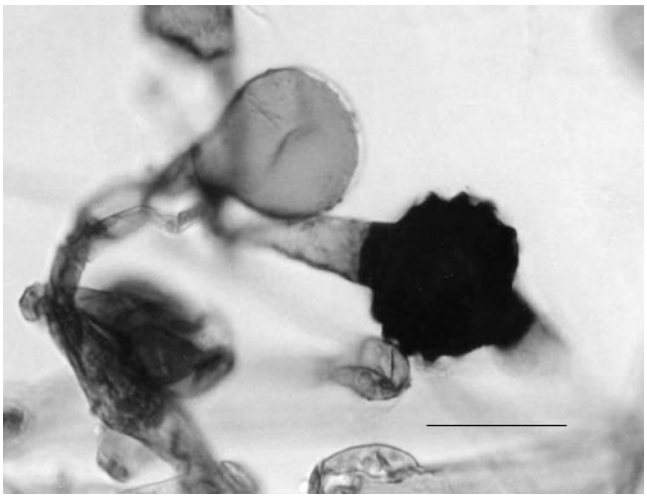

FIG. 1. Globose zygospores of *Rhizomucor pusillus* with blunt spines detected following mating studies of the case isolate with a minus mating strain. Bar = 50 μm. Magnification, ×400.

45°C to assess thermophilism. Mating experiments were performed to test for the production of zygospores with *R. pusillus* tester strains UAMH 8243(−) and UAMH 8244(+). The case isolate and a tester strain were inoculated onto separate halves of a Takashio agar plate (prepared in-house) (26).

The isolate (UAMH 10076) was identified as *R. pusillus* on the basis of colonial and microscopic features, thermophilism, and production of zygospores. Colonies on PDA exhibited rapid growth at 30°C, attaining a diameter of 35 mm in 6 days, and were velvety to floccose, initially yellowish brown and becoming grayish brown. Growth was faster at 37°C and at 45°C than at 30°C but was strongly inhibited at 25°C. On PDA after 3 days at 37°C, the hyphae were irregular, 11 to 14 μm in width, and nonseptate and formed sporangiophores having occasional rudimentary, yellowish-brown rhizoids. Sporangiophores were hyaline to pale brown, 8 to 18 μm wide, branched, and sometimes sparsely septate below the sporangium. Sporangia were yellowish brown and subglobose, measured up to 65 μm in diameter, and had obovoidal to subglobose columellae that were 35 μm long and 32 μm wide on average. Sporangiospores were yellow, subglobose, and 3.8 to 4.2 μm in diameter. Following formation of zygospores in a pairing with the minus mating strain UAMH 8243 within 7 days, the isolate was determined to be the plus mating type. Zygospores were dark brown, globose, and 60 μm in diameter on average and demonstrated a thick wall with blunt warts that were 2 to 3 μm high (Fig. 1).

Molecular testing. Extraction of DNA from the case isolate and from the sinus tissue was done following the procedures previously reported by Henry et al. (10). The PCR assay as performed by Henry et al. was utilized to test a 5-μl sample in a total reaction volume of 50 μl (10). Two oligonucleotide fungal primers (ITS 1 and ITS 4) previously described by White et al. were used for amplification (30). Primers were synthesized by the UNMC Eppley Molecular Biology Core Laboratory. DNA sequencing on the PCR products was done at the UNMC Eppley Molecular Biology Core Laboratory on a Perkin-Elmer/ABI model 373 DNA sequencer with protocols supplied by the manufacturer. The PCR products were directly sequenced using the ITS 1 and ITS 4 primers. The resultant nucleotide sequences were aligned with the MacVector sequence analysis software version 6.5 (Oxford Molecular Group, Inc., Campbell, Calif.) alignment application. Following amplification, an approximately 650-base-pair PCR product was detected from both culture and sinus extract. The resulting sequences aligned with a >99% similarity (535 of 537 bases) to sequences of *R. pusillus* following a BLAST search of the GenBank databases (2). The similarities of the sequences were determined with the expectation frequency minimized to 0.0001. Sequences were not filtered for low complexity.

Conclusions. *Rhizomucor pusillus* is a thermophilic saprophytic zygomycete with a wide distribution, but it is uncommonly associated with human disease (8, 9, 17, 20). Sporadic literature reports have shown this species to be mostly associated with patients who are severely immunocompromised, especially those patients undergoing therapy for leukemia or those who have uncontrolled diabetes (17, 25). Infection types include primary cutaneous disease with or without dissemination (14, 18, 21, 31), primary pulmonary disease with or without dissemination (3, 7, 15, 17, 23, 25), and sinusitis with or without orbital participation or involvement of the brain (1, 5, 6). In all cases, the identification of *Rhizomucor* species was based on morphological observations, with biochemical tests used in some cases. Kontoyiannis et al. compared the reliability of morphology and DNA-based methods for the identification of clinically important zygomycetes and found the highest disagreement among the four isolates of *R. pusillus*, with all four determined by sequence analysis as belonging to other genera (13). This suggested that some prior reports concerning *R. pusillus* may represent other zygomycetes (13, 27).

Mating studies and specialized phenotypic assays have been used for the identification of *Rhizomucor* species and uncommon zygomycetes (16, 27, 29). Weitzman et al. showed that mating studies leading to the production of zygospores could be used reliably to confirm the identity of the three *Rhizomucor* isolates demonstrating atypical growth characteristics, but they also recognized that this method may be limited to reference laboratories due to the need to maintain a library of testing strains (29). Zygospore production may be used also to differentiate the two *Rhizomucor* species associated with human disease, *R. pusillus* and *R. miehei*, because the latter has homothallic zygospore production (20). Lukács et al. showed that these two species could be reliably differentiated by the higher tolerance of *R. miehei* to lovastatin (16). Since no other zygomycetes were included in this evaluation, it is unknown whether this method would help to differentiate *Rhizomucor* from other genera, a problem that has been observed in several instances (13, 22).

In 1978, the genus *Rhizomucor* was taken up for *Mucor*-like fungi forming stolons and rudimentary rhizoids and expressing thermophilism (24). Molecular phylogenetic studies based on nuclear small- and large-subunit rRNA gene sequences support the distinction of *R. pusillus* from *Mucor* species (19). Species of *Mucor*, *Rhizopus*, *Cunninghamella*, and *Absidia* are more closely related to each other than any is to *R. pusillus*,

with the exception of *Absidia corymbifera*, which also shares antigenic similarities (19, 20, 28).

The ITS regions of the rRNA gene complex have been shown to be species specific and useful for the differentiation of a variety of fungal pathogens, including zygomycetes from culture isolates (4, 11–13, 32). Yeo and Wong also reviewed reports showing that the highly variable ITS regions were useful for the detection and identification of fungal pathogens such as *Candida* species and *Aspergillus* species from clinical specimens (32). Our study expands on the usefulness of the ITS target and shows its reliability in the identification of *R. pusillus* from both a culture isolate and a clinical specimen. The results also showed that the ITS target sequences can differentiate *R. miehei* from *R. pusillus* since sequence alignment analysis revealed only a 94% similarity between the case isolate and the two *R. miehei* sequences that are publicly available for comparison testing (GenBank accession numbers AJ278360 and AF205941).

This report of sinus-orbital zygomycosis highlights the value of mating studies and ITS sequence comparison for identification of *R. pusillus*. Identification of zygomycetous fungi is a challenge for clinical laboratories. The ability to identify this fungus and other uncommon pathogenic fungi to the species level by ITS sequencing will continue to emerge as a viable alternative for confirmation diagnosis.

Nucleotide sequence accession numbers. The sequence from the culture isolate was deposited into the GenBank database under accession number AF461764. A subculture was deposited into the American Type Culture Collection under accession number ATCC MYA-2881.

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