JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 2007, p. 568–571 Vol. 45, No. 2
0095-1137/07/$08.00+0 doi:10.1128/JCM.01684-06

Discovering Potential Pathogens among Fungi Identified as Nonsporulating Molds▽

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Received 15 August 2006/Returned for modification 12 October 2006/Accepted 15 November 2006

Fungal infections are increasing, particularly among immunocompromised hosts, and a rapid diagnosis is essential to initiate antifungal therapy. Often fungi cannot be identified by conventional methods and are classified as nonsporulating molds (NSM).We sequenced internal transcribed spacer regions from 50 cultures of NSM and found 16 potential pathogens that can be associated with clinical disease. In selected clinical settings, identification of NSM could prove valuable and have an immediate impact on patient management.

Fungal infections are increasing, particularly among immunocompromised hosts, and a rapid, accurate diagnosis is essential for the initiation of targeted antifungal therapy. Diagnosis of fungal infections usually depends on recovery of fungi from culture of clinical specimens, and their identification requires the presence of reproductive structures. Often fungi cannot be characterized fully because the mold does not sporulate, making identification by microscopic morphology not possible and potentially increasing the time to report an inconclusive result to 21 days. While many laboratorians and clinicians assume that these fungal isolates are environmental organisms and not clinically significant, to our knowledge no study has systematically attempted to classify these previously unidentifiable fungi in a clinical microbiology laboratory.

Amplification and sequencing of target regions within the ribosomal DNA gene complex has emerged as a useful, adjunctive tool for the identification of fungi and does not depend on mold sporulation for identification (3, 5, 9, 11). The internal transcribed spacer (ITS) regions 1 and 2 located between the highly conserved small (18S) and large (28S) ribosomal subunit genes in the rRNA operon are known to have sufficient sequence variability to allow identification to the species level for many fungi (2, 3, 5, 9, 11, 15). The goals of this study were to determine if sequencing the ITS 1 and 2 regions of nonsporulating molds (NSM) could identify fungi that were not identifiable by conventional methods and could serve as an approach to detect clinically relevant pathogens.

Sample selection. Identification of molds directly from a specimen or submitted as an isolated culture to Associated Regional and University Pathologists, Inc., Laboratories (ARUP Laboratories) was attempted by growth on inhibitory mold agar, modified Sabouraud agars, or potato dextrose agar. Microscopic structures were observed on tease or tape preparations and slide cultures for up to 21 days. NSM were defined as molds without reproductive structures and that could not be further characterized. From 1 January 2005 to 1 January 2006, clinical isolates classified as NSM were randomly selected for gene amplification and sequencing.

DNA preparation. After growth for 1 to 7 days on potato dextrose agar slants, lysates were prepared from approximately 1 cm^2 of mycelia with IDI lysis kits (GeneOhm Sciences, San Diego, CA). Briefly, in a biological safety cabinet, mycelia were collected by scraping the slant with a sterile stick in 1 ml of sterile, molecular-grade H_2O. The material was transferred to a 2-ml screw-cap tube. The tubes were centrifuged for 1 min at 6,000 × *g*. If the mycelia did not pellet, the material was contained with a pediatric blood serum filter (Porex Corp., Fairburn, GA). Supernatant was removed. The material was resuspended in 200 μl of IDI sample buffer and transferred to the lysis tube, which contained glass beads. Lysis tubes were vortexed on the highest setting for 5 min. The tubes were placed in a boiling water bath for 15 min. Tubes were centrifuged for 5 min at 16,000 × *g*. The supernatant was stored at −20°C until amplification.

Amplification and sequencing. Real-time PCR with SYBR green DNA binding dye and amplicon melting temperature analysis was performed on the RotorGene 3000 (Corbett Robotics, Inc., Sydney, Australia). PCR mixtures contained the following: 1× Lightcycler FastStart DNA Master Hybridization Probes mixture (Roche Applied Science, Penzberg, Germany), which contained deoxynucleoside triphosphates, FastStart *Taq* DNA polymerase, and 1 mM $MgCl_2$ (additional $MgCl_2$ was added to a final concentration of 4.6 mM); 0.4 μM each of ITS1 forward (5′TCCGTAGGTGAACCTGCGG3′) and ITS4 reverse (5′TCCTCCGCTTATTGATATGC3′) primers (15); 1× SYBR green (Molecular Probes, Inc. Eugene, OR); and 3 μl template DNA. Thermal cycling parameters with RotorGene 3000 were 95°C for 10 min; 50 cycles of 95°C for 5 s, 60°C for 20 s, and 76°C for 30 s; and a final extension at 72°C for 2 min. The quality of the amplicon was determined using the derivative of the melt analysis curve (55°C to 99°C, 45-s hold at 55°C, 5 s/°C). The amplified product was processed for bidirectional sequencing using ExoSAP-IT (USB Corp.,

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▽ Published ahead of print on 29 November 2006.

Cleveland, OH). Five microliters of Big Dye Terminator Ready Reaction Mix v. 1.1 (Applied Biosystems, Inc., Foster City, CA) was added to 4 μl of each primer (0.8 pmol/μl) and 3 μl of purified PCR product. Cycle sequencing was performed with a 9700 thermal cycler (ABI), using 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Sequencing reaction products were passed through a Sephadex G-50 fine column to remove unincorporated dye terminators. Purified sequencing reaction products were read on an ABI Prism 3100 Genetic Analyzer with a 50-cm capillary array.

Sequences were analyzed with the SmartGene Integrated Database Network (SmartGene Inc., Raleigh, NC) software version 3.2.3 vr. SmartGene is a web-based software and database system with reference sequences derived from the National Center for Biological Information (NCBI) GenBank repository. Phylogenetic trees and alignments of reference sequences with ≥98% identity to NSM isolate sequences were created using Clustalx v1.83 (University of British Columbia Bioinformatics Centre).

Nucleotide sequences with match lengths of ≥400 bp were analyzed. Sequence-based identifications were defined by percent identity: species, ≥99%; genus, 93 to 99%; and inconclusive, ≤93%. Molds were classified as fungi often associated with clinical disease (potential pathogens), emerging pathogens with more than three cases reported (emerging pathogens), or plant/soil-associated fungi with no or few published cases of human disease. Sequence information was independently analyzed by the Mycotic Diseases Branch of the Centers for Disease Control and Prevention (CDC) specifically to confirm microorganism identification.

The number of NSM reported was 215, which comprised 3.27% of the total number of fungal isolates identified at ARUP in 2005. A representative subset of 50 isolates reported as NSM (23%) was randomly selected for gene sequencing. The sources of these isolates were mostly respiratory (38 isolates) or skin, hair, and nails (11 isolates), with the source for 1 isolate not provided (Table 1).

Forty-eight of the isolates had ITS sequence and match lengths of ≥400 bp, with an average sequence length of 572.3 ± 74.9 bp and an average match length of 560.3 ± 86.5 bp. The two isolates with sequence lengths of <400 bp were not included in the analysis. For the 48 sequences, gene sequencing identified 44 (92%) to genus level and 38 (79%) to species level, with 4 (8%) being inconclusive.

Sixteen isolates had reference sequences that shared ≥98% identity for more than one species, involving 12 genera. As a representative example, two isolates identified as being in the *Pestalotiopsis* genus had 10 and 20 reference sequences sharing ≥98% identity with multiple, different species, respectively. Phylogenetic tree construction for *Pestaloptiopsis* spp. also demonstrated a high degree of interspecies similarity and an absence of well-characterized strains within this genus. Similar results were found for 11 other genera.

Eight NSM were classified as fungi with pathogenic potential; these were *Trichophyton verrucosum*, *Ajellomyces capsulatus* (anamorph *Histoplasma capsulatum*), *Aureobasidium pullulans*, *Exophiala dermatitidis*, *Exophiala jeanselmei*, *Fusarium* sp., *Lewia infectoria* (anamorph *Alternaria infectoria*), and *Phialophora* sp. Eight NSM were classified as potential emerging pathogens: *Botryosphaeria rhodina* (anamorph *Lasiodiplodia theobromae*) (one isolate), *Coniothyrium* sp. (one isolate), *Coprinus* sp. (one isolate), and *Schizophyllum commune* (five isolates). Thirty-two isolates (66.7%) were identified as plant- and soil-associated fungi, mostly as filamentous basidomycetes.

Independent analysis of the NSM sequences by the CDC using the GenBank database correlated with our identifications. All eight potential pathogens were identified by the CDC, as were the five isolates identified as *Schizophyllum commune*, an emerging pathogen. Six additional fungi were identified to the genus or species level by the CDC method. For the remaining 29 isolates, the CDC reported them as filamentous basidomycetes (26 isolates) or coelomycetes (3 isolates) without further identification, which corroborated our classifications.

Molds are ubiquitous organisms in the environment and often transiently colonize the respiratory, integument, and gastrointestinal systems of healthy hosts. Traditionally, NSM recovered in the laboratory have been dismissed as insignificant environmental organisms without further testing. Additionally, for those laboratories that attempt to augment sporulation specifically for identification, the process can require up to 3 weeks of incubation and often is without success. To date, sequencing of NSM has been limited to case reports. To our knowledge, this study is the first to systematically evaluate a large number of NSM clinical isolates with ITS sequencing for further species identification. With sequencing the ITS 1 and ITS 2 regions, we identified the majority of NSM that could not be identified by conventional mycological methods and classified over one-third of these molds as well-recognized or emerging pathogens with potential for invasive disease in the appropriate clinical setting. As a national reference laboratory, we were unable to obtain clinical histories for these patients, but we presume that for the majority of these NSM, clinicians felt that the isolates had clinical relevance and referred them to our laboratory for fungal identification. We acknowledge that the classification of fungal pathogenicity with clinical relevance was difficult in the absence of patient data, but we based our classifications on standard practices and the current literature (1–3, 6–11, 13, 14). For example, ITS sequencing identified *Histoplasma capsulatum* (from bronchoalveolar lavage [BAL]), and although clinical correlation was not possible, the significance of this pathogen is well recognized. Similarly, we identified pathogens such as *Schizophyllum commune* (3, 10), *Coniothyrium* (6, 7), *Coprinus* (8, 13), and *Botrysphaeria rhodina* (1, 14), which can be considered emerging pathogens capable of causing disease when there is histopathological or other corroborating evidence. For many isolates identified by ITS sequencing, we classified them as environmental fungi because these microorganisms are commonly encountered in the clinical laboratory and usually have no clinical importance in healthy subjects (12). Yet, for immunocompromised hosts, the pathogenicity of such environmental fungi has not been well defined, and the capacity to characterize these organisms by ITS sequencing may prove useful when there is a high index of suspicion that the environmental NSM is a putative pathogen.

The taxonomy of many fungi is evolving, and names of organisms may change rapidly, while obsolete nomenclature may exist in databases; this may contribute to the results that we and others have observed in fungal identification using ITS sequences (4, 5). In attempts to minimize the risk for misiden-

TABLE 1. Identification of 48 nonsporulating molds by ITS sequencing

Source	ARUP identification (GenBank accession no.)	Identity (%)	Sequence length (bp)	Match length (bp)	No. of species with >98% homology	CDC identification	Microorganism classification
BAL	*Inonotus cuticularis* (AF237730)	99.73	753	747	1	Filamentous basidiomycete	Plant/soil
BAL	*Ceriporiopsis aneirina* (AY219362)	91.41	660	489	0	Filamentous basidiomycete	Plant/soil
BAL	*Polyporus* sp. (AF516545)	94.43	659	646	0	Filamentous basidiomycete	Plant/soil
Scalp	*Trichophyton verrucosum* (AF168126)	99.85	655	655	1	*Trichophyton verrucosum*	Pathogen
Lung	*Irpex lacteus* (AB079265)	99.69	651	651	1	Filamentous basidiomycete	Plant/soil
Sputum	*Irpex lacteus* (AB079265)	99.85	650	648	1	Filamentous basidiomycete	Plant/soil
Bronchi	*Pleurotus ostreatus* (AY636055)	99.53	644	640	5	Filamentous basidiomycete	Plant/soil
Bronchi	*Oxyporus latemarginatus* (AF232721)	99.83	633	576	2	Filamentous basidiomycete	Plant/soil
BAL	*Coprinus* sp. (AF345808)	98.08	625	624	1	Filamentous basidiomycete	Potential pathogen
BAL	*Pycnoporus* sp. (AF363759)	94.36	623	618	0	Filamentous basidiomycete	Plant/soil
BAL	*Pycnoporus* sp. (AF363759)	94.35	619	618	0	Filamentous basidiomycete	Plant/soil
Sputum	*Amauroderma subresinosum* (AJ627583)	91.16	612	611	0	Filamentous basidiomycete	Plant/soil
Ankle	*Exophiala jeanselmei* (AJ866273)	99.67	608	607	4	*Exophiala* sp.	Pathogen
BAL	*Phellinus robinae* (AY558646)	99.17	607	603	1	Filamentous basidiomycete	Plant/soil
BAL	*Trametes versicolor* (AY309018)	99.84	607	607	6	Filamentous basidiomycete	Plant/soil
BAL	*Pycnoporus* sp. (AF363759)	93.73	606	606	0	Filamentous basidiomycete	Plant/soil
BAL	*Hyphodontia flavipora* (AF455399)	99.48	603	572	1	Filamentous basidiomycete	Plant/soil
BAL	*Schizophyllum commune* (AF348142)	99.33	603	597	1	*Schizophyllum commune*	Potential pathogen
Sinus	*Schizophyllum commune* (AF348142)	99.33	603	600	1	*Schizophyllum commune*	Potential pathogen
BAL	*Thanatephorus cucumeris* (AF455463)	99.00	600	599	2	Filamentous basidiomycete	Plant/soil
Sinus	*Phlebia subserialis* (AB084620)	99.83	598	572	1	Filamentous basidiomycete	Plant/soil
BAL	*Oxyporus latemarginatus* (AF232721)	99.83	592	573	2	Filamentous basidiomycete	Plant/soil
Bronchi	*Schizophyllum commune* (AY573544)	99.66	588	588	1	*Schizophyllum commune*	Potential pathogen
Sinus	*Schizophyllum commune* (AF348142)	99.32	585	585	1	*Schizophyllum commune*	Potential pathogen
Sinus	*Schizophyllum commune* (AF348142)	99.32	584	584	1	*Schizophyllum commune*	Potential pathogen
Scalp	*Aphanoascus fulvescens* (AF038357)	99.13	575	575	2	*Aphanoascus* sp.	Plant/soil
Toe	*Lewia infectoria*[a] (AF397239)	99.65	574	574	2	*Alternaria infectoria*[a]	Pathogen
Sputum	*Eutypella scoparia* (AF373064)	99.48	573	573	1	*Eutypella scoparia*	Plant/soil
BAL	*Phlebia* sp. (AY787680)	81.75	572	572	0	Filamentous basidiomycete	Plant/soil
Lung	*Phanerochaete chrysosporium* (AF475147)	88.93	572	572	0	Filamentous basidiomycete	Plant/soil
Bronchi	*Oxyporus latemarginatus* (AF232721)	99.46	570	557	2	Filamentous basidiomycete	Plant/soil
Sputum	*Arthrinium* sp. (AY425967)	100.00	570	570	2	*Arthrinium* sp.	Plant/soil
Nail	*Botryosphaeria obtusa* (AY259094)	99.82	564	560	2	Coelomycete fungus	Plant/soil
Sinus	*Myrothecium* sp. (AJ619957)	99.28	563	556	1	Possible coelomycete fungus	Plant/soil
Sputum	*Arthrographis cuboidea* (AB213444)	99.29	563	563	1	*Arthrographis* sp.	Plant/soil
Skin	*Aureobasidium pullulans* (AY225167)	99.82	559	556	1	*Aureobasidium pullulans*	Pathogen
Bronchi	*Phlebia subserialis* (AB084620)	99.66	558	588	1	Filamentous basidiomycete	Plant/soil
Lung	*Ceriporiopsis* sp. (AY781250)	99.46	557	557	1	Filamentous basidiomycete	Plant/soil
Nail	*Coniothyrium* sp. (AY157492)	100.00	542	516	2	Coelomycete fungus	Potential pathogen
Other	*Pestalotiopsis* sp. (AF409979)	99.08	541	541	10	*Pestalotiopsis* sp.	Plant/soil
Pleural fluid	*Botryosphaeria rhodina*[b] (AY568635)	100.00	537	504	1	Filamentous basidiomycete	Potential pathogen
BAL	*Oxyporus latemarginatus* (AF232721)	99.80	536	512	2	Filamentous basidiomycete	Plant/soil
BAL	*Amanita tenuifolia* (AF085492)	99.39	517	494	1	Filamentous basidiomycete	Plant/soil
Lung	*Fusarium* sp. (U61695)	99.22	516	515	1	*Fusarium* sp.	Pathogen
Nail	*Pestalotiopsis* sp. (AF409967)	99.60	498	498	20	*Pestalotiopsis* sp.	Plant/soil
BAL	*Ajellomyces capsulatus*[c] (U18363)	100.00	485	467	1	*Histoplasma capsulatum*[c]	Pathogen
Nail	*Phialophora* sp. (AF083206)	96.98	430	464	1	*Phialophora lignicola*	Pathogen
Scalp	*Exophiala dermatitidis* (AY213650)	100.00	404	404	3	*Exophiala* sp.	Pathogen

[a] *Lewia infectoria* teleomorph of *Alternaria infectoria.*
[b] *Botryosphaeria rhodina* teleomorph of *Lasiodiplodia theobromae.*
[c] *Ajellomyces capsulatus* teleomorph of *Histoplasma capsulatum.*

tification (2), we specifically selected a conservative match length of ≥400 bp and a 93% identity cutoff for identification to the genus level. The 93% cutoff was empirically determined to allow identification to the genus level of additional plant/soil-associated fungi. Despite these criteria, we found some clinical isolates sharing a high percent identity for multiple species, which suggests inadequate variation in the ITS regions and thereby prevention of species discrimination. When definitive fungal identification is clinically indicated, additional targets may be necessary to identify certain genera and species (5, 9). Four NSM were not conclusively identified by ITS sequencing in our study; these may represent novel species that do not have representative ITS reference sequences in GenBank.

In summary, ITS sequencing provides a fast alternative to conventional identification for nonsporulating molds. For specific specimen sources (e.g., tissue or BAL) and in the appropriate clinical setting, sequencing the ITS region for nonsporulating molds that cannot be conclusively characterized by conventional means could serve as a valuable tool to identify clinically relevant pathogens and enable the timely initiation of appropriate antifungal treatment.

We thank Dewey Hansen and the staff of the ARUP Mycology Laboratory for technical support. We thank Sam Page and Wei Tang for sequencing and technical advice for this project.

Support for this study was provided by the ARUP Institute for Clinical and Experimental Pathology, an enterprise of the University of Utah and its Department of Pathology.

The findings and conclusions in this article are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

We have no conflict of interest regarding the software, products, or concepts used in this study.

REFERENCES

1. **Borderie, V. M., T. M. Bourcier, J.-L. P. Poirot, M. Baudrimont, P. P. de Saint-Maur, and L. Larouche.** 1997. Endophthalmitis after *Lasiodiplodia theobromae* corneal abscess. Graefe's Arch. Clin. Exp. Opthalmol. **235:**259–261.
2. **Brandt, M. E., D. Gaunt, N. Iqbal, S. McClinton, S. Hambleton, and L. Sigler.** 2005. False-positive *Histoplasma capsulatum* Gen-Probe chemiluminescent test result caused by a *Chrysosporium* species. J. Clin. Microbiol. **43:**1456–1458.
3. **Buzina, W., D. Lang-Loidolt, H. Braun, K. Freudenschuss, and H. Stammberger.** 2001. Development of molecular methods for identification of *Schizophyllum commune* from clinical samples. J. Clin. Microbiol. **39:**2391–2396.
4. **Guarro, J., J. Gene, and A. M. Stchigel.** 1999. Developments in fungal taxonomy. Clin. Microbiol. Rev. **12:**454–500.
5. **Iwen, P. C., S. H. Hinrichs, and M. E. Rupp.** 2002. Utilization of the internal transcribed spacer regions as molecular targets to detect and identify human fungal pathogens. Med. Mycol. **40:**87–109.
6. **Kiehn, T. E., B. Polsky, E. Punithalingam, F. Edwards, A. E. Brown, and D. Armstong.** 1987. Liver infection caused by *Coniothyrium fuckelii* in a patient with acute myelogenous leukemia. J. Clin. Microbiol. **25:**2410–2412.
7. **Miele, P. S., C. S. Levy, M. A. Smith, E. M. Dugan, R. H. Cooke, J. A. Light, and D. R. Lucey.** 2002. Primary cutaneous fungal infections in solid organ transplantation: a case study. Am. J. Transplant. **2:**678–683.
8. **Nenoff, P., T. Freidrich, H. Schwenke, M. Mierzwa, L. C. Horn, and U. F. Haustein.** 1997. Rare fatal simultaneous mould infection of the lung caused by *Aspergillus flavus* and the basidiomycete *Coprinus* sp. in a leukemic patient. J. Med. Vet. Mycol. **35:**65–69.
9. **Rakeman, J. L, U. Bui, K. La Fe, Y.-C. Chen, R. J. Honeycutt, and B. T. Cookson.** 2005. Multilocus DNA sequence comparisons rapidly identify pathogenic molds. J. Clin. Microbiol. **43:**3324–3333.
10. **Rihs, J. D., A. A. Padhe, and C. B. Good.** 1996. Brain abscess caused by *Schizophyllum commune*: an emerging basidiomycete pathogen. J. Clin. Microbiol. **34:**1628–1632.
11. **Schwarz, P., S. Bretagne, J.-C. Gantier, D. Garcia-Hermoso, O. Lortholary, F. Dromer, and E. Dannaoui.** 2006. Molecular identification of zygomycetes from culture and experimentally infected tissues. J. Clin. Microbiol. **44:**340–349.
12. **Sigler, L., and S. P. Abbott.** 1997. Characterizing and conserving diversity of filamentous basidiomycetes from human sources. Microbiol. Cult. Coll. **13:**21–27.
13. **Surmont, I., F. Van Aelst, J. Verbanck, and G. S. De Hoog.** 2002. A pulmonary infection caused by *Coprinus cinereus* (*Hormographiella aspergillata*) diagnosed after a neutropenic episode. Med. Mycol. **40:**217–219.
14. **Thomas, P. A.** 2003. Current perspectives on ophthalmic mycoses. Clin. Microbiol. Rev. **16:**730–797.
15. **White, T. J., T. Bruns, S. Lee, and J. Taylor.** 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, p. 315–322. *In* M. A. Innis, D. H. Gelfand, J. J. Snisky, and T. J. White (ed.), PCR protocols: a guide to methods and applications. Academic Press, San Diego, CA.