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Development and Clinical Application of a Panfungal PCR Assay To Detect and Identify Fungal DNA in Tissue Specimens▽

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Given the rise in the incidence of invasive fungal infections (IFIs) and the expanding spectrum of fungal pathogens, early and accurate identification of the causative pathogen is essential. We developed a panfungal PCR assay that targets the internal transcribed spacer 1 (ITS1) region of the ribosomal DNA gene cluster to detect fungal DNA in fresh and formalin-fixed, paraffin-embedded (PE) tissue specimens from patients with culture-proven ($n = 38$) or solely histologically proven ($n = 24$) IFIs. PCR products were sequenced and compared with sequences in the GenBank database to identify the causal pathogen. The molecular identification was correlated with results from histological examination and culture. The assay successfully detected and identified the fungal pathogen in 93.6% and 64.3% of culture-proven and solely histologically proven cases of IFI, respectively. A diverse range of fungal genera were identified, including species of *Candida*, *Cryptococcus*, *Trichosporon*, *Aspergillus*, *Fusarium*, *Scedosporium*, *Exophiala*, *Exserohilum*, *Apophysomyces*, *Actinomucor*, and *Rhizopus*. For five specimens, molecular analysis identified a pathogen closely related to that identified by culture. All PCR-negative specimens ($n = 10$) were PE tissues in which fungal hyphae were visualized. The results support the use of the panfungal PCR assay in combination with conventional laboratory tests for accurate identification of fungi in tissue specimens.

The frequency of invasive fungal infections (IFIs) in critically ill and immunocompromised patients is continuing to increase. Epidemiological studies now indicate that the spectrum of fungal pathogens has expanded well beyond *Aspergillus fumigatus* and *Candida* species (28). Contributory factors include an increase in the patient population at risk of IFI, better appreciation that unusual fungi can cause disease, and selection pressures from current practices of antifungal use (8, 28). Early, rapid, and accurate identification of pathogenic fungi is important in order to guide the selection of appropriate antifungal therapy and thus improve patient outcomes, as well as for epidemiologic purposes (17). However, current culture-based phenotypic methods are insensitive and slow, may initially be nonspecific, and require considerable expertise for correct morphological identification of less common or unusual fungi (1, 5). Additional drawbacks of conventional culture include the failure of zygomycetes to grow when hyphal cells have been damaged during processing (21) and the collection of tissue biopsy specimens directly into formalin fixative for paraffin embedding when IFIs are not suspected clinically (19) or when limited material is available.

Recent efforts to improve the sensitivity and specificity of diagnostic tests have focused on culture-independent methods, in particular nucleic acid-based methods, such as PCR assays. These can be applied to fresh and formalin-fixed, paraffin-embedded (PE) sections. Numerous studies have highlighted the advantages of using PCR technology to detect viable and nonviable fungal pathogens in a variety of clinical specimens. The majority of assays target multicopy genes, in particular the ribosomal DNA (rDNA) genes (18S, 28S, and 5.8S) and the intervening internal transcribed spacer (ITS) regions (ITS1 and ITS2), in order to maximize sensitivity and specificity. To date, most assays have been designed to detect *Candida* or *Aspergillus* species only (14, 16, 17, 22, 24, 35, 38, 39, 43). Given that more than 200 fungal species have been reported to cause disease in humans and companion animals (11), the clinical utility of a species-specific or even a genus-specific assay is limited. Panfungal PCR assays, on the other hand, have the potential to detect all fungal species, but many rely on additional, time-consuming techniques, such as species-specific probes and hybridization, to identify the pathogen (10, 12, 15, 25, 29, 34, 37). Furthermore, probe design is restricted to known pathogens and does not allow the identification of new and emerging agents. Sequence-based identification of PCR products is a sensitive alternative, provided that accurate sequences have been submitted to public databases, e.g., GenBank (5, 32, 42).

In this study, we developed and evaluated a panfungal PCR assay to detect and identify fungal pathogens directly from fresh and PE tissue specimens obtained from patients with culture-proven and/or histologically proven IFIs. We chose to target the ITS1 region, located between the 18S and 5.8S rRNA genes, because (i) it is multicopy (≥100 copies in the fungal genome), (ii) universal fungal primers are available, and (iii) it contains highly variable regions for species identification (6, 15, 17, 18, 20, 23, 24, 26), and we used DNA sequence analysis for species identification. The

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results of the molecular identification were correlated with those obtained by histological examination and culture.

MATERIALS AND METHODS

Clinical specimens. Seventy-five tissue specimens from 62 patients (43 humans and 19 companion animals) with culture-proven (n = 38) (see Table 1) and histologically documented but culture-negative (n = 24) (see Table 2) IFIs were evaluated using the panfungal PCR assay. In cases of culture-positive IFI, the species was identified by standard phenotypic and morphological characteristics (9, 21). Specimens were obtained from a variety of body sites, including both sterile and nonsterile locations; 37 were fresh tissue biopsy specimens, and 38 were PE (see Tables 1 and 2). In addition, we tested a skin biopsy specimen from a patient with disseminated nodular skin lesions, where there was a high index of suspicion for IFI (patient 63) but culture and histology results were negative. Fresh (n = 14) and PE (n = 4) tissue specimens from a variety of body sites (brain, skin and soft tissue, eye, bone, lung, cardiac valve, and bone marrow) from 12 patients without IFIs were used as controls.

DNA extraction. Specimen manipulations and DNA extractions were performed in a class II laminar flow cabinet. For each PE tissue sample, 10 sections (thickness, 10 μm) were cut using a sterile microtome blade and transferred to a 10-ml centrifuge tube (Sarstedt Australia, Technology Park, Australia). To remove paraffin wax, 5 ml of histolene (Fronine Laboratory Supplies, Riverstone, Australia) was added, mixed by inversion, incubated at room temperature overnight, and centrifuged (at 3,838 × g for 15 min) in a Beckman Coulter (Fullerton, CA) GS-15 centrifuge. The supernatant was removed, and the pellet was washed with 5 ml of 100% ethanol (Ajax Finechem, Seven Hills, Australia), mixed by inversion, and centrifuged (at 3,838 × g for 15 min). The tissue pellet was transferred to a 2-ml microcentrifuge tube (Eppendorf AG, Hamburg, Germany) and washed in 1 ml of 100% ethanol, followed by 1 ml of 70% ethanol. The ethanol was removed by centrifugation (at 5,900 × g for 10 min), and the pellet was air dried at room temperature in preparation for DNA extraction. For DNA extraction, all tissue samples were incubated for ≥3 h in proteinase K and lysis buffer at 55°C, and the DNA was extracted using the MagNAPure LC instrument with the MagNAPure LC DNA isolation kit II (Tissue) (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The DNA was stored at −20°C prior to use. To monitor contamination, each specimen was shadowed by a negative control containing molecular-biology-grade water (Eppendorf AG).

PCR amplification and DNA sequencing. PCRs were performed in a 25-μl volume consisting of 1× PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, and 0.001% gelatin) (Applied Biosystems, Foster City, CA), 2 mM $MgCl_2$ solution (Roche Diagnostics), 5% glycerol (Sigma Chemical Co., St. Louis, MO), 0.25 mM deoxynucleoside triphosphates (Roche Diagnostics), 0.8 μM primers ITS 1 (5′-TCC GTA GGT GAA CCT GCG G) and ITS 2 (5′-GCT GCG TTC TTC ATC GAT GC) (40) (Sigma-Genosys, Australia), 1.25 U of AmpliTaq Gold DNA polymerase (Applied Biosystems), and 10 μl of DNA. Amplification was performed on a Mastercycler gradient thermocycler (Eppendorf AG). The thermal cycling conditions were 95°C for 10 min, followed by 60 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 5 min. A negative control of molecular-biology-grade water and an inhibition control composed of an equal mixture of specimen DNA and *Candida parapsilosis* (strain no. ATCC 22019) positive-control DNA were included for each specimen. The inhibition control was used to exclude the presence of inhibitory substances. PCR products were separated by 1.5% agarose gel electrophoresis, stained with SYBR Safe DNA gel stain (Molecular Probes, Eugene, OR), and visualized by UV light transillumination. PCR products were purified using the GFX PCR DNA and gel band purification kit (Amersham Biosciences, Castle Hill, Australia) and were sequenced using the ITS 1 primer and the BigDye Terminator (version 3.1) cycle sequencing kit in the ABI PRISM 3100 genetic analyzer (Applied Biosystems). Sequences were edited using Chromas (version 2.23) software (Technelysium Pty. Ltd.) and entered into a BLASTN sequence analysis search (2) provided by BioManager, ANGIS (http://www.angis.org.au), for species identification.

Contamination control. Prior to any experimental procedure, all work surfaces and equipment, including cabinets, pipettes, and racks, were wiped down with NucleoClean decontamination solution (Chemicon International, Temecula, CA). DNA extraction, PCR setup, PCR amplification, and agarose gel electrophoresis were performed in separate, independently equipped laboratories, set out in a unidirectional workflow to prevent carryover contamination. PCR master mixes were prepared using a PCR cabinet cleaned with 70% alcohol and UV irradiated for 20 min, positive-displacement pipettes, and aerosol-resistant pipette tips.

RESULTS

Specimens from patients without IFIs. No fungal DNA was amplified from 18 control tissue specimens obtained from 12 patients without IFIs.

Specimens from patients with culture-proven IFIs. Thirty-one fresh and 16 PE tissue specimens from 38 patients with culture-proven IFIs were tested. Patients were infected with a diverse range of yeasts and molds, including the uncommon pathogens *Metarhizium anisopliae* and *Microsphaeropsis arundinis* (Table 1). A PCR product was obtained from each specimen; however, DNA sequencing was unsuccessful for three (patients 36 to 38). For two of these, DNA was extracted from PE sections in which only scant fungal hyphae were seen upon histological examination. For the remaining specimen (patient 37), multiple bands were present on the gel, suggesting a mixed fungal infection; however, a single isolate recovered by culture was identified as *Glomerella lagenaria* by ITS sequence analysis.

Sequencing results correlated with culture identification for 39 of 44 (88.6%) specimens (Table 1). For the remaining five specimens, analysis yielded sequences with 98 to 100% identity to sequences of fungi that are phylogenetically closely related to the species identified by culture-based methods (given in parentheses): patient 18, *Neosartorya pseudofischeri* (*Aspergillus fumigatus*); patient 27, *Exophiala spinifera* (*Exophiala jeanselmei*); patient 35, *Rhizomucor pusillus* (*Absidia corymbifera*) (Table 1). Forty-one of 44 (93.2%) sequences showed ≥98% identity to sequences deposited in the GenBank database. The other three demonstrated 96 to 97% identity. Sequence analysis of the ITS1 region was unable to differentiate between members of the *Cryptococcus neoformans* complex (*Cryptococcus gattii*, *Cryptococcus neoformans* var. *neoformans*, and *Cryptococcus neoformans* var. *grubii*); *Fusarium* species, including *Fusarium verticillioides*, *F. subglutinans*, and *F. proliferatum*; *Neosartorya* spp. and *Aspergillus lentulus*; *Rhizopus oryzae* and *Amylomyces rouxii*; and *Exserohilum rostratum* and *Exserohilum mcginnisii*.

Specimens from patients with histologically proven IFIs. Six fresh and 22 PE tissue specimens from 24 patients with histologically proven IFIs were analyzed by PCR. Nine of these specimens (six patients) were obtained at postmortem examination. Fungal DNA was amplified from 18 (64.3%) samples, and DNA sequencing identified a variety of fungal pathogens, including *Candida* spp., *Trichosporon* spp., *Aspergillus* spp., and zygomycetes (Table 2). With the exception of one specimen (patient 51), sequence analysis showed ≥98% identity to sequences in the GenBank database. The molecular identification was consistent with the histological diagnosis in 17 of 18 (94.4%) cases. The exception was patient 40, where *C. parapsilosis* DNA was detected in a nasopharyngeal biopsy specimen but histopathology (showing narrow-necked budding, encapsulated yeasts) was consistent with a *C. neoformans* complex infection. All of the PCR-negative specimens (n = 10) were PE sections; six of these (patients 59 to 62) had morphological characteristics indicative of a zygomycete, while scant fungal elements were seen in three of the remaining four (patients 56 to 58). *Cryptococcus albidus* and *Cryptococcus albidosimilis* (patient 41) were not distinguished by sequence analysis of the ITS1 region.

TABLE 1. Results of culture, PCR, and DNA sequence analysis of tissue samples from patients with culture-proven IFIs[a]

Patient	Tissue site (type of specimen)	Clinical diagnosis	Identification by culture	Histology result	PCR result	Molecular identification (% identity with GenBank sequence)
1	Vocal cord (F)	IC	*Candida albicans*	NP	+	*C. albicans* (99)
2	Thigh (F)	IC	*C. albicans*	NP	+	*C. albicans* (99)
3	Skin (F)	IC	*Candida glabrata*	+	+	*C. glabrata* (98)
4	Urinary bladder (F)	IC	*Candida krusei*	−	+	*C. krusei* (99)
5	Carotid artery (F)	IC	*Candida tropicalis*	+	+	*C. tropicalis* (100)
6[b]	Skin (PE)	Cryptococcosis	*Cryptococcus gattii*	+	+	*C. neoformans*/*C. gattii* (99)
7	Lung (F)	Cryptococcosis	*Cryptococcus neoformans*	NP	+	*C. neoformans*/*C. gattii* (100)
8	Nasal mucosa (F)	Cryptococcosis	*C. neoformans*	+	+	*C. neoformans*/*C. gattii* (99)
9[b]	Nasal cavity (PE)	Cryptococcosis	*C. neoformans*	+	+	*C. neoformans*/*C. gattii* (98)
10	Paranasal sinus (F)	IA	*Aspergillus flavus*	−	+	*A. flavus* (99)
11	Lung (F)	IA	*Aspergillus fumigatus*	+	+	*A. fumigatus* (100)
12	Nasal mucosa (F)	IA	*A. fumigatus*	+	+	*A. fumigatus* (99)
13[b]	Nasal cavity (F)	IA	*A. fumigatus*	+	+	*A. fumigatus* (100)
14[b]	Frontal sinus (F)	IA	*A. fumigatus*	−	+	*A. fumigatus* (99)
15[b]	Nasal cavity (PE)	IA	*Aspergillus* spp.	+	+	*A. fumigatus* (100)
16[b]	Air sac (PE)	IA	*A. fumigatus*	+	+	*A. fumigatus* (100)
17[b]	Paranasal sinus (PE)	IA	*A. fumigatus*	+	+	*A. fumigatus* (100)
18A[b]	Retrobulbar space (F)	IA	*A. fumigatus*	+	+	*Neosartorya pseudofischeri* (100)
18B[b]	Retrobulbar space (PE)	IA	*A. fumigatus*	+	+	*N. pseudofischeri* (100)
18C[b]	Retrobulbar space (F)	IA	*A. fumigatus*	+	+	*N. pseudofischeri* (98)
19	Sinus (F)	IFI	*A. fumigatus*	+	+	*A. fumigatus* (100)
20A	Lung (F)	IFI	*A. fumigatus*	+	+	*A. fumigatus* (100)
20B	Spleen (F)	IFI	*A. fumigatus*	−	+	*A. fumigatus* (99)
21	Brain (F)	IFI	*A. fumigatus*	+	+	*A. fumigatus* (99)
22A	Lung (PE)	IFI	*Neosartorya* spp./*Aspergillus lentulus*	+	+	*Neosartorya* spp./*A. lentulus* (99)
22B	Lung (F)	IFI	*Neosartorya* spp./*A. lentulus*	+	+	*Neosartorya* spp./*A. lentulus* (99)
22C	Lung (F)	IFI	*Neosartorya* spp./*A. lentulus*	+	+	*Neosartorya* spp./*A. lentulus* (99)
23[b]	Abdominal mass (PE)	IFI	*Scedosporium apiospermum*	+	+	*S. apiospermum* (99)
24	Costal cartilage (F)	IFI	*Scedosporium prolificans*	−	+	*S. prolificans* (99)
25	Skin (PE)	Fusariosis	*Fusarium* spp.	+	+	*Fusarium* spp. (99)[c]
26	Synovial fluid (F)	IFI	*Exophiala* spp.	NP	+	*Exophiala* spp. (97)
27[b]	Skin (PE)	Dematiaceous IFI	*Exophiala jeanselmei*	+	+	*Exophiala spinifera* (98)
28[b]	Skin (PE)	IFI	*E. jeanselmei*	+	+	*Exophiala* spp. (99)
29	Nasal mucosa (F)	IFI	*Exserohilum rostratum*	+	+	*E. rostratum*/*Exserohilum mcginnisii* (99)
30[b]	Nasal bones (PE)	IFI	*Metarhizium anisopliae*	+	+	*M. anisopliae* (99)
31[b]	Nasal cavity (PE)	IFI	*Microsphaeropsis arundinis*	+	+	*M. arundinis* (100)
32A	Buttock (F)	Zygomycosis	*Apophysomyces elegans*	+	+	*Apophysomyces elegans* (96)
32B	Buttock (F)	Zygomycosis	*Apophysomyces elegans*	+	+	*Apophysomyces elegans* (97)
33A	Paranasal sinus (F)	Zygomycosis	*Rhizopus* spp.	+	+	*Rhizopus oryzae*/*Amylomyces rouxii* (99)
33B	Paranasal sinus (F)	Zygomycosis	*Rhizopus* spp.	+	+	*R. oryzae*/*A. rouxii* (98)
33C	Paranasal sinus (F)	Zygomycosis	*Rhizopus* spp.	+	+	*R. oryzae*/*A. rouxii* (99)
33D	Paranasal sinus (F)	Zygomycosis	*Rhizopus* spp.	+	+	*R. oryzae*/*A. rouxii* (99)
34	Paranasal sinus (F)	Zygomycosis	*R. oryzae*	+	+	*R. oryzae*/*A. rouxii* (100)
35[b]	Nasal turbinate (PE)	Zygomycosis	*Absidia corymbifera*	+	+	*Rhizomucor pusillus* (99)
36[b]	Nasal cavity (PE)	IA	*A. fumigatus*	+	+	No result
37[b]	Flank (F)	IFI	*Glomerella lagenaria*	+	+	No result
38[b]	Eye (PE)	Cryptococcosis	*C. neoformans*	+	+	No result

[a] F, fresh tissue; PE, paraffin embedded; IA, invasive aspergillosis; IC, invasive candidiasis; NP, histology not performed.
[b] Specimen from animal patient.
[c] Differentiation of *Fusarium* species by ITS sequence analysis is complicated by the presence of >1 ITS sequence variant in a single strain (27).

Specimen from a patient with a suspected IFI. PCR and DNA sequence analysis of a skin biopsy specimen from patient 63 identified the causative pathogen as *Trichophyton verrucosum*, with 100% identity to *T. verrucosum* sequences in the GenBank database.

DISCUSSION

Rapid and precise identification of fungal pathogens to species level is critical to improving the management of IFIs. The results from this study indicate that the application of a panfungal PCR to amplify the ITS1 region of the rDNA gene cluster followed by DNA sequencing is a highly sensitive and useful tool for the detection and identification of a wide range of fungi from both fresh (>97% sensitivity) and PE (68% sensitivity) tissue specimens.

The validity and clinical applicability of the assay were confirmed by testing specimens from patients with culture-proven IFIs (Table 1). Although the PCR amplified fungal DNA for

TABLE 2. Results of histology, PCR, and DNA sequence analysis of tissue samples from patients with histologically proven IFIs

Patient	Tissue site[a]	Clinical diagnosis[b]	Historical characteristic (estimate of quantity[c])	PCR result	Molecular identification (% identity with GenBank sequence)
39	Liver (F)	IFI	Pseudohyphae (+++)	+	*Candida dubliniensis* (99)
40[d]	Nasopharynx (PE)	Cryptococcosis	Yeast-like organisms with capsule and narrow-necked budding (++)	+	*Candida parapsilosis* (98)
41	Neck (PE)	IFI	Yeast-like organisms with capsule (++)	+	*Cryptococcus albidus*/*Crypotococcus albidosimilis* (99)
42A	PM pericardium (PE)	IFI	Prominent hyphae (+++)	+	*Trichosporon asahii* (100)
42B	PM kidney (PE)	IFI	Prominent hyphae (+++)	+	*T. asahii* (99)
43	Muscle (F)	IFI	Yeast-like organisms (+++)	+	*Trichosporon cutaneum* (99)
44	PM lung (PE)	IA	Septate branching hyphae (+++)	+	*A. fumigatus* (100)
45	PM lung (PE)	IA	Septate branching hyphae (+++)	+	*A. fumigatus* (100)
46	Lung (F)	IFI	Septate branching hyphae (+++)	+	*A. fumigatus* (100)
47	Lung (F)	IA	Septate branching hyphae (+++)	+	*A. fumigatus* (99)
48	Lung (PE)	IFI	Septate branching hyphae (+++)	+	*A. fumigatus* (99)
49[d]	Paranasal sinus (PE)	IFI	Fungal elements (+++)	+	*Neosartorya* spp./*Aspergillus lentulus* (100)
50	Brain (F)	IFI	Branching, septate hyphae (++)	+	*Neosartorya* spp./*A. lentulus* (99)
51	Knee biopsy (F)	IFI	Fungal elements (+)	+	*Phoma* spp. (97)
52A	PM large bowel (PE)	IFI	Broad, irregular aseptate hyphae (++)	+	*Apophysomyces elegans* (98)
52B	PM liver (PE)	IFI	Broad, irregular aseptate hyphae (++)	+	*Apophysomyces elegans* (98)
53	Nasal mucosa (PE)	Zygomycosis	Broad, irregular aseptate hyphae (++)	+	*Actinomucor elegans* (99)
54	PM lung (PE)	Zygomycosis	Broad, irregular aseptate hyphae (++)	+	*Rhizopus microsporus* (99)
55	Unspecified (PE)	Coccidioidomycosis	Unknown	−	−
56	Lung (PE)	Histoplasmosis	Intracellular yeast (+)	−	−
57	Skin (PE)	Penicilliosis marneffei	Intracellular fungal elements (+)	−	−
58	Nasal mucosa (PE)	IFI	Hyphae and spores (+)	−	−
59A	PM brain (PE)	IFI	Broad, irregular aseptate hyphae (+++)	−	−
59B	PM brain (PE)	IFI	Broad, irregular aseptate hyphae (+++)	−	−
60	Pterygopalatine fossa (PE)	IFI	Broad aseptate hyphae (++)	−	−
61	Lung (PE)	IFI	Moderately thick branching hyphae (++)	−	−
62A	Paranasal sinus (PE)	IFI	Broad, irregular aseptate hyphae (++)	−	−
62B	Paranasal sinus (PE)	IFI	Fungal elements (++)	−	−

[a] F, fresh tissue; PM, postmortem examination specimen; PE, paraffin embedded.
[b] IA, invasive aspergillosis.
[c] +++, numerous; ++, moderate; +, scant.
[d] Specimen from animal patient.

all 47 culture-positive specimens, DNA sequencing was unsuccessful in 3 cases. In one case, this was probably due to the presence of mixed fungal species, since multiple bands were present on the gel. In the other two, insufficient DNA may have been extracted from the paraffin sections, or nonspecific PCR products may have been generated.

Comparative sequence analysis confirmed the conventional culture-based identification for 39 of the 44 (88.6%) remaining specimens. Discordant results were observed for five specimens from three patients (Table 1). The cultures of three retrobulbar specimens from one patient yielded *A. fumigatus* by conventional identification, but subsequent DNA sequence analysis of the complete ITS region identified the culture as *N. pseudofischeri*. Although genetically distinct, the asexual state of *N. pseudofischeri* (and the closely related *Neosartorya fischeri*) is morphologically similar to *A. fumigatus*. Until recently, *Neosartorya* spp. have rarely been reported to have caused disease, although the difficulty in morphologically distinguishing *A. fumigatus* from *Neosartorya* spp. may have led to underestimation of the frequency of infection caused by the latter (3). Accurate identification is clinically important, since it has been reported that *Neosartorya* spp. are less susceptible in vitro to antifungal agents than *A. fumigatus* (3).

For the second patient, molecular analysis of the fungal pathogen from a skin biopsy specimen (PE) identified it as *Exophiala spinifera*, while the isolate was identified morphologically from culture of fresh tissue as *Exophiala jeanselmei*. Comparison of ITS1 sequences of multiple strains of *E. spinifera* and *E. jeanselmei* deposited in GenBank revealed that the two species can be distinguished easily. The isolate from patient 27 showed 98% sequence identity to six *E. spinifera* strains from the Centraalbureau voor Schimmelcultures. The variable morphological characteristics of *Exophiala* spp. make definitive identification difficult; as a result, molecular analyses are increasingly used for species confirmation (30).

PCR amplification and DNA sequencing twice identified the fungal pathogen from a nasal turbinate biopsy specimen (PE) from the third patient as *Rhizomucor pusillus* rather than *Absidia corymbifera*. Although these two organisms share antigenic similarities, they are morphologically distinct, and comparison of their ITS1 sequences reveals substantial differences (33). Given that the organism was isolated by another labora-

tory in 1992, it was not possible to check the details of the original identification, but it is likely to be a case of misidentification.

The panfungal PCR assay performed well on specimens where fungal elements were visualized but no pathogen was grown. A molecular identification could be assigned to 18 of 28 (64.3%) specimens, including all 5 fresh specimens, and the identification was consistent with the histological findings for all but 1. In this case, *C. parapsilosis* DNA was detected from a nasopharyngeal biopsy specimen, but histologically the pathogen was identified as *C. neoformans* complex. It is likely that *C. parapsilosis* was present as a commensal, rather than as the infecting agent, in the nasopharynx of this patient and was amplified by the PCR assay. This case and those reported by others (42) demonstrate that the application of a sensitive, broad-range nucleic acid test to specimens from nonsterile sites should always be interpreted in the appropriate clinical context. The PCR was negative for 10 specimens from eight patients with histologically proven IFIs (Table 2, patients 55 to 62). Since all of these specimens were PE, the quality of DNA is likely to have been compromised by the routine processes required for histological examination, in particular the duration of contact with formalin fixative prior to paraffin embedding (41, 42). Additionally, insufficient amounts of DNA may have been present for detection by PCR, since at least three of the specimens had scant fungal elements upon histological examination (Table 2).

DNA sequencing proved useful in two additional clinical contexts. First, tissue obtained at autopsy, where IFI is not clinically suspected antemortem, is frequently not submitted for culture. In our study, the fungal pathogen was identified by PCR and DNA sequencing in specimens obtained at postmortem examination from five of six patients (Table 2). Second, for a patient with follicular skin lesions for whom fungal infection was suspected, the diagnosis of *T. verrucosum* infection achieved in the absence of either culture or histology results was central to selecting appropriate antifungal therapy.

Studies have demonstrated that the most promising targets for molecular identification of fungi are the ITS1 and/or the ITS2 region, followed by the D1–D2 region of the large-subunit DNA gene (28S rDNA) (7, 17, 23, 31). For this reason, we targeted the ITS1 region. Nevertheless, our results indicate that there is insufficient sequence variation to differentiate between species of certain genera by using the ITS1 region alone (e.g., *C. neoformans* complex, *C. albidus* and *C. albidosimilis*, *Neosartorya* spp. and *A. lentulus*, and *Fusarium* spp.). These results were not unexpected, since it has been reported that ITS sequence variations among some species of fungi, including *Aspergillus* spp., are minimal (13, 17, 19, 36), and the differentiation of *Fusarium* species is complicated by the presence of >1 ITS sequence variant in a single strain (27). For those species that cannot be discriminated using the ITS1 region alone, future work may involve sequence analysis of additional genes, such as ITS2, the D1–D2 region of 28S rDNA, or intergenic spacer regions (19).

Despite the usefulness of the panfungal PCR assay for the identification of fungal pathogens in histologically positive but culture-negative tissue specimens, the limitations of the assay must also be considered. Environmental contamination of specimens and/or the PCR master mixture by ubiquitous fungal spores is a possible cause of "false-positive" results in a sensitive, broad-range PCR assay. This was not likely to have occurred in the present study, because strict precautions were taken throughout the whole procedure, including processing each specimen with its own negative control during DNA extraction and PCR amplification and performing all procedures using a unidirectional workflow pattern. The turnaround time for results is realistically a minimum of 48 h for fresh specimens and 4 to 5 days for PE sections. Two working days are required to remove the wax from PE tissue specimens, and one working day is required to extract the DNA and perform PCR amplification and product detection. Additionally, we rely on an external DNA sequencing facility, and its turnaround time is 24 to 48 h. Importantly, accurate sequence-based identification of fungal pathogens is dependent on the quality and accuracy of sequences in existing databases. GenBank sequence submissions are not peer reviewed, and it has been estimated that 10 to 20% of fungal sequences in GenBank are misidentified organisms (4; R. Summerbell, presented at the 16th Congress of the International Society for Human and Animal Mycology, 2006). Given that in our study, the molecular identification was consistent with the histological diagnosis for 17 of 18 specimens for which histology only was positive, the inaccuracy of some GenBank entries should not have affected our results. However, the development of an accurate sequence database for fungal species would be beneficial for this assay.

In conclusion, this study illustrates the potential benefits of using the panfungal PCR assay in combination with conventional laboratory tests for sensitive and specific identification of fungal pathogens in both fresh and PE tissue specimens. For rapid diagnosis of IFIs from fresh tissue, we recommend that the panfungal PCR be performed as soon as fungal elements are seen on microscopy. For PE specimens, we envisage the assay to be most valuable in cases where fungal hyphae are visualized and where either the culture result is negative or culture is not performed. Evaluation of the panfungal PCR assay on specimens from other sterile body sites, e.g., blood, is indicated.

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