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Pulmonary Infection Caused by *Gymnascella hyalinospora* in a Patient with Acute Myelogenous Leukemia

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We report the first case of invasive pulmonary infection caused by the thermotolerant ascomycetous fungus *Gymnascella hyalinospora* in a 43-year-old female from the rural midwestern United States. The patient was diagnosed with acute myelogenous leukemia and treated with induction chemotherapy. She was discharged in stable condition with an absolute neutrophil count of 100 cells per μl. Four days after discharge, she presented to the Cancer Clinic with fever and pancytopenia. A solitary pulmonary nodule was found in the right middle lobe which was resected by video-assisted thoracoscopy (VATHS). Histopathological examination revealed septate branching hyphae, suggesting a diagnosis of invasive aspergillosis; however, occasional yeast-like cells were also present. The culture grew a mold that appeared dull white with a slight brownish tint that failed to sporulate on standard media. The mold was found to be positive by the AccuProbe *Blastomyces dermatitidis* Culture ID Test (Gen-Probe Inc., San Diego, Calif.), but this result appeared to be incompatible with the morphology of the structures in tissue. The patient was removed from consideration for stem cell transplant and was treated for 6 weeks with amphotericin B (AmB), followed by itraconazole (Itr). A VATHS with biopsy performed 6 months later showed no evidence of mold infection. In vitro, the isolate appeared to be susceptible to AmB and resistant to fluconazole and 5-fluorocytosine. Results for Itr could not be obtained for the case isolate due to its failure to grow in polyethylene glycol used to solubilize the drug; however, MICs for a second isolate appeared to be elevated. The case isolate was subsequently identified as *G. hyalinospora* based on its formation of oblate, smooth-walled ascospores within yellow or yellow-green tufts of aerial hyphae on sporulation media. Repeat testing with the *Blastomyces* probe demonstrated false-positive results with the case isolate and a reference isolate of *G. hyalinospora*. This case demonstrates that both histopathologic and cultural features should be considered for the proper interpretation of this molecular test and extends the list of fungi recognized as a cause of human mycosis in immunocompromised patients.

Invasive mold infections have emerged as major causes of morbidity and mortality in immunocompromised patients, including patients with hematological malignancies undergoing treatment (7, 23, 37). *Aspergillus* species are the most common cause of invasive mold infection, but other opportunistic molds such as *Fusarium* species, *Pseudallescheria boydii*, and *Rhizopus* species have frequently been reported as causes of invasive disease (1, 3, 4, 13, 16, 20). The number of mold species causing invasive infection continues to expand, with the addition of fungi once thought incapable of causing human disease (12, 14, 18, 19, 26). This report describes the first case of an invasive pulmonary mycosis caused by the thermotolerant ascomycete *Gymnascella hyalinospora* in a patient undergoing therapy for acute myelogenous leukemia.

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CASE REPORT

A 43-year-old female childcare employee presented with a 3-month history of sinusitis. A complete blood count showed pancytopenia plus circulating blasts and a diagnosis of acute myelogenous leukemia (FAB-M1) was made. Induction chemotherapy, consisting of idarubicin and cytarabine, was administered, and the patient was discharged in stable condition 8 days following chemotherapy with no evidence of cancer. At discharge, an absolute neutrophil count of <100 cells per μl was noted. Four days after discharge, she presented to the Cancer Clinic with fever and pancytopenia. A chest radiograph at that time showed a 2.5-cm right-middle-lobe opacity. A computerized tomography (CT) scan of the thorax demonstrated a 2.5-by-1.8-cm pleural-based peripheral nodule. A wedge resection of the right upper lobe, along with a biopsy of the parietal pleura, was accomplished with video-assisted thoracostomy (VATHS). Histopathology of the lung and pleural tissues revealed hemorrhagic infarcts and numerous septate hyphae with *Aspergillus*-like characteristics (Fig. 1). Also present were numerous solitary yeast-like cells, some of which appeared to be budding (Fig. 2).

Tissue from the right upper lobe was plated on Sabouraud dextrose agar with chloramphenicol (SAB-C; Remel, Lenexa, Kans.) and incubated at 30°C. Bacterial cultures were also performed on this tissue by using sheep blood agar, chocolate agar, MacConkey agar, and thioglycolate broth (all from Remel). On SAB-C, a rapidly growing mold with a white colony grew which subsequently became gray (Fig. 3A, left). The same white mold grew on all bacterial media except MacConkey

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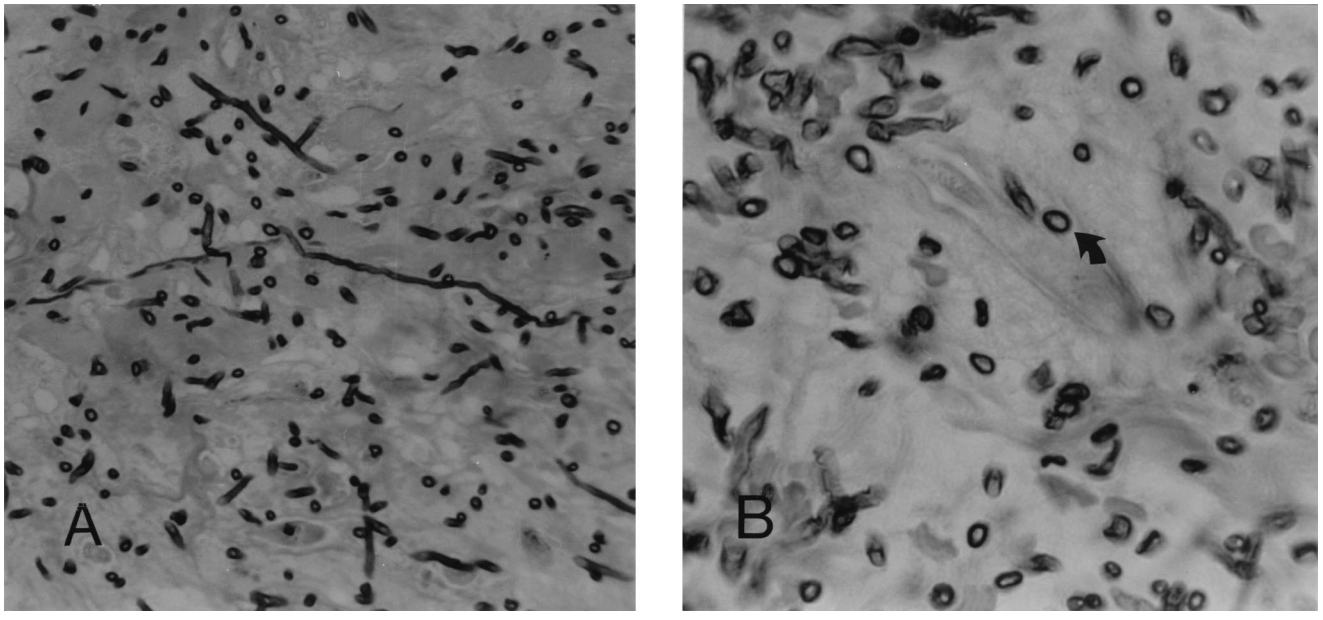

FIG. 1. Methenamine silver stain of lung tissue. (A) Hyphal elements and small, oval ascospores that resemble yeast cells can be seen. Magnification, ×580. (B) Higher-magnification view of solitary ascospores (arrow). Magnification, ×1,120.

agar. A 10-day-old slide culture prepared on plain SAB (Remel) incubated at 30°C revealed septate hyphae but no reproductive structures. The mold was positive by the AccuProbe *Blastomyces dermatitidis* Culture ID Test. In consideration of possible *Aspergillus* or *Blastomyces* infection, the patient was removed as a candidate for stem cell transplantation. A serum sample, submitted to a reference laboratory for serological studies, was subsequently reported as negative for antibodies to *Aspergillus* and *Blastomyces* as determined by immunodiffusion testing.

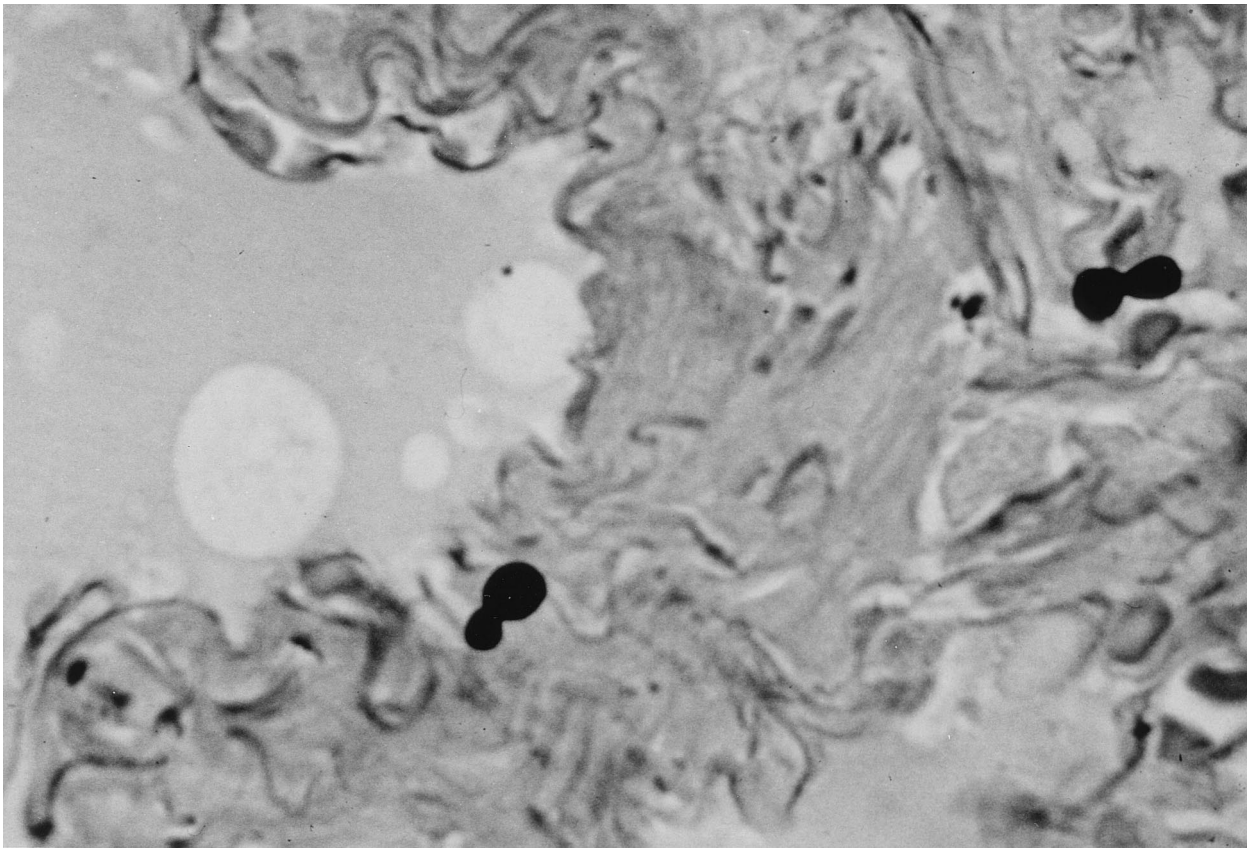

FIG. 2. Methenamine silver stain of lung tissue showing what appears to be germinating ascospores that resemble budding yeast cells. Magnification, ×400.

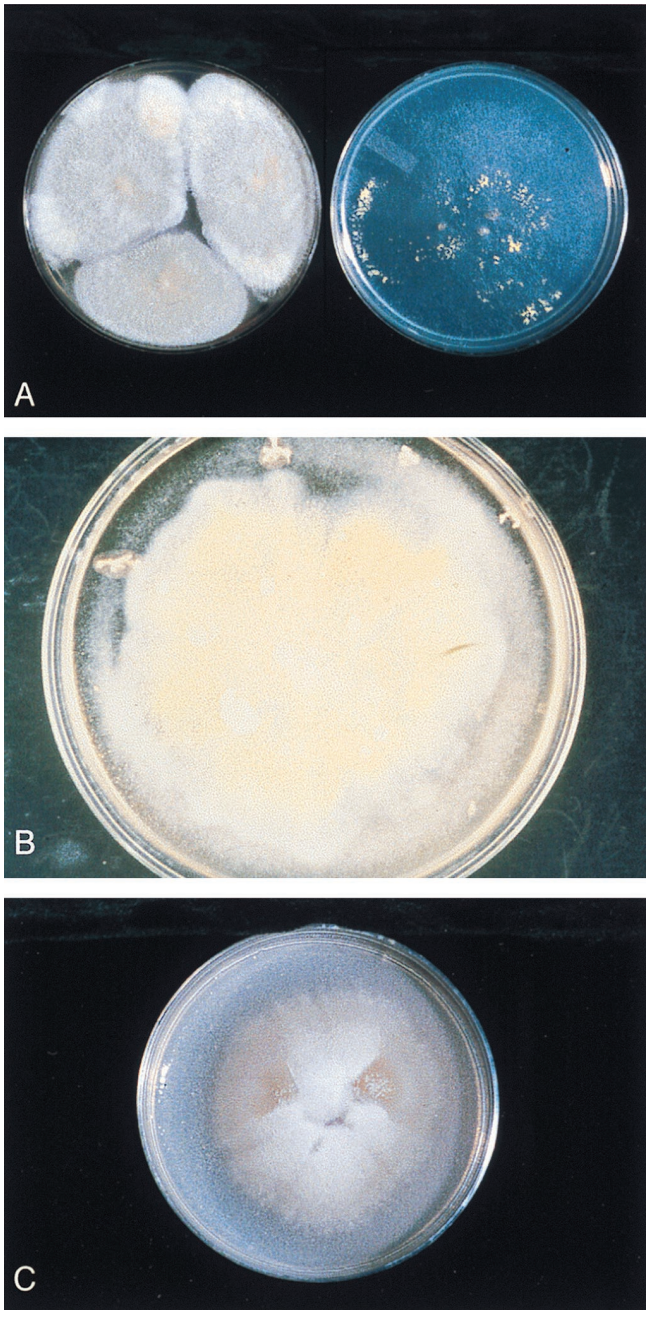

FIG. 3. *G. hyalinospora* case isolate (UAMH 9359) on SAB-C (left) and Czapek agar (right) at 21 days at 30°C (A), on PDA showing the confluent yellow-white mycelium after 4 weeks at 30°C (B), and on PFA showing sectors of different colonial color and texture at 21 days at 30°C (C).

Based on a diagnosis of fungal pneumonia, intravenous amphotericin B (AmB) was started at a dose of 1 mg/kg of body weight/day. At 8 days after the operation, the patient was discharged and continued to receive AmB for a 6-week period. Therapy with itraconazole (Itr) was administered orally initially at a dose of 200 mg daily and then increased to 400 mg for an additional 6 weeks because of low serum levels. At the completion of AmB treatment, a CT scan of the thorax was performed which demonstrated scar tissue in the right upper lobe of the lung. A repeat VATHS was performed 112 days following the completion of AmB therapy, with biopsy of the pleura and resection of the lung scar to verify that the mold infection had resolved prior to any consolidation chemotherapy. There was no evidence of fungal elements by histopathology of this tissue, and no growth was obtained by culture.

The patient subsequently relapsed at approximately 9 months after induction chemotherapy and was reinduced with idarubicin and cytarabine. A second remission was achieved and, although the patient developed prolonged neutropenic fever and was treated with AmB, there was no evidence of invasive fungal infection noted. The patient was subsequently placed on a protocol for stem cell transplantation.

MATERIALS AND METHODS

Mycology. The lung isolate was forwarded to the Fungus Testing Laboratory, Department of Pathology, University of Texas Health Science Center (UTHSC) at San Antonio, Tex., for characterization and susceptibility testing. It was entered into the UTHSC stock collection under accession number UTHSC 98-1356. Due to the absence of sporulation, the isolate was subsequently referred for additional testing to the University of Alberta Microfungus Collection and Herbarium (UAMH), where it was deposited as strain UAMH 9359. To induce sporulation, the isolate was subcultured onto a variety of media, including potato dextrose agar (PDA; Difco Laboratories, Detroit, Mich.), potato flakes agar (PFA), Czapek agar, cereal agar, and oatmeal salts agar (OAT), the latter prepared in-house (15). The case isolate was identified by its micromorphological features and confirmed by morphological and molecular comparison with a reference isolate from a human source (strain UAMH 7366, repeatedly isolated from the respiratory tract of an immunosuppressed patient, deposited by I. Weitzman, New York, N.Y.). Colonial features were examined on PDA and OAT with growth rates evaluated at 25, 30, and 37°C. The microscopic morphology was examined from colonies on OAT or Czapek agar or from slide culture mounts by using cereal agar. A subculture of the case isolate was deposited in the American Type Culture Collection under accession number ATCC 204275.

Antifungal susceptibility testing. Susceptibility testing was performed on both the case isolate and UAMH 7366, utilizing the National Committee for Clinical Laboratory Standards macrobroth dilution method M27-A, modified for mold testing (21). Briefly, the isolates and the *Paecilomyces* control strain (UTHSC 90-459) were grown on PFA for 7 days at 25°C, and the inocula were standardized spectrophotometrically. The PFA slants were overlaid with sterile distilled water, and suspensions were made by gently scraping the colonies with the tip of a Pasteur pipette. Heavy hyphal fragments were allowed to settle, and the upper, homogenous suspensions were removed. Suspensions were adjusted to a 95% transmission at 530 nm and then diluted 1:10 in medium to provide a 1.0×10^4 final inoculum concentration as determined by plate counts. Final drug concentration ranges were as follows: for AmB (E.R. Squibb & Sons, Princeton, N.J.), 0.03 to 16 μg per ml; for 5-fluorocytosine (5-Fc; Roche Laboratories, Nutley, N.J.), 0.125 to 64 μg per ml; for fluconazole (Flu; Pfizer, Inc., New York, N.Y.), 0.125 to 64 μg per ml; for Itr (Janssen Pharmaceutica, Titusville, N.J.), 0.015 to 8 μg per ml; and for ketoconazole (Ket; Janssen Pharmaceutica, Titusville, N.J.), 0.03 to 16 μg per ml. AmB was tested in Antibiotic Medium 3 (Difco, Detroit, Mich.); other antifungal agents were tested in RPMI 1640 with L-glutamine and morpholinepropanesulfonic acid (MOPS) buffer at a concentration of 165 mM and without sodium bicarbonate (American Biorganics, Inc., Niagara Falls, N.Y.). Previously prepared, frozen drug tubes containing 0.1 ml of drug were allowed to thaw and were inoculated with 0.9 ml of the hyphal medium suspension. The tubes were incubated at 35°C, and MICs were read at the first 24-hr interval when growth was observed in the drug-free growth control. MICs were defined as the drug concentration of the first tube that yielded a score of 0 (optically clear) for AmB and a score of 2 (reduction in turbidity of ≥80% in contrast to the drug-free control tube) for 5-Fc, Flu, Itr, and Ket. Minimum lethal concentrations (MLCs) for AmB were determined by plating 100-μl samples onto SBA plates from tubes containing the following: drug-free control, AmB at the MIC, and AmB at concentrations above the MIC. All plates were incubated at 35°C. The MLC was defined as the lowest concentration of antifungal compound resulting in five or fewer colonies on the SBA plate, which represented 99.9% killing (27).

Molecular testing. The commercially available DNA probe for identification of *Blastomyces dermatitidis* (AccuProbe; Gen-Probe, Inc., San Diego, Calif.) was used per the manufacturer's instructions. A signal greater than or equal to 50,000 relative light units (RLU) was considered positive. The test was performed three times on the case isolate and once on the reference strain, UAMH 7366. Each isolate was also tested once with the probe for *Histoplasma capsulatum*. Comparison of the ribosomal DNA (rDNA) internal transcribed spacer regions (ITS1 and ITS2) was used to confirm identity between the case isolate and the reference strain (P. C. Iwen, T. J. Henry, S. R. Tarantolo, and S. H. Hinrichs, Abstr. Focus Fungal Infect. No. 9, abstr. 26, 1999).

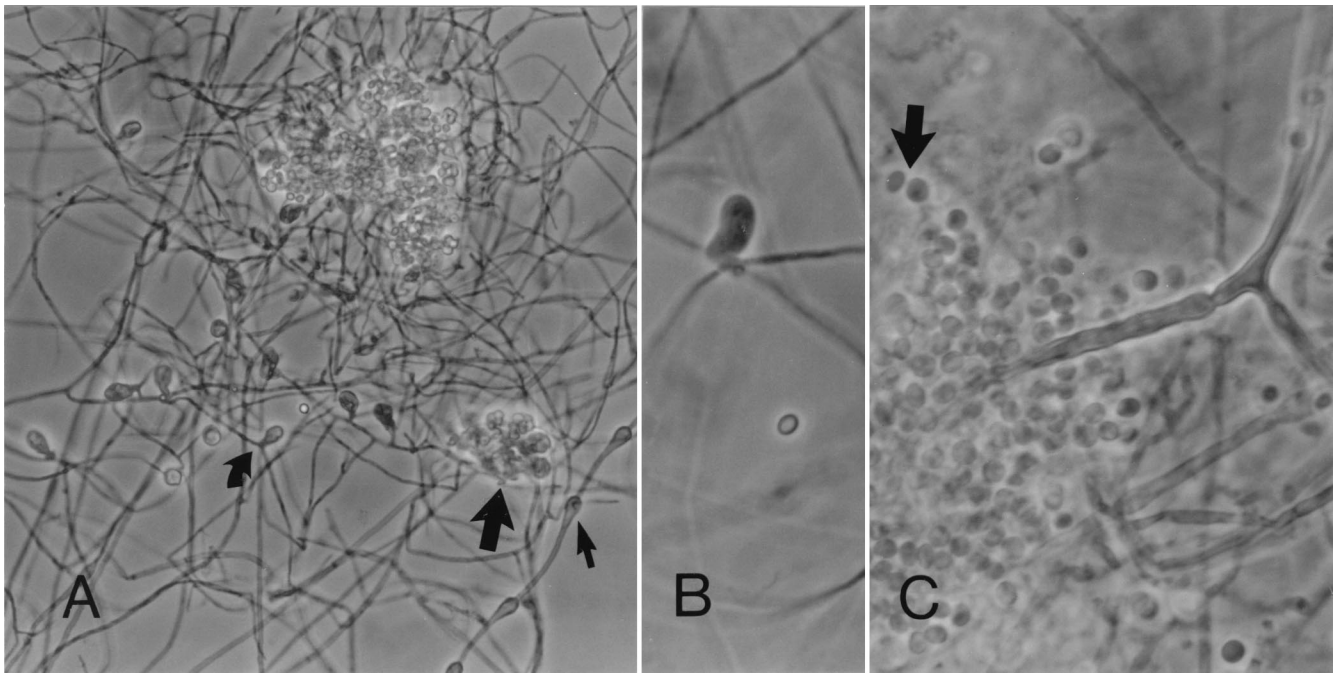

FIG. 4. Development of asci and ascospores in *G. hyalinospora*. (A) Solitary asci (curved arrow) and asci in clusters (large arrow). Racquet hyphae also may be seen (small arrow). Magnification, ×460. (B) Solitary ascus and single ascospore in side view. Magnification, ×1,120. (C) Mature oblate ascospores associated with thick-walled hyphae. The arrow points to two ascospores in different orientations, showing that they appear round in the face view and slightly flattened in the side view. Magnification, ×1,120.

RESULTS

The case isolate (UAMH 9359) was identified as *G. hyalinspora* (Kuehn, G.F. Orr & G.R. Ghosh) Currah by its colonial and macroscopic characteristics, as well as micromorphological features. Colonies on PDA at 30°C were initially flat, with a glabrous (smooth) yellow-gray surface mycelium overlaid with sparse tufts of cottony-white aerial hyphae and an orange-brown reverse. A tan pigment diffused into the agar. After 4 weeks, the colony developed raised and more confluent yellow-white woolly mycelium, and this mycelium darkened to pale yellow or olive green after 10 weeks (Fig. 3B). On oatmeal agar at 30°C after 14 days, the colony was 100 mm in diameter, flat, thinly cottony, and yellow-white in the center; after 10 weeks, the center became golden yellow with a few tufts of green mycelium, while the margin was thin and white. The case isolate and reference isolate (UAMH 7366) demonstrated similar growth rates at 30 and 37°C, attaining colony diameters of 65 to 75 mm on PDA after 14 days. Both isolates grew slower at 25°C (colony diameters of 35 mm for the case isolate and of 55 mm for UAMH 7366 in 14 days on PDA). Colonies of the reference strain were more velvety and bright colored, becoming golden yellow to deep green on PDA and gray to deep green on oatmeal agar after 10 weeks. The case isolate differed from UAMH 7366 in its tendency to develop sectors of different color or texture on all media (Fig. 3C), but sectoring is common among isolates of *G. hyalinospora* (5). Changes in colony color from white to yellow or green occurred with the commencement of ascosporulation and the maturation of ascospores (sexual spores or meiospores) that formed within loosely aggregated naked clusters. In age, the ascospore masses were associated with loosely arranged, thicker-walled yellow hyphae, which were especially prominent on Czapek agar (Fig. 3A, right). The vegetative hyphae were narrow, commonly measuring 2 to 3 μm wide and sometimes showing swellings up to 4 or 5 μm wide at one end of a cell (racket hyphae). Asci measured 8 to 11 μm long by 5.5 to 8.5 μm wide. Ascospores were 2.8 to 3.2 μm long and 2.2 to 2.5 μm wide, oblate (spherical in the face view and flattened in the side view), and pale yellow en masse. They appeared smooth under light microscopy (Fig. 4), but by scanning electron microscopy they appeared to be covered with a warty membrane that sloughed off (Fig. 5). In addition to their morphological similarities, the case and reference isolates demonstrated >99% homology between the ITS region sequences.

The case isolate was tested with the DNA probe for *B. dermatitidis* because it appeared to be unusual at initial presentation, having sterile glabrous colonies with a slight brownish tint as is occasionally seen with atypical isolates of *B. dermatitidis*. Although the test was done to rule out *B. dermatitidis*, two tests performed 15 days apart gave positive results, with RLU readings of 85,686 and 111,548. Additionally, the probe test was positive for the UAMH reference isolate of *G. hyalinospora* (246,835 RLU). However, a third test done on the case isolate approximately 5 months later recorded a negative result, with an RLU reading of 40,065. Neither isolate demonstrated a positive reaction when tested with the AccuProbe assay for *H. capsulatum*.

The drug susceptibility data for the case isolate and UAMH 7366 indicated that both isolates have a similar pattern of susceptibility (Table 1). Based upon serum drug concentrations achievable by standard dosing regimens, the isolates appeared to be susceptible to AmB, both by MICs and MLCs. In vitro data for Ket and Itr differed for the two isolates. The case isolate demonstrated an MIC of 2 μg/ml for Ket but failed to grow in the polyethylene glycol used to stabilize Itr for drug dilutions, and thus results were not obtained. The 48-h MICs of 4 μg/ml for Ket and Itr from the UAMH 7366 isolate appeared elevated. Both isolates showed resistance to 5-Fc and Flu.

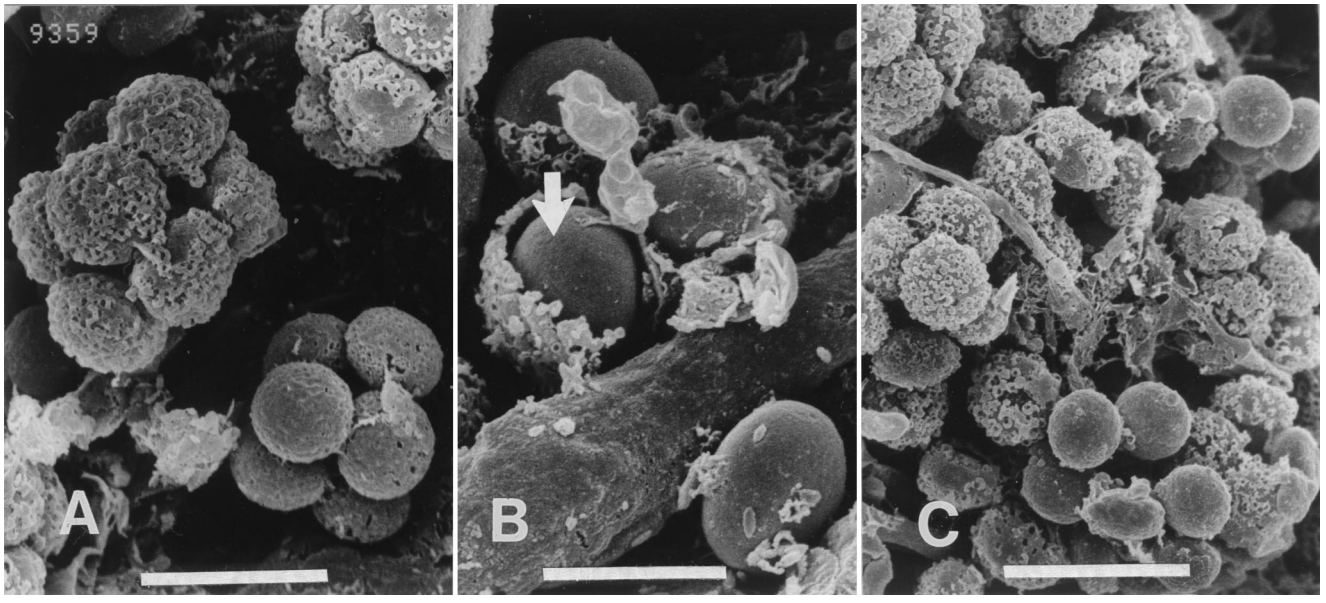

FIG. 5. Ascospores examined by scanning electron microscopy. (A) Ascospores released from the ascus in the case isolate. Ascospores in one group appear to be covered with a warty membrane covering (perispore). Bar, 5 μm. (B) Ascospore shedding the outer membrane in the case isolate. Bar, 2.5 μm. (C) Ascospores of the reference isolate showing features identical to those of the case isolate. Bar, 6 μm.

DISCUSSION

G. hyalinospora is a member of the ascomycetes order *Onygenales*, family *Gymnoascaceae*. Most human pathogenic members of the order are placed in the classifications *Arthrodermataceae* (dermatophytes) and *Onygenaceae* (systemic dimorphic pathogens and some other species) (2, 15, 30). Currah (5) redescribed the genus *Gymnascella* and included a number of species that had formerly been included in the genera *Arachniotus*, *Rollandina*, and *Gymnoascus*. The genus is distinguished by asci and ascospores that are naked or surrounded by scarcely differentiated hyphae and ascospores that are smooth to irregularly lumpy and oblate (5, 15). *Gymnascella dankaliensis* has been reported as a rare agent of onychomycosis (15, 35). Other members of the genus have not been confirmed as pathogens of humans or other animals, even though they are occasionally isolated from associated materials (dung, litter, and soil associated with animals) or from the animals themselves (lung, skin scrapings, hair, etc.) (5, 32). Based on prior records of its isolation and its ability to grow well at 37°C, *G. hyalinospora* has been considered a possible cause of infection. Strain UAMH 7366 was isolated from tracheal aspirate, bronchial washings, and sputum from an immunosuppressed patient; however, histopathological documentation of invasive disease could not be obtained (32). The present study demonstrates the pathogenic potential of this ascomycete species in a patient with acute myelogenous leukemia undergoing chemotherapy.

TABLE 1. In vitro susceptibility data for *G. hyalinospora*[a]

Isolate	MIC (μg/ml)					MLC (μg/ml), AmB
	AmB	5-Fc	Flu	Itr	Ket	
Case isolate	0.5	>64	64		2	0.5
UAMH 7366	0.25	>64	>64	4[b]	4[b]	0.25

[a] All results are 48-h readings.
[b] The 24-h readings for Itr and Ket were 1 and 2 μg/ml, respectively.

The absence of sporulation on standard media may present difficulties in identifying an isolate of *G. hyalinospora* in the clinical setting. However, ascosporulation is induced on sporulation media such as OAT within 2 to 4 weeks at 30 or 37°C, and the smooth, oblate ascospores develop within yellow to yellow-green sectors or patches. Although the case isolate was identified by micromorphological features, analysis of sequences from the rDNA internal transcribed spacer regions ITS1 and ITS2 provided another method to confirm the identity with the reference isolate and to further investigate the potential of this technique to classify difficult-to-identify clinical isolates (Iwen et al., Abstr. Focus Fungal Infect.).

Our patient's infection was not evident upon discharge from the hospital following induction chemotherapy, but it appeared radiologically in the lung 4 days after discharge. It is not known where the patient acquired the infection, but it is suspected that she may have inhaled ascospores prior to admission and that her induced immunosuppression allowed in situ germination to the hyphal form (Fig. 2). The histopathologic findings are consistent with this hypothesis. Although the individual cells observed in the tissue section were initially thought to be yeast-like, retrospective reexamination revealed that the cells were similar in size (3 μm long by 2 to 2.5 μm wide) and shape to ascospores formed in culture, although they were not quite as regular in shape (Fig. 4). *G. hyalinospora* has been isolated from agricultural soils and once from a lesion on the comb of a rooster in India (UAMH 6510). Our patient came from a rural background and had resided in the country, where she raised chickens, geese, and guinea hens. Attempts to isolate the organism from the soil where the fowl had resided failed because of other saprophytic fungal overgrowth.

AmB remains the treatment of choice for prophylaxis and treatment of invasive mold infections, even though Itr has been shown to be effective in the treatment of some cases (6, 8, 9, 10, 29, 36). It had been shown previously that leukemia and solid organ transplant patients who develop a solitary pulmonary nodule benefit from aggressive surgical resection of the nodule with improved survival (25, 28). In our patient's case, the lesion

was surgically removed prior to the administration of an antifungal, so the efficacy of the antifungals is difficult to evaluate. Susceptibility data (Table 1) suggested that the organism was susceptible to AmB. Our patient was treated also with Itr, but susceptibility of the isolate to this drug could not be determined due to its failure to grow in the drug dilution tubes; the MIC for the reference strain appeared to be elevated. The role of antifungal therapy in the overall clinical response of this patient is unclear since surgery was a critical part of this patient's management. A second VATHS with biopsy was performed 112 days following the completion of AmB therapy after the Itr had been started. The tissue culture from this sample was negative for fungus; however, this may have been compromised because of antifungal therapy, although no histological evidence of fungus was observed.

Two unusual findings made diagnosis in this case difficult. The first was the histopathological appearance of the fungus. The hyphae resembled those of *Aspergillus* species, leading to an initial diagnosis of suspected invasive aspergillosis, but the presence of small oval yeast-like cells were inconsistent with this diagnosis. Since *Aspergillus* species are vasculopathic and their presence may imply a grave disease process, there was an urgent need for an accurate diagnosis (6), but diagnosis was impeded by the difficulty in interpreting the histopathology and by the initial failure of the isolate to sporulate. Because some aspects of the cultural features suggested that it might be an atypical isolate, the DNA probe for *B. dermatitidis* was performed to rule out this species. The second unusual finding was the positive probe result for *B. dermatitidis*. Once the isolate produced ascospores and was identified as *G. hyalinospora* it was retested, and positive results were obtained with both the case and the reference isolates. Care was taken to ensure that the product had not exceeded its expiration date and that the manufacturer's instructions were followed. Although tests were twice positive for the case isolate, a third test done approximately 5 months after the original two tests recorded a negative result, with an RLU reading of 40,065. It is unknown why the *B. dermatitidis* probe hybridized the rDNA released from this species. Although false-positive results have occurred with *Paracoccidioides brasiliensis* (21), these taxa are close relatives (11, 24). In contrast, current taxonomic concepts place *G. hyalinospora* and *B. dermatitidis* in different families (5). In their phylogeny of the *Onygenales*, Leclerc et al. found that two members of the *Gymnoascaceae* clustered with some members of the *Onygenaceae* and apart from a group including the dimorphic pathogens (17).

While the gene probes have been reported to be highly specific (22, 34), the probe was not reliable in confirming the identity of the isolate in this case. The presence of hyphae in tissue, together with the absence of structures that are characteristic of *B. dermatitidis*, suggested to us that the probe results were misleading. *B. dermatitidis* produces a yeast phase characterized by large (8 to 10 μm in diameter), thick-walled, broad-based budding cells and in culture forms solitary conidia borne on stalks (15, 31). Sterile isolates are rarely encountered that would be difficult to distinguish from *G. hyalinospora*, but ascosporulation can be induced in the latter by the use of appropriate media. Our results show that it is wise to follow the manufacturer's direction, which states that "results from the [*Blastomyces* Culture Identification Test] should be interpreted in conjunction with the laboratory and clinical data." This case further illustrates that septate branching hyphae identified in tissue should not be immediately interpreted as *Aspergillus* species and that a combination of tests is needed to make an accurate diagnosis.

This study adds the ascomycete *G. hyalinospora* to the list of opportunistic human pathogens. As immunosuppressive therapies become more widely used, environmental fungi with low pathogenicity will continue to emerge as causes of invasive disease, and it is important that the pathogen be recognized in a timely manner for effective management of the patient.

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