Real-Time PCR Method for Detection of Zygomycetes[†]

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Zygomycete infections can be devastating in immunocompromised hosts. Difficulties in the histopathologic differentiation of this class from other filamentous fungi (e.g., *Aspergillus* spp., *Fusarium* spp.) may lead to delays in diagnosis and initiation of appropriate treatment, thereby significantly affecting patient outcome. A real-time PCR assay was developed to detect species of the zygomycete genera *Absidia*, *Apophysomyces*, *Cunninghamella*, *Mucor*, *Rhizopus*, and *Saksena* in culture and tissue samples. Primers and fluorescence resonance energy transfer hybridization probes were designed to detect a 167-bp conserved region of the multicopy zygomycete cytochrome *b* gene. A plasmid containing target sequence from *Mucor racemosus* was constructed as a positive control. The analytical sensitivity of the assay is 10 targets/μl, and a specificity panel consisting of other filamentous fungi, yeasts (*Candida* spp.), and bacteria demonstrated no cross-reactivity in the assay. The clinical sensitivity and specificity of the assay from culture isolates were 100% (39/39) and 92% (59/64), respectively. Sensitivity and specificity determined using a limited number of fresh tissue specimens were both 100% (2/2). The sensitivity seen with formalin-fixed, paraffin-embedded tissues was 56% (35/62), and the specificity was 100% (19/19). The speed, sensitivity, and specificity of the PCR assay indicate that it is useful for the rapid and accurate detection of zygomycetes.

Zygomycetes are common environmental fungi capable of producing serious disease in immunocompromised hosts. The vast majority of infections occur in individuals who are neutropenic, those receiving cytotoxic therapies, or those who have underlying metabolic acidosis. Although previously considered a relatively rare cause of disease, the incidence of opportunistic infections due to zygomycetes has increased over the last decade. Studies from multiple centers have indicated infection rates from 2.5% in bone marrow transplant recipients to 5.7% in solid-organ transplant recipients (10). Depending on the patient population, underlying disease, clinical presentation, and an early, accurate diagnosis, overall mortality rates due to zygomycete infections have ranged from 20% in localized infections to 100% in cases of disseminated disease (5, 13).

Members of the order *Mucorales* include species of the genera *Rhizopus*, *Absidia*, *Mucor*, *Rhizomucor*, *Apophysomyces*, *Cunninghamella*, and *Saksena*, and all have been implicated in human disease. Approximately 90% of all infections are caused by members of the genus *Rhizopus*, most commonly, *R. arrhizus*, followed by *R. microsporus* var. *rhizopodiformis*.

Currently, a diagnosis of zygomycosis is based upon identification of broad, ribbon-like, pauciseptate hyphae by histopathology or the use of macroscopic and microscopic morphology analysis following fungal culture. Histopathology determinations suffer from subjectivity that is dependent upon the experience of the reader. In addition, tissue processing, fixation, and staining may require several days, especially when the specimen is large and requires extended formalin fixation or

when fungal stains are not initially ordered. When culture is used, the distinctive hyphal elements of zygomycetes may be difficult to distinguish visually from those of other filamentous fungi, especially during the early growth period. Although the zygomycetes grow quite rapidly on solid media, sporulation and identification may still take several days. In addition, when a zygomycetous infection is not specifically suspected at the time of specimen submission, tissues may undergo extensive processing (e.g., grinding) prior to culture, which ruptures the pauciseptate hyphae. It is therefore not unusual to have negative fungal culture results when histopathology results suggest the presence of zygomycete infection.

Molecular detection of zygomycetes has been infrequently reported in the literature. Imhof et al. developed a real-time PCR assay that detected, among other fungi, a single *Conidiobolus* isolate in culture (11). Recently, Schwarz et al. reported the molecular identification of zygomycetes in culture and experimentally infected mouse tissue by use of PCR followed by sequencing of the target amplicon (19).

We have developed a real-time PCR assay for the detection of zygomycetes in culture and tissue specimens (fresh and fixed). The assay targets a region of the multicopy cytochrome *b* gene that is found in *Absidia*, *Apophysomyces*, *Cunninghamella*, *Mucor*, *Rhizopus*, and *Saksena* spp. Genus-level identification of members of these six zygomycete genera can be achieved in approximately 4 h, providing essential and timely information to guide appropriate therapy. The specificity of PCR assay is such that the presence of other filamentous fungi (e.g., *Aspergillus* spp., *Fusarium* spp.), yeasts (*Candida* spp.), and bacteria may be excluded. The closed-system format of real-time PCR reduces the potential for contamination from environmental zygomycetes.

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TABLE 1. Zygomycete culture isolates evaluated with LightCycler PCR ($n = 44$)

Organism	Accession no. or source	No. of isolates
<i>Absidia coerulea</i>	DSMZ 3018	1
<i>Absidia corymbifera</i>	DSMZ 1144	1
<i>Absidia glauca</i>	DSMZ 63295	1
<i>Absidia repens</i>	ATCC 14849	1
<i>Absidia repens</i>	DSMZ 812	1
<i>Absidia</i> sp.	Clinical isolate	1
<i>Apophysomyces elegans</i>	ATCC 64704	1
<i>Apophysomyces elegans</i>	ATCC 64705	1
<i>Apophysomyces elegans</i>	ATCC 46557	1
<i>Apophysomyces</i> sp.	Clinical isolate	1
<i>Conidiobolus coronatus</i>	ATCC 32801	1
<i>Cunninghamella bertholletiae</i>	ATCC 42115	1
<i>Cunninghamella blakesleeana</i>	DSMZ 1906	1
<i>Cunninghamella echinulata</i>	DSMZ 1905	1
<i>Cunninghamella elegans</i>	DSMZ 1908	1
<i>Cunninghamella</i> sp.	Clinical isolate	1
<i>Mucor circinelloides</i>	DSMZ 2183	1
<i>Mucor flavus</i>	DSMZ 2184	1
<i>Mucor hiemalis</i>	DSMZ 2656	1
<i>Mucor mucedo</i>	DSMZ 809	1
<i>Mucor racemosus</i>	DSMZ 62760	1
<i>Mucor</i> sp.	Clinical isolate	6
<i>Rhizomucor miehei</i>	ATCC 22064	1
<i>Rhizomucor miehei</i>	DSMZ 1330	1
<i>Rhizomucor pusillus</i>	DSMZ 1331	1
<i>Rhizopus microsporus</i> var. <i>chinensis</i>	DSMZ 1834	1
<i>Rhizopus microsporus</i> var. <i>chinensis</i>	DSMZ 2195	1
<i>Rhizopus microsporus</i> var. <i>oligosporus</i>	DSMZ 1964	1
<i>Rhizopus oryzae</i>	DSMZ 5772	1
<i>Rhizopus oryzae</i>	DSMZ 853	1
<i>Rhizopus oryzae</i>	DSMZ 905	1
<i>Rhizopus</i> sp.	Clinical isolate	3
<i>Rhizopus stolonifer</i>	ATCC 12938	1
<i>Rhizopus stolonifer</i>	DSMZ 2194	1
<i>Rhizopus stolonifer</i>	DSMZ 63011	1
<i>Saksenaia vasiformis</i>	ATCC 44101	1
<i>Syncephalastrum racemosum</i>	DSMZ 859	1

MATERIALS AND METHODS

Culture isolates. Thirty-two isolates of zygomycetes belonging to the genera *Absidia*, *Apophysomyces*, *Conidiobolus*, *Cunninghamella*, *Mucor*, *Rhizopus*, *Rhizomucor*, *Saksenaia*, and *Syncephalastrum* were obtained from the American Type Culture Collection (ATCC) or from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ). An additional 12 zygomycete isolates were obtained from clinical specimens submitted to the Mayo Clinic Mycology Laboratory during the period 2003 to 2004. All zygomycete stock isolates were cultured on inhibitory mold agar plates and incubated at 30°C until hyphal growth was observed. The identity of stock isolates was confirmed on the basis of microscopic morphology (Table 1). Fungal cultures were lysed using PrepMan Ultra reagent (Applied Biosystems, Foster City, CA) following the method of Hall et al. (7). DNA was extracted using a MagNA Pure platform (Roche Applied Sciences, Indianapolis, IN), a total nucleic acid isolation kit, and a final elution volume of 100 μ l.

Formalin-fixed tissue. A review of archived pathology specimens from the Mayo Clinic in Rochester collected from 1995 to 2004 revealed nine cases with diagnoses consistent with zygomycete infection, as determined by pathologist interpretations of fungal morphology and staining characteristics. From the nine cases with positive results, a total of 81 formalin-fixed, paraffin-embedded tissue blocks were available for evaluation. All slides from the cases were subsequently reviewed by an independent pathologist (B.S.P.) to confirm the initial histopathologic diagnosis.

One 50- μ m-thick section from each block was obtained using a microtome and placed into a 1.5-ml screw-top tube. Tissue lysates were prepared by washing the tissue sections with xylene to remove wax followed by washing with 95% alcohol. The tissues were then placed in a mixture of 500 μ l of Tris-EDTA buffer, 50 μ l

of 10% sodium dodecyl sulfate, and 100 μ l of proteinase K. The tissues were incubated for 24 h at 55°C by use of a Thermomixer (Eppendorf AG, Hamburg Germany) at 500 rpm. Fresh tissue specimens were also obtained prospectively from specimens submitted to the Mayo Clinic Surgical Pathology service. They were placed directly in the Tris-EDTA-sodium dodecyl sulfate-proteinase K mixture without the xylene-alcohol step.

Primer and probe design. The cytochrome *b* gene is a highly conserved, multicopy gene which has exhibited utility in the classification, identification, and phylogenetic analysis of yeasts and filamentous fungi (23, 25). The National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov>) was searched using the keywords "zygomycete" and "cytochrome *b*" to identify available cytochrome *b* sequences in the zygomycete class. Data for fluorescence resonance energy transfer (FRET) probe construction were also supplemented by sequence analysis of PCR products from culture-confirmed zygomycete isolates from the Mayo Clinic. The sequences were further examined using OligoPrimer analysis v. 6.61 software (Molecular Biology Insights, Inc.), BioEdit sequence alignment editor v. 7.0 software (Isis Pharmaceuticals, Inc.), and Sequencher v. 4.0.5 software (Gene Codes, Inc., Ann Arbor, MI) to identify suitable regions for primer and hybridization probes based on sequence homologies among six genera (*Rhizopus*, *Absidia*, *Mucor*, *Apophysomyces*, *Cunninghamella*, and *Saksenaia*). Forward (5'-TAGGAATTACAGCAAAT-3') and reverse (5'-CCAATGCAAACTCC-3') oligonucleotide primers were designed to amplify a specific 167-bp region of the cytochrome *b* gene. FRET hybridization probes were composed of the following sequences: 5'-ACAATTTTCTTATCTTCTTAGTATTAG-3' (anchor probe) (3' fluorescein labeled) and 5'-TTTATCTTATTCATATGCTCCAAATA-3' (donor probe) (5' LCRED-640 labeled). Primers and probes were synthesized by TIB Molbiol (Berlin, Germany).

Positive-control plasmid. Using the primers described above, a plasmid containing a 167-bp region of the *Mucor racemosus* (accession no. DSMZ 62760) cytochrome *b* consensus sequence was constructed using a pCR 2.1 TOPO TA cloning kit (Invitrogen Corp., Carlsbad, CA). *Mucor racemosus* was selected for the positive control because it had the most complete sequence data available via GenBank and supplemental sequence analysis. Briefly, nucleic acid from *M. racemosus* was extracted as described by Hall et al. by use of a Roche MagNA Pure kit and a total nucleic acid isolation kit (Roche Applied Sciences, Indianapolis, IN) (6). Purified nucleic acid was subjected to PCR using the primers described above and targeting a 167-bp region of the cytochrome *b* gene. The PCR product was then ligated into the PCR 2.1 vector by use of a TOPO TA cloning kit, following the instructions of the manufacturer. The expected size of the recombinant plasmid was confirmed with 2% agarose gel electrophoresis, and the reaction mixture was purified using a Roche High Pure plasmid isolation kit (Roche Applied Science, Indianapolis, IN). The positive-control plasmid was diluted into Tris-EDTA buffer (pH 8.0) and stored at 4°C.

LightCycler PCR. Real-time PCR amplification and amplicon detection using extracted DNA template was performed with using a Roche LightCycler system. Each reaction mixture contained 15 μ l of the PCR master mix plus 5 μ l of the extracted DNA from clinical specimens or control material. The PCR mixture consisted of 1 $\times$ LightCycler FastStart DNA master hybridization probe buffer, 3 mM MgCl₂, 0.5 μ M forward and reverse primers, 0.2 μ M fluorescein-labeled probe, and 0.4 μ M RED 640-labeled probe. The total volume per reaction mixture was 20 μ l (15 μ l of master mix plus 5 μ l of sample). Amplification conditions consisted of 1 cycle of 95°C for 10 min followed by 45 cycles of 95°C for 10 s (denaturation), 48°C for 15 s (annealing), and 72°C for 15 s (extension). A melting curve was generated using the following profile: 95°C for 0 s, 59°C for 20 s, and 34°C for 20 s, with a 0.3°C/sec transition, and 85°C for 0 sec, with a 0.3°C/s transition. Analysis was performed using a LightCycler v. 1.2 system with LightCycler v. 3.5 software.

Analytical sensitivity and specificity. Dilutions of the plasmid control were prepared to assess the analytical sensitivity of the PCR assay. A range of 5 to 500,000 copies of target DNA per reaction were tested in triplicate. The sensitivity of the PCR assay from culture was tested using DNA extracted as described above and 32 zygomycete stock isolates and 12 confirmed clinical zygomycete isolates (Table 1). The analytical specificity of the assay was determined by performing a BLAST search of the primer and probe sequences on the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov>). Further, a specificity panel containing DNA extracted from clinical isolates of yeast, filamentous fungi, and bacteria was tested for potentially cross-reacting organisms (Table 2). Amplification and sequencing of 16S (bacteria) and D2 large ribosomal subunit (fungi) or specific PCR assays were utilized to confirm the presence of amplifiable nucleic acid in the specificity panel and organism identification (6, 7).

Accuracy. Sections from formalin-fixed, paraffin-embedded tissue blocks that were either positive or negative for zygomycetes by histopathology were also

TABLE 2. Specificity panel of isolates tested for cross-reactivity using a Zygomycete LightCycler PCR assay

Isolate	Isolate
Fungi and algae	<i>Klebsiella pneumoniae</i>
<i>Alternaria</i> sp.	<i>Legionella jordanis</i>
<i>Aspergillus flavus</i>	<i>Legionella micdadei</i>
<i>Aspergillus fumigatus</i>	<i>Legionella pneumophila</i>
<i>Aspergillus glaucus</i>	<i>Listeria monocytogenes</i>
<i>Aspergillus nidulans</i>	<i>Moraxella catarrhalis</i>
<i>Aspergillus niger</i>	<i>Morganella morganii</i>
<i>Aspergillus terreus</i>	<i>Mycoplasma pneumoniae</i>
<i>Aspergillus versicolor</i>	<i>Neisseria gonorrhoeae</i>
<i>Beauveria</i> sp.	<i>Neisseria meningitidis</i>
<i>Bipolaris</i> sp.	<i>Proteus mirabilis</i>
<i>Blastomyces dermatitidis</i>	<i>Proteus vulgaris</i>
<i>Blastoschizomyces capitatus</i>	<i>Pseudomonas aeruginosa</i>
<i>Candida albicans</i>	<i>Pseudomonas cepacia</i>
<i>Candida dublinensis</i>	<i>Pseudomonas fluorescens</i>
<i>Candida glabrata</i>	<i>Rothia mucilaginosa</i>
<i>Candida guilliermondii</i>	<i>Staphylococcus aureus</i>
<i>Candida krusei</i>	<i>Staphylococcus capitis</i> subsp. <i>capitis</i>
<i>Candida kefyr</i>	<i>Staphylococcus caprae</i>
<i>Candida lambica</i>	<i>Staphylococcus cohnii</i> subsp. <i>cohnii</i>
<i>Candida lipolytica</i>	<i>Staphylococcus epidermidis</i>
<i>Candida lusitanae</i>	<i>Staphylococcus haemolyticus</i>
<i>Candida parapsilosis</i>	<i>Staphylococcus hominis</i> subsp. <i>hominis</i>
<i>Candida rugosa</i>	<i>Staphylococcus lentus</i>
<i>Candida tropicalis</i>	<i>Staphylococcus lugdunensis</i>
<i>Candida zeylanoides</i>	<i>Staphylococcus saccharolyticus</i>
<i>Cladophialophora bantiana</i>	<i>Staphylococcus saprophyticus</i>
<i>Coccidioides immitis</i>	<i>Staphylococcus sciuri</i> subsp. <i>sciuri</i>
<i>Cokeromyces recurvatus</i>	<i>Staphylococcus simulans</i>
<i>Cryptococcus albidus</i>	<i>Staphylococcus warneri</i>
<i>Cryptococcus curvatus</i>	<i>Staphylococcus xylosus</i>
<i>Cryptococcus laurentii</i>	<i>Stenotrophomonas maltophilia</i>
<i>Cryptococcus neoformans</i>	<i>Streptococcus pneumoniae</i>
<i>Curvularia</i> sp.	<i>Streptococcus pyogenes</i>
<i>Epicoccum</i> sp.	Mycobacteria and actinomycetes
<i>Epidermophyton floccosum</i>	<i>Mycobacterium abscessus</i>
<i>Exophiala dermatitidis</i>	<i>Mycobacterium africanum</i>
<i>Exophiala jeanselmei</i>	<i>Mycobacterium avium</i>
<i>Fusarium</i> sp.	<i>Mycobacterium bovis</i>
<i>Histoplasma capsulatum</i>	<i>Mycobacterium celatum</i>
<i>Kloderia</i> sp.	<i>Mycobacterium chelonae</i>
<i>Malassezia pachydermatis</i>	<i>Mycobacterium fortuitum</i>
<i>Microsporum canis</i>	<i>Mycobacterium goodii</i>
<i>Microsporum nanum</i>	<i>Mycobacterium gordonae</i>
<i>Nigrospora</i> sp.	<i>Mycobacterium haemophilum</i>
<i>Paecilomyces lilacinus</i>	<i>Mycobacterium intracellulare</i>
<i>Penicillium marnettii</i>	<i>Mycobacterium kansasii</i>
<i>Penicillium</i> sp.	<i>Mycobacterium lentiflavum</i>
<i>Phoma</i> sp.	<i>Mycobacterium leprae</i>
<i>Prototheca wickerhamii</i>	<i>Mycobacterium marinum</i>
<i>Rhinocladia</i> sp.	<i>Mycobacterium paratuberculosis</i>
<i>Saccharomyces cerevisiae</i>	<i>Mycobacterium scrofulaceum</i>
<i>Scedosporium apiospermum</i>	<i>Mycobacterium smegmatis</i>
<i>Scedosporium prolificans</i>	<i>Mycobacterium szulgai</i>
<i>Scopulariopsis</i> sp.	<i>Mycobacterium terrae</i>
<i>Sporothrix schenckii</i>	<i>Mycobacterium tuberculosis</i>
<i>Stachybotrys</i> sp.	<i>Mycobacterium ulcerans</i>
<i>Trichophyton mentagrophytes</i>	<i>Mycobacterium xenopi</i>
	<i>Actinomyces madurae</i>
<i>Trichophyton rubrum</i>	<i>Gordonia sputi</i>
Bacteria	<i>Nocardia brasiliensis</i>
<i>Acinetobacter lwoffii</i>	<i>Nocardia brevicatena</i>
<i>Aeromonas hydrophila</i>	<i>Nocardia cavia</i>
<i>Bordetella bronchiseptica</i>	<i>Nocardia farcinica</i>
<i>Bordetella holmesii</i>	<i>Nocardia nova</i>
<i>Bordetella parapertussis</i>	<i>Nocardia oitidiscaviarum</i>
<i>Bordetella pertussis</i>	<i>Nocardia transvalensis</i>
<i>Campylobacter jejuni</i>	<i>Rhodococcus equi</i>
<i>Citrobacter freundii</i>	<i>Rhodococcus rhodococcus</i>
<i>Corynebacterium haemolyticum</i>	<i>Streptomyces griseus</i>
<i>Corynebacterium diptheriae</i>	<i>Tsukamurella inehonensis</i>
<i>Corynebacterium pseudodiptheriae</i>	<i>Tsukamurella pulmonis</i>
<i>Escherichia coli</i>	
<i>Haemophilus influenzae</i>	Human DNA
<i>Klebsiella oxytoca</i>	

were evaluated using the PCR method. A specimen was considered to be histopathology positive when the pathology report and independent pathology review indicated the presence of fungal elements consistent with the presence of a zygomycete.

Inhibition studies. Extraction inhibition and PCR inhibition were assessed by spiking tissue lysates not containing zygomycetes with known concentrations of the plasmid control prior to and after DNA extraction. Plasmid concentrations used were within 1 log of the limit of sensitivity of the assay.

RESULTS

Analytical sensitivity and specificity. The analytical sensitivity was determined to be 10 targets/ μ l (50 targets/reaction) by use of the plasmid control. Melt curve analysis demonstrated an average melting temperature of $49.3^{\circ}\text{C} \pm 0.16^{\circ}\text{C}$ (mean $\pm$ standard deviation) for the plasmid control (Fig. 1). The assay did not demonstrate cross-reactivity with any of the organisms included in the specificity panel (Table 2).

Clinical sensitivity and specificity. A total of 44 zygomycete culture isolates were tested using a LightCycler PCR assay (Table 1 and Table 3). Thirty-nine of 44 culture isolates were detected in the assay. All specimens belonging to the genera *Rhizopus*, *Mucor*, *Absidia*, *Cunninghamella*, *Apophysomyces*, and *Saksenaia* were detected with the PCR assay. As expected, isolates of *Rhizomucor* ($n = 3$), *Condiobolus* ($n = 1$), and *Syncephalastrum* ($n = 1$) spp. were not detected by the assay since the selected probes were not specific for these organisms. The six genera exhibited a range of melt temperatures from 41°C to 55°C , with three genera (*Absidia*, *Apophysomyces*, and *Mucor*) having melt temperatures within approximately 1°C of each other (Fig. 1). DNA isolated from fifty-nine cultures growing nonzygomycetous fungi (Table 2) was also tested. All results were negative using the PCR assay, corresponding to an assay specificity of 100%.

Eighty-one formalin-fixed, paraffin-embedded tissue samples from nine patients were tested with the LightCycler PCR assay. Thirty-five tissue samples were positive by both LightCycler PCR and histopathology, while 19 specimens were negative by both techniques. Twenty-seven specimens were positive by histopathology but negative by the PCR assay, resulting in a sensitivity of 56.5% (Table 3).

Two fresh sinus cavity tissue samples from a patient with positive histopathology results were available for analysis. DNA isolated from two of two fresh tissue samples were positive with the PCR assay, and fungal culture samples from the samples were positive for *Apophysomyces elegans*.

Inhibition study. To assess potential extraction inhibition and PCR inhibition by use of formalin-fixed, paraffin-embedded tissues, digested samples from tissue not containing zygomycetes were spiked in triplicate with known concentrations of the plasmid control prior to automated nucleic acid extraction. No inhibition was observed in the spiked tissue specimens, despite a drop in fluorescence intensity of the melting peaks. In a separate PCR inhibition experiment, when a positive plasmid control was added to negative tissue extracts after MagNA Pure extraction, there was again no inhibition observed.

DISCUSSION

It has been estimated that the incidence of zygomycete infections is relatively low, but they nevertheless constitute a significant area of concern, especially in immunosuppressed patient populations. Although overall survival rates in cases of zygomycosis have increased from 50% to 80% over the last 10 years (5), the overall incidence rate has shown a disturbing

FIG. 1. Melt curves for the zygomycete genera detected by the LightCycler PCR assay. Average melting-temperature values were as follows: for *Absidia* spp., 49.9°C; for *Apophysomyces* spp., 51.0°C; for *Cunninghamella* spp., 44.3°C; for *Mucor* spp., 49.9°C; for *Rhizopus* spp., 55.0°C; and for *Saksenaia vasiformis*, 41.9°C.

upward trend (13). Delays in accurate identification of zygomycete infections negatively affect patient survival. In a retrospective series from a single institution, premortem diagnosis of zygomycosis was performed with only 22% of cases prior to 1970 and with 93% of cases after 1970, and survival rates were 20% and 73%, respectively (13). Inappropriate antifungal therapy, secondary to incomplete identification or misidentification of zygomycete infections, has clearly led to a reduction in survival rates (5, 24). Recent reports of a zygomycete “break-through” in treatment of patients on long-term prophylaxis with voriconazole presents an additional need for the accurate and rapid identification of these organisms (16, 21, 22).

Diagnosis of zygomycosis may be made on the basis of morphological identification of characteristic hyphae present in tissue or other clinical specimens (5). However, extensive necrosis, hyphal fragmentation, and poor staining of the thin hyphal walls may make microscopic visualization difficult. Finally, a pathologist may mistakenly conclude that the presence of septations excludes the diagnosis of zygomycosis, since it

was previously thought that zygomycete hyphae are aseptate instead of pauciseptate (4).

Fungal culture is necessary for genus identification, because histopathologic diagnosis can be difficult. However, the recovery of zygomycetes in culture can be a difficult proposition. Blood and urine culture results are rarely positive in the diagnosis of zygomycosis (5). Tissue samples may undergo aggressive initial processing, thereby making hyphal elements nonviable. In one study, 33% of histologically proven zygomycete infections failed to grow in culture (13). In addition, fungal hyphae may be present in only a portion of the specimen, again resulting in poor recovery for culture (18). Although zygomycetes are commonly found in the environment, recovery from cultures from sterile sites should never be dismissed as laboratory contamination but should be considered to represent a medical emergency in cases involving immunocompromised patients (5). However, their presence in nonsterile sites requires accurate clinical correlation.

Molecular methods are potentially helpful for the detection and identification of zygomycetes. Methods such as in situ hybridization (8), conventional PCR (1, 3, 12, 14, 15, 17) and nucleic acid sequencing (9, 19) have been reported, but all suffer from a relatively long turnaround time compared to the time required for the clinical progression of a zygomycete infection. The application of real-time PCR methodologies is especially attractive as a choice for clinical diagnostics due to increases in detection sensitivity, reduction of outside contamination, and decreased test turnaround time.

We have developed a real-time PCR assay using the LightCycler platform that is highly specific for species of six clinically significant zygomycetes genera (*Rhizopus*, *Mucor*, *Absidia*,

TABLE 3. Detection of zygomycetes in culture isolates and in fixed tissue

LightCycler PCR result	No. of culture isolates with indicated result		No. of isolates with indicated result by histopathology of fixed tissue	
	Positive	Negative	Positive	Negative
Positive	39	0	35	0
Negative	5 ^a	59	27	19

^a Cultures grew species of *Rhizomucor* ($n = 3$), *Syncephalastrum* ($n = 1$), and *Conidiobolus* ($n = 1$), which are genera not detected by this PCR assay.

Apophysomyces, *Cunninghamella*, and *Saksenaea*). These species comprise the majority of isolates noted in our clinical practice. The PCR is highly sensitive and detects as few as 10 copies of the zygomycete cytochrome *b* gene in samples from culture isolates. The overlapping melt curves obtained with *Absidia*, *Apophysomyces*, and *Mucor* spp. prevent identification to the genus level using the assay; however, initiation of appropriate therapy does not depend on specific identification to this level. Although it is highly conserved compared with other zygomycete targets considered, there was enough variation with the cytochrome *b* target region that the construction of a single set of FRET hybridization probes that detected all genera of zygomycetes is impractical. Therefore, *Rhizomucor*, *Conidiobolus*, and *Syncephalastrum* spp. are not detected with this PCR assay. Although these species are not commonly seen in our clinical practice, future design of additional FRET probes may allow for detection of these genera.

The performance of the PCR assay using samples from culture and a limited number of organisms in fresh tissue was excellent, with 100% sensitivity and no cross-reactivity demonstrated with the tested species of other filamentous fungi, yeasts, or bacteria. Sensitivity of the assay using samples from formalin-fixed, paraffin-embedded tissue was lower at 57%. Several factors may contribute to reduced sensitivity in fixed tissue. It is possible that subjectivity associated with the histopathology interpretation may have contributed to decreased sensitivity. More likely, extensive cross-linking of tissue proteins after fixation in formaldehyde may have resulted in nucleic acid fragmentation and inhibition of the PCR process (2). Although our intentional use of a short (167-bp) amplicon as the target somewhat mitigated this effect, DNA fragmentation may still have contributed to lower sensitivity with fixed tissue. Interestingly, for a single patient with invasive zygomycosis due to *A. elegans*, fixed frontal sinus tissue was PCR assay negative, whereas fresh tissue was PCR positive. Finally, the success of recovery of organism DNA might vary due to sampling issues associated with the use of discrete tissue sections from each tissue block rather than use of the entire specimen, as can often be done with fresh specimens.

The greatest advantage for the use of real-time PCR in the diagnosis of zygomycete infection is the reduction of turnaround time for results from days to hours, a difference that can have a significant positive impact on patient care. The growth of zygomycetes in culture is often quite brisk, causing many mycologists to refer to these organisms as "lid-lifters" in reference to their ability to rapidly escape the confines of an agar plate within a few hours. While identification of zygomycetes after growth in culture by use of microscopic morphology can be relatively simple at times, there are instances in which the identification is unclear due to poor sporulation or an inexperienced reader. The PCR assay described herein can be utilized to provide an objective result either as a stand-alone test or as a complement to microscopy. In instances in which no culture assay is performed, the ability to perform a rapid identification using samples of fixed tissue is vital to patient management. Tissue fixation, processing, and histopathology examination of biopsy specimens may take several days. The combination of automated DNA extraction with real-time PCR significantly reduces turnaround time to 2 to 4 h. In addition, the lack of cross-reactivity demonstrated by the PCR

assay may make it extremely useful in excluding infections due to fungi other than zygomycetes, thereby reducing the risk of inappropriate empirical therapy. Incorporation of this assay into the normal laboratory workflow is quite practical, as exemplified by other molecular tests performed in our laboratory (20). Further studies are required to determine the utility of this test in the detection of zygomycetes in samples from other specimen types.

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