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Identification and First Report of *Inonotus* (*Phellinus*) *tropicalis* as an Etiologic Agent in a Patient with Chronic Granulomatous Disease

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Although isolates of filamentous basidiomycetes can usually be recognized in a clinical laboratory setting, identification is problematic, as they seldom exhibit diagnostic morphological features formed in nature. This paper is the first report of *Inonotus* (*Phellinus*) *tropicalis* inciting human disease and describes the methods used to support the identification.

CASE REPORT

A 21-year–old Hispanic man with X-linked chronic granulomatous disease (CGD) and a long history of bacterial and fungal infections presented after 2 days of painless swelling over the lower back with no history of trauma, travel outside of his lifelong home in Houston, Tex., unusual environmental exposures, or systemic problems. Previous infections had included *Aspergillus* osteomyelitis of the ribs and vertebral bodies (28). lymphadenitis with *Staphylococcus aureus,* and cutaneous and systemic infections with *Serratia marcescens.* Routine X rays were negative, but computed tomography and magnetic resonance imaging studies showed paraspinal and presacral inflammation and extensive sacral bony changes. He had been on chronic daily intravenous antibiotics and maintenance interferon, co-trimoxazole, and itraconazole. Between July 2001 and January 2004, a rapidly growing golden-brown filamentous mold was isolated on seven occasions. Isolation media were incubated between 25 and 30°C and consisted of Sabouraud dextrose agar. Mycosel agar, and brain heart infusion agar supplemented with 5% sheep cells. chloramphenicol. and gentamicin (Becton Dickinson, Cockeysville, Md.). Six of these isolates were forwarded to the Fungus Testing Laboratory, University of Texas Health Science Center (UTHSC) at San Antonio, for identification. Between July 2001 and March 2002, isolates were obtained from a back abscess (UTHSC 01-1419), a back lesion (UTHSC 01-1652), a bone marrow aspirate (UTHSC 02-617), and paraspinal abscess tissue (isolate not accessioned into the UTHSC stock collection). Bath the bone marrow aspirate and the paraspinal abscess tissue were positive for hyphal elements histopathologically. From February 2003 to January 2004, the organism was recovered from a paraspinal abscess (UTHSC 03-453), another back wound (UTHSC 113-1972), and the paraspinal area (UTHSC 04-212). The organisms recovered were thought to represent basidiomycetes on the basis of their cultural features (rapid growth and a golden-brown color) and their growth an 10% benomyl agar (58), which paralleled that observed on potato flakes agar (PFA) (42). Somewhat unusual in our experience, however, was the ability of the isolates to grow on media containing cycloheximide. Isolate numbers UTHSC 01-1419, UTHSC 01-1652, and UTHSC 03-2972 have been deposited in the University of Alberta Microfungus collection and Herbarium, Edmonton, Alberta, Canada, as UAMH 10436, UAMH 10437, and UAMH 10376, respectively. Complete clinical details and therapeutic regimens for this case will be presented elsewhere.

The six isolates submitted to the Fungus Testing Laboratory were initially examined on PFA and benomyl agar prepared in-house and on commercially available cycloheximide agar (Remel, Lenexa, Kans.). When grown on PFA, isolates were woolly and yellowish orange and exhibited rapid growth, filling a 60-mm-diameter plate in 10 days at 25°C (Fig. 1A). Growth also occurred on media containing 10% benomyl and 0.04% cycloheximide at 25 and 35°C but not at 40°C. Hyphae lacked clamp connections and were either thin walled and hyaline or thick walled and yellow, and 3-week-old cultures produced golden, thick-walled, pointed setal hyphae up to 100 μm in length (Fig. 1B). When morphological and internal transcribed spacer (ITS) region sequence data (described below) indicated that the fungus was a wood-decaying basidiomycete, three isolates, i.e.. UTHSC 01-1419, UTHSC 01-1652, and UTHSC 02-617, were sent to the Forest Products Laboratory, Madison, Wis., for further study. The Center for Forest Mycology Research at the Forest Products Laboratory specializes in the study of wood-decaying basidiomycetes. Strains were grown separately on 2% Bacto malt extract agar (MEA; Difco Laboratories, Detroit, Mich.), 0.5% gallic acid agar (GAA), and 0.5% tannic acid agar (TAA) (Mallinckrodt, St. Louis, Mo.) (12) according to standard protocols for cultures of decay fungi (35, 37). GAA and TAA are used in the study of wood-decaying fungi to test for the presence of polyphenol oxidases that are associated with the decay of wood (12). Colony diameters on MEA at 25°C were 43 to 46 mm at 1 week and 83 to 85 mm at 12 weeks. Mats at 2 weeks were moderately thick, raised, and

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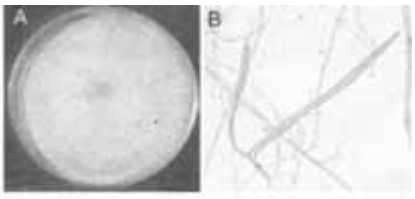

FIG. 1. (A) Woolly, yellow-orange colony of the case isolate on a 60-mm-diameter PFA plate after a 2-week incubation at 25°C; (B) thick-walled setal hyphae produced by the case isolate after 3 3-week incubation at 25°C on potato flakes agar.

densely woolly, especially around the inoculum. Mats were orange-yellow or yellowish orange with yellowish brown streaks and became thinner and arachnoid to downy toward the margin. The margin was even to slightly uneven and appressed. and the agar beneath the mat was stained a dark brown or reddish brown. No odor was noticed at 2 weeks. Advancing zone hyphae of the margin were 2 to 3.5 μm in diameter, simply septate (as opposed to having a nodose septum or developing clamp connections), and rarely branched. with thin. smooth, and hyaline walls. Aerial hyphae from the mar surface were 1.5 to 3 μm in diameter and simply septate or aseptate with thin to slightly thick and smooth walls that were hyaline at first before becoming yellowish brown. Hyphae growing in the agar were 2 to 3.5 μm in diameter. simple septate, and moderately to frequently branched, with thin, smooth, hyaline to dark-yellow walls. By 4 week, numerous irregular swellings were observed on the submerged hyphae (Fig. 2). No growth or reactions were observed on GAA at 1 or 2 weeks; however, a strong reaction (i.e., a dark-brown discoloration of 3 to 4 cm in diameter) was observed on TAA, with colony diameters after 1 and 2 weeks reaching 11 to 15 and 28 to 33 mm, respectively.

Extraction of DNA from the hone marrow isolate, UTHSC 02-617, was done using hyphae collected from a 7-day-old culture on Sabouraud dextrose agar (Remel) after incubation at 30◦C according to procedures explained by Henry et al. (19).

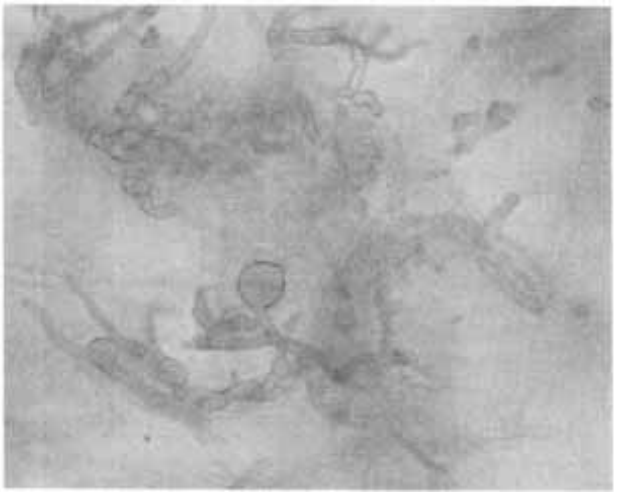

FIG 2. Hyphal swellings produced by UTHSC 01-1419 after 4 weeks of incubation at 25°C on malt extract agar.

DNA was purified using the QIAmp blood kit (QIAGEN Inc., Valencia, Calif.) and protocols for crude cell lysates supplied by the manufacturer. Two oligonucleotide fungal primers (ITS 1 and ITS 4) described by White et al. (63) were used for amplification. Primers were synthesized by the University of Nebraska Medical Center Eppley Molecular Biology Core Laboratory. The PCR assay was performed with 5 μl of the test sample in a 50-μl total reaction volume consisting of the following: PCR buffer (20 mM Tis-HCl [pH 8.4], 50 mM KCl); 0.1 mM (each) dATP, dGTP, dCTP, and dTTP; 1.5 mM $MgCl_2$; 0.3 μM concentrations of each primer; and 1.5 U of PlatinumTaq high-fidelity DNA polymerase (Gibco BRL Life Technologies, Gaithersburg, Md.). 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. DNA sequencing was performed with a Perkin-Elmer/ABI model 373 DNA sequencer by following protocols supplied by the manufacturer. The PCR products were directly sequenced using the ITS 1 and ITS 4 primers for the ITS region. The resultant nucleotide sequences were aligned with the MacVector sequence analysis software, version 6.5 (Oxford Molecular Group, Inc., Campbell, Calif.), alignment application. Amplification of the ITS 1-5.8S rRNA-ITS 2 region of the case isolate genome resulted in the production of a 680-bp PCR product. A BLAST search of the NCBI GenBank database resulted in the identification of the top eight sequences producing significant alignments as *Inonotus* (*Phellinus*) *linteus* strains. In the closest match, the identification showed an alignment homology of only 91% (307 of 334 bp) between the database strain and the case isolate.

DNA sequence data for 18S rRNA, 26S rRNA, ITS, and mitochondrial rRNA have frequently been used in recent phylogenetic studies of eukaryotic cells. Among the sequenced genetic material, the ITS, which has the fastest evolutionary speed, is appropriate for the molecular identification or delimitation of fungi and is now commonly used in the systematics of species within a genus (34). A phylogenetic analysis of ITS sequences compared to sequences in the H. S. Jung *Inonotus* (*Phellinus*) database, Seoul National University (now registered sequences in GenBank), demonstrated that the clade including the case isolate UTHSC 02-617 (AY641432) was composed of two subclades representing *Inonotus* (*Phellinus*) *tropicalis* and *I.* (P.) *linteus-Phellinus repandus.* The former subclade was fully supported by the 100% bootstrap value, suggesting that the isolate is statistically identical to *I. tropicalis* (Fig. 3). The overall sequence similarity between *I. tropicalis* CBS 617.89 and UTHSC 02-617 was 97.8%, which was slightly lower than expected for isolates of the same species.

Mitochondrial small-subunit rRNA was amplified with the primers BMSO5 and BMS173 described by Hong et al. (21). Subsequent sequence determination and phylogenetic evaluation were also done by following the method of Hong et al. (21). In the phylogenetic analysis of the mitochondrial small-subunit rRNA sequences, the two new sequences of UTHSC 01-1419 (AY598828) and UTHSC 01-1652 (AY598829) were identical, having 100% (582 of 582) similarity in sequence and forming a clade with *I. tropicalis* with a bootstrap value of 90%

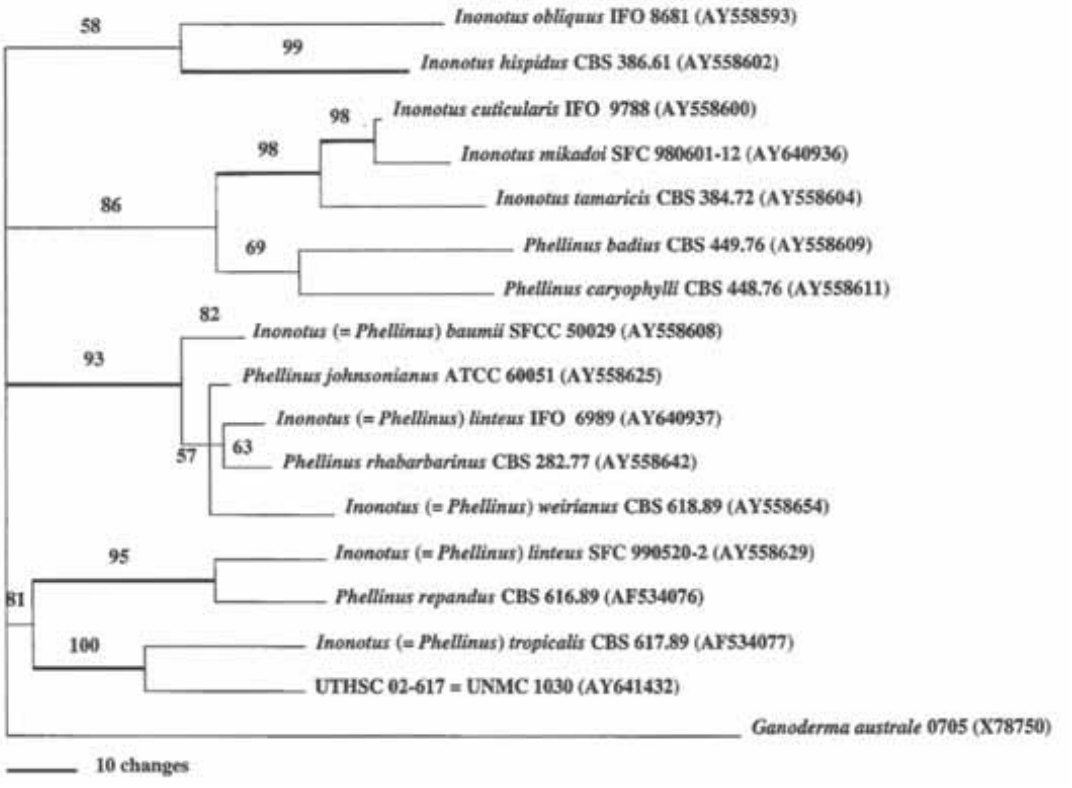

FIG. 3. Phylogenetic tree of *Inonotus* and related taxa based on 16 newly sequenced ITS regions. This is a 50% majority rule consensus tree (tree length, 573 steps; confidence interval, 0.665; retention index. 0.682) that was produced by use of the stepwise-addition option of the heuristic method of PAUP* 4.0b4a. Bootstrap values above 50%, based on 1,000 replicates, are indicated on or next to branches. *Ganoderma australe* X78750 was retrieved from GenBank as an out-group to root the tree.

(data not shown). When UTHSC 01-1419 and UTHSC 01-1652 were compared with *I. tropicalis* CBS 617.89 (AY558713), the sequence similarity was 99.2% (512 of 516), suggesting that the causal fungus was actually a strain of *I. tropicalis.* The strain of *I. tropicalis* used in the analysis, CBS 617.89 from the Centraalbureau voor Schimmelcultures, was sent to the Centraalbureau voor Schimmelcultures by F. F. Lombard of the Center for Forest Mycology Research, Forest Products laboratory, Madison. Wis., in 1989. It was collected from hardwood in Tacares, Heredia Province, Costa Rica, on 16 August 1963 by J. L. Lowe; the original collection number is L-13465. It was identified by M. J. Larsen (30).

The nuclear large-subunit rRNA of the casual fungus was also sequenced and analyzed. Extraction of the total DNA of isolates UTHSC 01-1419 and UTHSC 01-1652 and the amplification and sample preparation of the nuclear large-subunit rRNA for sequencing was done according to the procedure described by Greslebin et al. (17). The bone marrow isolate UTHSC 02-617 was not successfully amplified and thus was not evaluated. Prepared samples were sent to the University of Wisconsin Biotechnology Center for sequencing on an Applied Biosystems/Hitachi model 3730 xl DNA analyzer. Nuclear large-subunit rRNAs of UTHSC 01-1419 and UTHSC 01-1652 (AY598826 and AY598827, respectively) were identical and, when compared with *I. tropicalis* CBS 617.89 (AY059037), differed at only 6 out of 868 bases (99.3% sequence similarity), When both nuclear large-subunit rRNA sequences were added into the multiple sequence alignment of Wagner and Fischer (61) and analyzed with PAUP* (59), they formed a clade with *I. tropicalis* that was fully supported by a 100% bootstrap value (data not shown), strongly indicating that the causal fungus in this investigation was *I. tropicalis.*

Few filamentous basidiomycetes have been authenticated as human etiologic agents. Two species documented to invade tissue include *Schizophyllum commune* and *Coprinopsis cinerea* (Formerly *Coprinus cinereus*). *Schizophyllum commune* has elicited serious invasive disease in the brain (5, 41), lungs (6, 41, 53), and hard and soft palates (40). It has also been implicated in allergic bronchopulmonary disease (1, 22), chronic sinusitis (4, 25, 33, 45, 51, 52), and less serious disease of the nails (27). *Coprinopsis cinerea* (anamorph, *Hormographiella aspergillata*) (14, 18) has been the causative agent in a case of prosthetic valve *endocarditis* (13, 57) and in a fatal pulmonary infection (60). A *Coprinus* species was reported to cause a fatal infection in a leukemia patient 5 years after bone marrow transplantation (36). Another basidiomycete thought to cause pulmonary disease is *Phanerochaete chrysosporium* (animorph, *Sporotrichum pruinosum*), although no tissue invasion has been noted (26, 56). *Bjerkandera adusta*, frequently isolated from pulmo-

nary sites, may also he pathogenic in some settings (16). Fungal infections in patients with CGD are most frequently caused by *Aspergillus fumigatus* (7). More-recent reports have cited additional etiologic agents. These include the basidiomycete *Trichosporon inkin* reported by Kenney et al. in 1990 as *Sarcinosporon inkin* (24); the homothallic ascomycete *Pseudallescheria boydii* (39); *Chrysosporium zonatum,* an anamorph of the heterothallic ascomycete *Uncinocarpus orisii* (44); and other hyphomycetous species, i.e., *Acremonium strictum* (2), *Exophiala* (*Wangiella*) *dermatitidis* (23), *Paecilomyces lilacinus* (55), *Paecilomyces variotii* (8, 62), and a *Paecilomyces* species (54). To our knowledge, filamentous basidiomycetes have not been reported to occur in patients with CGD. This report adds the wood-destroying *I. tropicalis* to the list of basidiomycetes previously cited as being invasive and extends the genera of fungal organisms inciting disease in CGD patients.

Filamentous basidiomycetes are being recovered from clinical specimens with increasing frequency, and their identification in this setting remains problematic. González et al. (16), reporting in vitro antifungal susceptibility data for 44 clinical isolates of basidiomycetous fungi, indicated that, in addition to *Schizophyllum* commune and *Coprinopsis cinerea,* many arthroconidium-forming molds resembling *Bjerkandera adusta* were being recovered. Many more isolates, however, remained sterile and failed to produce clamp connections and/or lacked spicules suggestive of *Schizophyllum commune* and were reported as unidentified basidiomycetes (16). The characteristics that facilitate recognition of a basidiomycete include rapid growth; colonies that are usually white, cream, or yellow–orange pigmented; the presence of clamp connections and/or crystals; the formation of spicules along the hyphae; and mushroom- or basidiocarp-type fruiting bodies (50). Since most clinical isolates are monokaryons, neither clamps nor fruiting structures are produced, and mating studies may be required to induce these diagnostic features. The ability of most basidiomycetes to grow on agar containing 2 to 10 μg of benomyl/ml has also been employed as a screening test, although many ascomycetous fungi grow on this medium as well (58). Several basidiomycetes also fail to grow on media containing cycloheximide, a substrate commonly found in typical mycology laboratories. The isolate presented here was typical of a basidiomycete in that it exhibited rapid filamentous growth, had a yellowish to orange pigmentation, and was tolerant of benomyl. Also typical was the lack of clamp connections and basidiocarps. Somewhat unusual was the ability of the organism to grow equally well on medium containing cycloheximide. Since the organism had been isolated on numerous occasions from a profoundly compromised patient, molecular characterization was pursued.

When the sequence data of the isolates analyzed were compared to ITS and mitochondrial small-subunit rRNA sequences in GenBank, 97.8 and 99.2% matches, respectively, were made with *I. tropicalis.* In the sequence analysis of nuclear large-subunit rRNAs, they again showed high sequence similarity, i.e., of 99.3%. with *I. tropicalis* and were positively concluded to be *I. tropicalis.*

I. tropicalis is a poroid (having pores rather than gills) wood-decaying fungus that is associated with white-rot decay of various woody angiosperms. As is typical of many fungi, *I. tropicalis* has a complex taxonomic history. First described as *Poria rickii* Bres. in 1920 (3), it was given the new name *Phellinus tropicalis* M. Larsen et Lombard in 1988 for nomenclatural reasons (30). *Phellinus ferrugineo-velutinus* (Henn.) Ryv. is considered by some mycologists to be identical to *Phellinus tropicalis* (9, 15, 48), and its name is preferred for this taxon, based on priority. However, because the type specimen of *Phellinus ferrugineo-velutinus* is sterile (11), others prefer the name *Phellinus tropicalis.* Some mycologists also consider *Phellinus flavomarginata* (Murr.) Ryv. to be a synonym for *Phellinus ferrugineo-velutinus* (15, 48), but this is disputed by others (30, 31). *I. tropicalis* is common in Brazil, the type locality (30, 47), and it is reported also to occur in Mississippi (32), Florida, Georgia (15), Jamaica (46), Guadeloupe (11), Costa Rica (30), Colombia (49), East Africa (48), and Malaya, Johore, and Mawai, Malaysia (9). *Phellinus* is placed in the family *Hymenochaetaceae* and is one of the largest basidiomycetous genera of wood-destroying fungi, with over 220 named species worldwide (29). Traditionally, *Phellinus* is distinguished from *Inonotus,* a closely related genus, by its hard, woody, perennial fruiting bodies and dimitic hyphal system. *Inonotus,* in contrast, produces softer, annual fruiting bodies with a monomitic hyphal system. However, the morphological distinction between these two genera has become blurred over time (9, 15). Recent phylogenetic studies show that although the members of *Hymenochaetaceae* are a well-defined group (20), genera within this family, including *Phellinus* and *Inonotus,* are not monophyletic (43, 61). Thus, the taxonomy of *Phellinus* and allied genera is in a state of flux and is an area of active research (9, 10, 31, 38, 61). Wagner and Fischer's (61) phylogenetic study of *Phellinus* and *Inonotus* placed *Phellinus tropicalis* in the *Inonotus* sensu stricto clade. Thus, they transferred *Phellinus tropicalis* to the genus *Inonotus.* With the data available at this time, the best name for the casual fungus is *Inonotus tropicalis*; however, because most of the literature on this species refers to it as being of the genus *Phellinus*, we use both generic names.

This report extends the genera of basidiomycetous fungi inciting human disease and highlights the utility of molecular methods in the identification or verification of these often-unidentifiable molds when conventional taxonomic practices fail. Although the sequences from the case isolates have now been registered in GenBank, the Fungus Testing Laboratory was initially faced with locating individuals with expertise in poroid fungi who may have held genus-specific databases not in the public domain. Private and institutional databases appear to be a growing trend, and this case illustrates their usefulness for the identification of a wood-decaying fungus not previously known as a human etiologic agent.

Nucleotide sequence accession numbers. The following sequences have been deposited in the GenBank database (accession number): UTHSC-01-1419 small subunit rRNA gene (AY598828), UTHSC-01-1419 25S rRNA gene (AY598826), UTHSC-01-1652 small subunit rRNA gene (AY598829), UTHSC-01-1652 25S rRNA gene (AY598827), UTHSC-02-617 18S rRNA gene partial-ITS1 complete-5.8S rRNA complete-ITS2 complete-28S rRNA partial (AY641432), and UTHSC-03-2972 18S rRNA gene partial-ITS1 complete-5.8S rRNA complete-ITS2 complete-28S rRNA partial (AY599487).

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