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**Mucor circinelloides** Was Identified by Molecular Methods as a Cause of Primary Cutaneous Zygomycosis

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A case of primary cutaneous zygomycosis caused by *Mucor circinelloides* is described. Histopathology showed typical hyphae along with chlamydospores. The isolate was identified by molecular and phenotypic methods. The utility of sequence analysis of the internal transcribed spacer region is highlighted; however, further studies are needed to assess species genetic heterogeneity.

CASE REPORT

A 90-year-old male presented to his primary-care physician with fever, fatigue, and lower-right-leg cellulitis following trauma to the leg. The trauma, which occurred about 1 month prior to presentation, was described by the patient as being “struck by a shrub branch while trimming brush.” He had a 2-year history of progressive pancytopenia subsequently diagnosed as myelodysplastic syndrome (MDS) and a 20-year history of diabetes mellitus type II controlled by insulin injections.

On physical examination, the temperature was 38°C, and laboratory examination showed pancytopenia with an absolute neutrophil count of 1.0 × 10³ per mm³ (normal range, 1.3 × 10³ to 7.5 × 10³ per mm³) and hyperglycemia with a serum glucose level of 525 mg per dl (normal range, 70 to 125 mg per dl). He was hospitalized at the Nebraska Medical Center and started on filgrastim (Neupogen; Amgen, Inc., Thousand Oaks, CA) to improve neutropenia and on insulin to control the serum glucose level. Intravenous ceftriaxone and ciprofloxacin were given for the lower-right-leg skin/soft tissue infection. The patient was discharged on day 7 post-primary admission (PPA) with improvement of the leg cellulitis.

A follow-up exam on day 14 PPA showed progression of the leg wound, now with a black eschar developing. The patient was readmitted to the hospital with neutropenia (absolute neutrophil cell count, 0.9 × 10³ per mm³) and hyperglycemia (serum glucose level, 1,160 mg per dl). He was taken to the operating room for debridement of the wound, and tissue was submitted to the hospital clinical microbiology laboratory for bacterial and fungal cultures. The results of a histopathological examination of this tissue were reported as “hemorrhage and extensive necrosis, focal formation of granulation tissue, and numerous invasive fungal organisms (septate hyphae and thick-walled yeast forms)” (Fig. 1). Based on a diagnosis of an invasive fungal (mold) infection, liposomal amphotericin B (AMB) at 5 mg per kg of body weight per day (Abelcet; ENZON Pharmaceuticals, Piscataway, NJ) was begun (day 16 PPA). A fungus grew within 4 days on Sabouraud dextrose (Emmons) agar (Remel Labs, Lenexa, KS) but did not grow on Mycobiotic agar (Remel Labs) after incubation at 30°C. The isolate, which was morphologically identified as a *Mucor* species, was subsequently sent to the Molecular Mycology Laboratory located at the University of Nebraska Medical Center (UNMC) for species identification. A DNA sequence of the isolate obtained from the internal transcribed spacer (ITS) regions of the rRNA gene was submitted for a BLAST search of the GenBank database (National Center for Biotechnology Information, Washington, DC). The molecular product was identified as most closely related to *Mucor circinelloides* (1). The isolate was also sent to the University of Alberta Microfungus Collection and Herbarium (UAMH) for phenotypic characterization and mating competence.

On day 17 PPA, a radiograph of the lower leg showed a small soft-tissue ulcer but no evidence of osteomyelitis. A wide surgical debridement of the involved area followed. The right-leg wound gradually healed completely with no further clinical evidence of infection. The liposomal AMB was continued for 25 days (total dose, 5.25 g). The patient was discharged from the hospital on day 47 PPA (33 days after readmission) in stable condition. A follow-up examination at 3 months after discharge showed no further clinical evidence of the right-lower-extremity infection.

**Myological studies.** The isolate cultured from debrided tissue was deposited at the UAMH under accession number UAMH 10385. The morphology was examined on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) at 30°C in the dark, and temperature tolerance was assessed on Sabouraud dextrose agar (SAB; Remel). The optimum temperature was 30°C, and there was no growth at 40°C. Colony diameters after 48 h on SAB were 5.9 cm at 25°C, 6.3 cm at 30°C, 5.6 cm at 35°C, and 4 cm at 37°C. Colonies on PDA at 30°C after 6 days were yellowish brown with raised mycelium. Microscopic examination revealed globose yellowish brown sporangia, measuring 35 to 75 μm in diameter, with finely roughened walls (Fig. 2). Columellae were subglobose to obvoid. Sporangiothecia either were long and branched sympodially or were shorter with slightly recurved lateral branches. Sporangiospores were hyaline, were ellipsoidal to slightly asym-

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metrical or obovoidal, and measured 5.5 to 7 μm long and 3.5 to 5.5 μm wide (average, 6 by 4 μm). Intercalary chlamydospores were produced in the basal mycelium; they were thick-walled, subglobose, and oval or irregularly shaped and measured 40 μm long and 30 μm wide, on average (Fig. 2). Rhizoids and stolons were absent. Mating experiments were performed to test for the production of zygospores with M. circinelloides tester strains UAMH 8306 (plus) and UAMH 8307 (minus). The case isolate was paired with each tester strain on an oatmeal salts agar plate (prepared in-house) and overlaid with a cellophane membrane (20, 27). Plates were incubated in the dark at 30°C and examined regularly for 21 days. Zygospores were produced in the pairing between UAMH 8306 and 8307 after 7 days, but no zygospores were produced in the test matings. The procedure was repeated with the same results.

**Molecular testing.** For DNA extraction, hyphae from a 48-h culture on SAB (case isolate) or from cultures on PDA (UAMH 8306 and 8307) were suspended in UltraPURE distilled water (Invitrogen Corporation, Carlsbad, CA) in 2-ml Eppendorf tubes, each containing one sterile 4.5-mm steel shot pellet. The samples were mixed for 4 min by being shaken 20 times/s using a Mixer Mill 300 (Retsch, Haan, Germany). The suspensions were extracted using the MagNa Pure LC instrument (Roche, Mannheim, Germany) and the MagNa Pure LC DNA isolation kit III for bacteria and fungi according to the manufacturer’s protocol. A modification of the universal fungal primer pairs as described by White et al. was used for amplification: ITS1-M (5’-GGA AGT AAA AGT CGT AAC AAG G-3’) and ITS4 (5’-TCC TCC GCT TAT TGA TAT GC-3’) (29). The primers were synthesized by the UNMC Eppley Molecular Biology Core Laboratory. The PCR assay was performed using 5 μl of the test DNA sample in a total reaction volume of 50 μl. The PCR master mix consisted of 31.2 μl of UltraPURE distilled water, 5 μl of 10× high-fidelity buffer, 2.5 μl of stock 2 mM deoxynucleoside triphosphate mix, 3 μl of 50 mM MgSO₄, 0.75 μl of 20 μM stock of each primer, 1.5 μl of stock 50% dimethyl sulfoxide, and 0.3 μl of 5-U/μl high-fidelity Platinum Taq DNA polymerase (Invitrogen Corporation, Carlsbad, CA). Thirty-five cycles of amplification were performed in a Stratagene Robocycler model 96 thermocycler after initial denaturation of DNA at 95°C for 4.5 min. Each cycle consisted of a denaturation step at 95°C for 30 s, an annealing step at 50°C for 30 s, and an extension step at 72°C for 1 min, with a final extension at 72°C for 3 min following the last cycle. Following amplification, a ca. 650-bp PCR product was detected from both the case isolate and the mating strains.

DNA sequencing of the PCR products was done at the Genomics Core Research Facility at the University of Nebraska—Lincoln on a Beckman Coulter CEQ2000XL 8-capillary DNA sequencer using dye-terminator chemistry and following the manufacturer’s protocols. The PCR products were directly sequenced using both the ITS1-M and ITS4 primers. The resultant nucleotide sequences were aligned with the MacVector sequence analysis software, version 6.5 (Oxford Molecular Group, Inc., Campbell, CA), to produce consensus sequences for analysis. The consensus sequence of the case isolate aligned with >98% sequence similarity to multiple sequences of M. circinelloides available in the GenBank database and with 99.0% and 97.5% similarity to sequences newly obtained from mating strains UAMH 8306 and 8307, respectively. The similarities of the sequences using the BLAST search program were determined with the expectation frequency minimized to 0.0001, and the sequences were not filtered for low complexity.

**Conclusions.** *Mucor* species are considered a distant third behind *Rhizopus* species and *Absidia corymbifera* as causes of zygomycosis (16). Although more than 50 valid *Mucor* species as well as subspecies are listed at the Index Fungorum website (www.indexfungorum.org/Names/Names.asp), only 5 species are suspected to cause human disease. These include the thermotolerant species *M. circinelloides*, *M. indicus* (M. rouxianus),...
and *M. ramosissimus*, as well as *M. hiemalis* and *M. racemosus*, which either do not grow or grow poorly at 37°C (4, 16). The inability of the latter species to grow at the elevated temperature makes their potential as causes of human disease questionable (5). Since most case reports of zygomycosis do not identify the species of *Mucor*, it is difficult to associate disease with species. Additionally, in cases where an isolate has been determined to species level, morphology alone has been shown to be unreliable for the species determination of some zygomycetes (12). The present report is the first to apply ITS region sequencing of the rRNA gene for identification of a pathogenic *Mucor* species.

Although *Mucor* species have been associated with a variety of invasive diseases, primary cutaneous zygomycosis has rarely been reported (16). Three cases of this disease caused by *M. circinelloides* are described in the literature (2, 7, 26). In these cases, patients were females ranging in age from 23 to 60 years, and all were known or suspected to have been exposed following trauma of either the left forearm (2), the chest (7), or the right hand (26). Hematological malignancy was the underlying disease in two cases (acute myelocytic leukemia and MDS, respectively), but no underlying disease was recognized in the third case. Although our patient was older (90 years old), the presence of a hematological malignancy (MDS) and trauma to the extremities were common findings. Leukemia and uncontrollable diabetes mellitus have been reported as the first and second most common underlying conditions, respectively, for patients who developed zygomycosis (13). Both risk factors were present in our patient, since MDS is considered to be a preleukemic condition in patients. Although hyperglycemia is were present in our patient, since MDS is considered to be a preleukemic condition in patients. Although hyperglycemia is frequently observed in patients who develop rhinocerebral or disseminated zygomycosis, primary cutaneous disease caused by a zygomycete has rarely been reported for patients with this underlying condition (15, 16). Chan-Tack et al. did describe a case of central venous catheter-associated fungemia caused by *M. circinelloides* in a 48-year-old male with hyperglycemia induced by total parenteral nutrition (3).

The mortality rate for rhinocerebral zygomycosis in immunocompromised patients has been shown to be >80%, even when treated; however, primary cutaneous disease has responded more favorably to therapy (2, 7, 17). Surgery alone was successful in one case (2), and AMB therapy alone was successful in another (7). Therapy with liposomal AMB together with surgical debridement was effective in treating our patient. *Mucor circinelloides*, as well as other zygomycetes, have been reported to be susceptible to AMB in vitro (22, 24). Posaconazole also may be considered for therapy of zygomycosis based on susceptibility and compassionate-treatment data (11, 24).

Phenotypic methods have traditionally been used to identify clinically important *Mucor* species (2, 7, 26). However, the fact that most published reports refer only to the genus *Mucor* underlines the difficulties in species identification (16). Accurate identification of heterothallic zygomycetes is enhanced by observation of zygospores in mating studies (9, 28). Weitzman et al. successfully employed this method to identify an isolate of *M. circinelloides* that lacked the characteristic circinate branching (28). However, the need to maintain a library of tester strains makes this method difficult for many laboratories. Additionally, mating tests do not always yield a positive result (18, 21). A study of fertility among *M. circinelloides* and its formae by Schipper (18) revealed that isolates from different formae could mate with each other, thus confirming the broad-species concept for *M. circinelloides*, but that some isolates within a forma failed to mate. The plus tester strain that we employed, UAMH 8306, was used in both of these studies, and the minus tester strain, UAMH 8307, was used only by Weitzman et al. (28). Zygospores in pairings between the tester strains in the present study were observed, but they were not observed in any pairing that included the case isolate.

To help understand why the mating was not successful, the tester strains were sequenced (Table 1). When the case isolate sequence (UAMH 10385) was compared with those from tester strains, the percentage of identity was 99.0% with UAMH 8306 but only 97.5% with UAMH 8307. The decreased similarity may indicate why mating was not successful. Percentages of similarity between sequences of strains that were successfully mated by Schipper were 98.7% for UAMH 8306 (CBS 192.68) paired with CBS 119.087 and 99.5% for UAMH 8306 paired with CBS 108.16 (Table 1). The tester strain sequences were also 99% identical to each other (data not shown).

Molecular techniques using genomic targets within the rRNA complex have been shown to be reliable for the species identification of the zygomycetes, including *Mucor* species (9, 10, 14, 19). A BLAST search of the GenBank database showed the case isolate to be closely related to *M. circinelloides*, followed by *M. racemosus* and *Amylomyces rouxii*. For the other three species of *Mucor* suspected to cause human disease (*M. indicus*, *M. ramosissimus*, and *M. hiemalis*), a sequence similarity of only <95% was observed. The sequence similarities of the case isolate with all strains of *M. circinelloides* now available in the GenBank database ranged from 97.5% (with UAMH 8307) to 99.7% (with IP 1873.89 or CBS 119.087) (Table 1). These data are in agreement with those of Schwarz et al., who reported higher ITS variability within *M. circinelloides* compared with other zygomycete species examined (19).

The case isolate had >99% sequence identity with 7 of 11 *M. circinelloides* sequences available in the GenBank database. This group includes type strains of species that Schipper placed into synonymy with *M. circinelloides* based on mating results and morphology (18). The case isolate and both tester strains were least similar to the neotype strain (CBS 195.68NT). Schipper selected this strain as the neotype and indicated it as a plus mating type but did not select this strain as a tester strain for matings involving other strains.

The close relationship observed between *M. circinelloides* and *M. racemosus* has been demonstrated by others using the 18S and 28S rRNA gene targets (25). Our data revealed a >99% sequence identity between the case isolate and two sequences of *M. racemosus* from the GenBank database (Table 1); however, the possibility that these strains are misidentified is supported by low sequence similarity (<96%) between *M. circinelloides* (both tester strains and the case isolate) and the type strain of *M. racemosus* (CBS 280.685), represented by two sequences (14, 19). The species also differ in thermotolerance: the case isolate of *M. circinelloides* grew well at 37°C, but *M. racemosus* is not known to grow at elevated temperatures (5, 18).

The three GenBank sequences of *Amylomyces rouxii*, which were all derived from the same strain (CBS 416.77), also
showed ≥99% sequence similarity to the case isolate and >98% similarity to the two tester strains (Table 1). Our data confirm those of Schwarz et al., who reported a >99% ITS sequence similarity between these two species (19). *Amylomyces rouxi*, also known as *Mucor rouxii*, has not been described as a cause of human disease, although the taxonomic positioning of this species may be questioned in light of this similarity in sequence. Additionally, the chlamydiopores associated with this species, which have been described by Ellis et al., are similar to the structures observed by us in the case isolate of *M. circinelloides* (6).

As noted by Schwarz et al. and confirmed here, *M. circinelloides* demonstrates variability within the ITS regions, potentially supporting subspecies groups (19). Schipper described four formae (*Mucor circinelloides* f. *circinelloides*, *Mucor circinelloides* f. *janssenii*, *Mucor circinelloides* f. *griseocyanus*, and *Mucor circinelloides* f. *lusitanicus*) based on differences in the shapes of spores and columellae and in thermotolerance; however, these subgroups have not been evaluated by molecular methods (18). In the GenBank database, complete ITS sequences are available only for *M. circinelloides* f. *circinelloides*, while a partial sequence (ITS1) is available for *M. circinelloides* f. *janssenii*. Five of the 10 sequences representing different strains of *M. circinelloides* to which the case isolate aligned closely were identified as *M. circinelloides* f. *circinelloides*, but more sequence data are needed to evaluate subgroups within this species (Table 1).

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<th>Reference</th>
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<sup>a</sup>CBS, Concentrable voor Schimmelcultures, Utrecht, The Netherlands; CNRMA, National Reference Center for Mycoses and Antifungals, Institut Pasteur, Paris, France; IP, Pasteur Institute Collection of Fungi, Institut Pasteur, Paris, France; UWFP, University of Washington Fungal Project, University of Washington Medical Center, Seattle; NP, not published; N/A, not applicable; T, type strain; NT, neotype strain.

<sup>b</sup>At least 550 bases were evaluated, including the 5' end of the 18S rRNA gene; the complete ITS1 region, 5.8S rRNA gene, and ITS2 region; and the 3' end of the 28S rRNA gene.

<sup>c</sup>Also referred to as CBS 192.68 and NRRL 3614 (Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, IL).

<sup>d</sup>Sequences obtained in this study; accession numbers for the tester strains are DQ837219 (UAMH 8306) and DQ837220 (UAMH 8307).

<sup>e</sup>Mating type designation according to Schipper (18) and Weitzman et al. (28).

<sup>f</sup>Identified as *Mucor circinelloides* f. *circinelloides* by Schipper (18) or in the GenBank database.

<sup>g</sup>A species considered to be a synonym of *Mucor circinelloides* f. *circinelloides* by Schipper (18).

The present report describes a case of primary cutaneous zygomycosis caused by *M. circinelloides* in a patient with MDS and hyperglycemia. Although our data highlight the utility of ITS sequence comparison analysis for the identification of *M. circinelloides*, they also reveal that further studies are needed to evaluate the genetic heterogeneity among isolates identified as this species. It is not yet clear whether this heterogeneity and failure to mate are suggestive of cryptic species within *M. circinelloides*.

**Nucleotide sequence and culture accession numbers.** Sequences from the case isolate (UAMH 10385) and the tester strains UAMH 8306 and UAMH 8307 have been deposited in GenBank under accession numbers DQ787159, DQ837219, and DQ837220, respectively. A subculture of the case isolate has been deposited in the American Type Culture Collection under accession number ATCC MYA-4072.
We thank the personnel at the Mycology Section of the Clinical Microbiology Laboratory at the Nebraska Medical Center for support in the laboratory evaluation of isolates from patients with unusual fungal pathogens.

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