

Molecular Detection and Species-Specific Identification of Medically Important *Aspergillus* Species by Real-Time PCR in Experimental Invasive Pulmonary Aspergillosis[∇]

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Diagnosis of invasive pulmonary aspergillosis (IPA) remains a major challenge to clinical microbiology laboratories. We developed rapid and sensitive quantitative PCR (qPCR) assays for genus- and species-specific identification of *Aspergillus* infections by use of TaqMan technology. In order to validate these assays and understand their potential diagnostic utility, we then performed a blinded study of bronchoalveolar lavage (BAL) fluid specimens from well-characterized models of IPA with the four medically important species. A set of real-time qPCR primers and probes was developed by utilizing unique ITS1 regions for genus- and species-specific detection of the four most common medically important *Aspergillus* species (*Aspergillus fumigatus*, *A. flavus*, *A. niger*, and *A. terreus*). Pan-*Aspergillus* and species-specific qPCRs with BAL fluid were more sensitive than culture for detection of IPA caused by *A. fumigatus* in untreated ($P < 0.0007$) and treated ($P \leq 0.008$) animals, respectively. For infections caused by *A. terreus* and *A. niger*, culture and PCR amplification from BAL fluid yielded similar sensitivities for untreated and treated animals. Pan-*Aspergillus* PCR was more sensitive than culture for detection of *A. flavus* in treated animals ($P = 0.002$). BAL fluid pan-*Aspergillus* and species-specific PCRs were comparable in sensitivity to BAL fluid galactomannan (GM) assay. The copy numbers from the qPCR assays correlated with quantitative cultures to determine the pulmonary residual fungal burdens in lung tissue. Pan-*Aspergillus* and species-specific qPCR assays may improve the rapid and accurate identification of IPA in immunocompromised patients.

Invasive pulmonary aspergillosis (IPA) is a leading cause of infectious pneumonic mortality in patients with hematological malignancies, aplastic anemia, myelodysplasia, and hematopoietic stem cell transplantation (HSCT) (14). *Aspergillus fumigatus* is the most common cause of invasive pulmonary aspergillosis in immunocompromised patients. Non-*Aspergillus fumigatus* species may also cause invasive disease (27). *Aspergillus terreus* is resistant to amphotericin B and causes pulmonary and disseminated infection (6, 15, 24, 28). *Aspergillus flavus* has been found to cause combined sinopulmonary disease and has been implicated in causing outbreaks of nosocomial aspergillosis. *Aspergillus niger* is usually a saprophytic organism but may cause invasive disease. Early diagnosis and initiation of antifungal therapy are important for improved outcomes.

The diagnosis of IPA remains challenging. Standard laboratory techniques for the diagnosis of IPA include direct examination, lung tissue histology, and culture of respiratory secretions. Serum galactomannan (GM) and (1→3)-β-D-glucan assays are FDA-approved assays for the diagnosis of invasive pulmonary aspergillosis. The measurement of galactomannan in bronchoalveolar lavage (BAL) fluid shows promise in enhancing the diagnosis of IPA (2, 4, 12, 18).

Under the best of circumstances, BAL fluid analysis yields a diagnosis by culture and direct exam in only approximately 50% of cases (13). Some centers report substantially lower frequencies. Saito et al. reported 0% diagnosis for patients who ultimately succumbed to IPA and whose infection was found postmortem (22).

We therefore developed rapid and sensitive real-time PCR assays for genus- and species-specific identification of *Aspergillus* infections by use of TaqMan technology. In order to validate these assays and understand their potential diagnostic utility, we then performed a controlled blinded study of BAL fluid and lung tissue biopsy specimens from well-characterized persistently neutropenic rabbit models of IPA with the four medically important species (15, 28). These studies establish

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TABLE 1. Primers and probes used in *Aspergillus* real-time PCR assays

Assay	Primer or probe	Primer sequence (5' → 3') ^a	GenBank accession no.	Nucleotide positions
Pan- <i>Aspergillus</i>	pan-Asp-For	GTGGAGTGATTTGTCTGCTTAATTG	AF548061 ^d	1215–1239
	pan-Asp-Probe	CGGCCCTAAATAGCCCGGTCCG ^b	AF548061 ^d	1258–1280
	pan-Asp-Rev	TCTAAGGGCATCACAGACCTGTT	AF548061 ^d	1345–1367
<i>A. fumigatus</i>	A. fum-For	GCCCGCCGTTTCGAC	GU319985	86–100
	A. fum-Probe	CCCGCCGAAGACCCCAACATG ^b	GU319985	136–156
	A. fum-Rev	CCGTGTTGAAAAGTTTTAACTGATTAC	GU319985	195–221
<i>A. flavus</i>	A. flav-For	CGAGTGTAGGGTTCCTAGCGA	HQ026744	59–79
	A. flav-Probe	TCCCACCCGTGTTTACTGTACCTTAGTTGCT ^b	HQ026744	88–118
	A. flav-Rev	CCGGCGGCCATGAAT	HQ026744	133–147
<i>A. niger</i>	A. nig-For	GCCGGAGACCCCAACAC	GU256739	95–111
	A. nig-Probe	AATCAACTCAGACTGCACGCTTTCAGACAG ^b	GU256739	117–146
	A. nig-Rev	TGTTGAAAAGTTTTAACTGATTGCATT	GU256739	148–173
<i>A. terreus</i>	A. terr-For	CATTACCGAGTGCGGGTCTTTA	GU256759	12–33
	A. terr-Probe	CCCAACCTCCCACCCGTGACTATTG ^b	GU256759	37–61
	A. terr-Rev	CCCGCCGAAGCAACAAG	GU256759	65–81
Lambda universal internal control	λ-UIC-For	CAGCAGAACACCCCCATC	U57606	1162–1179
	λ-UIC-Probe	AACCACTACCTGAGCACCCAGTCC ^c	U57606	1207–1230
	λ-UIC-Rev	GTGATCGCGCTTCTCGTT	U57606	1249–1266

^a Primer concentrations were optimized empirically. *Aspergillus* target and λ-UIC primers had final reaction concentrations of 800 and 200 nM, respectively.
^b All *Aspergillus* probes possessed a 5' 6-carboxyfluorescein (FAM) reporter dye and a 3' dark quencher.
^c The λ-UIC probe possessed a 5' VIC reporter dye and a 3' tetramethyl rhodamine isocyanate (TAMRA) quencher.
^d The pan-*Aspergillus* oligonucleotides were designed to hybridize to the 18S rDNA region of all *Aspergillus* species. The sequence accession number for the pan-*Aspergillus* assay is based on the *A. fumigatus* sequence (GenBank accession no. AF548061).

the experimental foundation for clinical investigation of this real-time PCR system in lung transplant recipients and for the clinical application of the system in the reference laboratory (16).

MATERIALS AND METHODS

Primer and probe design. For the design of species-specific assays, alignments of 27 to 67 ITS1 sequences for each *Aspergillus* species (*A. fumigatus*, *A. flavus*, *A. niger*, and *A. terreus*) were created using Geneious software (Biomatters Ltd., Auckland, New Zealand). The complete set of sequences available for each of the four species was used (National Center for Biotechnology Information [NCBI] public genetic database [GenBank]). Primers and probes that annealed to sequences of the target *Aspergillus* species but not to other *Aspergillus* spp., *Penicillium* spp., or other unrelated fungal genera were initially chosen. However, recent additions to GenBank revealed that the assay target region for *A. niger* has significant homology to five additional members of the section Nigri (*Aspergillus costaricensis*, *Aspergillus foetidus*, *Aspergillus piperis*, *Aspergillus vandenensis*, and *Aspergillus tubingensis* [one sequence]).

In order to detect the full spectrum of potential *Aspergillus* pathogens, a pan-*Aspergillus* real-time PCR assay was developed. For design of the pan-*Aspergillus* real-time PCR assay, BLAST (Basic Local Alignment Search Tool) searches for homology with the 18S rRNA genes of non-*Aspergillus* species of fungi were conducted. For the pan-*Aspergillus* primers, no significant homologies with the target area were identified with any fungal species other than *Penicillium* spp. BLAST searches for homology with the human 18S rRNA gene were also conducted. No significant homologies for the target area were identified within the human 18S rRNA gene. Primer Express 3.0 (Applied Biosystems, Foster City, CA) was used to help select primers and probes with optimal melting temperatures for TaqMan real-time PCR on a model 7500 Fast instrument (Applied Biosystems, Foster City, CA). The primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA), and the probes were synthesized by Biosearch Technologies, Inc. (Novato, CA). The sequences of the primers and probes are shown in Table 1, and the locations of real-time PCR assay targets are shown in Fig. 1.

Organisms. Isolates of *Aspergillus* spp. and other fungi were obtained for use in *in vitro* development of the real-time PCR assays (Tables 2 and 3). A different

panel of *Aspergillus* sp. isolates was studied in experimental IPA in order to validate the *in vitro* analytical PCR assay. Isolates of *Aspergillus fumigatus* (NIH 4215; ATCC MYA-1163), *A. flavus* (NIH 8B and 10B), *A. terreus* (NIH 68A and 2624; TX 94-559, 96-1290, and 98-87), and *A. niger* (NIH 120 and 141) were obtained from patients with invasive pulmonary aspergillosis and used in all experiments for *in vitro* calibration studies and experimental IPA.

Growth and harvest of fungal stock cultures. Conidia were isolated from *Aspergillus* spp. and *Penicillium* spp. grown on Sabouraud glucose agar (SGA) plates for 2 to 7 days, depending on the growth rate. Conidia were harvested by washing the cultures with phosphate-buffered saline (PBS). Conidia were then separated from hyphal elements by filtration through sterile gauze. Following centrifugation at 2,000 × g for 2 min, the conidia were washed and resuspended with PBS. Conidia were used directly as extraction positive-control material and as sources for specificity testing. Additionally, 1 × 10⁷ *A. fumigatus* and *A. terreus* conidia were inoculated into 10 ml yeast peptone glucose medium and incubated at 37°C with shaking. Morphological changes and quantification for *in vitro* molecular studies were determined by direct light microscopy. Once 100% of conidia had swelled and most had initiated germ tube formation (~5.5 h), these “germlings” were mixed in equal proportions, aliquoted, frozen at -80°C, and subsequently used in limit-of-detection (analytical sensitivity) experiments. The limit of detection is the concentration at which ≥95% of samples are positive.

Development of control for PCR inhibition and nucleic acid extraction efficiency. In order to control for PCR inhibition and poor DNA extraction, we designed a bacteriophage lambda universal internal control (λ-UIC). The λ-UIC gene target, *egfp* (enhanced green fluorescent protein), derived from the jellyfish *Aequifex aeolicus*, is an unlikely contaminant of clinical specimens and is sufficiently foreign for this purpose. A λ-UIC lysate was formed and assayed for



FIG. 1. Locations of real-time PCR assay targets. An rDNA single repeat is shown. The pan-*Aspergillus* assay targets the 18S rRNA gene region. The species-specific assays target the ITS1 region.

TABLE 2. Testing of species-specific real-time PCR assays against genomic DNAs of *Aspergillus* spp. and other fungi

Fungal species	Strain ^a	Real-time PCR assay result			
		<i>A. fumigatus</i>	<i>A. flavus</i>	<i>A. niger</i>	<i>A. terreus</i>
<i>Aspergillus fumigatus</i>	NRRL 163	+	-	-	-
	NRRL 164	+	-	-	-
	NRRL 165	+	-	-	-
	NRRL 166	+	-	-	-
	NRRL 5109	+	-	-	-
	NRRL 5587	+	-	-	-
<i>Aspergillus flavus</i>	NRRL 1957	-	+	-	-
	NRRL 3518	-	+	-	-
	NRRL 4818	-	+	-	-
<i>Aspergillus niger</i>	NRRL 3	-	-	+	-
	NRRL 326	-	-	+	-
<i>Aspergillus terreus</i>	NRRL 255	-	-	-	+
	NRRL 260	-	-	-	+
	NRRL 680	-	-	-	+
	NRRL 1913	-	-	-	+
	NRRL 2399	-	-	-	+
<i>Penicillium citrinum</i>	This study	-	-	-	-
<i>Penicillium chrysogenum</i>	This study	-	-	-	-
<i>Penicillium purpurogenum</i>	This study	-	-	-	-
<i>Candida albicans</i> (SC5314)	MRL 842	-	-	-	-

^a NRRL, Northern Regional Research Laboratories (part of the USDA Agricultural Research Service Culture Collection); MRL, Mycology Reference Laboratory (part of the Center for Medical Mycology at the University Hospitals of Cleveland). Three *Penicillium* strains were obtained as clinical isolates from the National Cancer Institute during the course of this study.

PFU/μl by utilizing pEGFP-C2 (Clontech, Mountain View, CA) and a Lambda Zap II predigested vector kit with Gigapack III Gold packaging extract (Agilent, Santa Clara, CA) as described in the package insert. Approximately 1 × 10⁸ copies of λ-UIC were spiked into every specimen and extraction negative control prior to nucleic acid extraction.

Animals and immunosuppression. Detailed methodology of the persistently neutropenic rabbit model of IPA is described elsewhere (4, 5, 19). This animal model system has been predictive of the clinical properties of other *Aspergillus* biomarkers, including galactomannan and (1→3)-β-D-glucan (8, 10, 17, 19, 30). Infection was confirmed by pulmonary lesion scores, histology, quantitative lung cultures, and, when applicable, computed tomography (CT) scans (29). Healthy female New Zealand White rabbits (Covance Research Products, Inc., Denver, PA) were used in all experiments. All animals were monitored under humane care and use standards in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, according to the guidelines of the National Research Council for the care and use of laboratory animals, and under the approval of the Institutional Animal Care and Use Committee of the National Cancer Institute, Bethesda, MD. Vascular access was established by surgical placement of a Silastic tunneled central venous catheter as previously described (26). Profound and persistent neutropenia (a neutrophil concentration of <100 neutrophils/μl) and immunosuppression were established and maintained using cytarabine and methylprednisolone, as described previously (5). Antibiotics (ceftazidime, gentamicin, and vancomycin) were used for prevention of opportunistic bacterial infections during neutropenia.

Nine experimental groups were studied (see Tables 4 and 5). Untreated and treated models of IPA were investigated for each of the four species of *Aspergillus*, as well as a ninth group of noninfected and untreated animals.

Inoculation. For each experiment, an inoculum of 1 × 10⁸ to 1.25 × 10⁸ conidia of an isolate of *A. fumigatus*, *A. flavus*, *A. terreus*, or *A. niger* in a volume of 250 μl to 350 μl was prepared and determined by colony counts. Endotracheal inoculation was performed on day 2 of experiments, while rabbits were under

TABLE 3. Pan-*Aspergillus* real-time PCR assay against genomic DNAs of *Aspergillus* spp. and other fungi

Fungal species	Strain ^a	Pan- <i>Aspergillus</i> real-time PCR result
<i>A. caesiellus</i>	NRRL 5061	+
<i>A. candidus</i>	NRRL 303	+
<i>A. carbonarius</i>	NRRL 348	+
<i>A. carneus</i>	NRRL 527	+
<i>A. clavotonicus</i>	NRRL 4741	+
<i>A. clavatus</i>	NRRL 1	+
<i>A. conicus</i>	NRRL 149	+
<i>A. deflectus</i>	NRRL 2206	+
<i>A. flavus</i>	NRRL 1957	+
<i>A. fumigatus</i>	NRRL 163	+
<i>A. glaucus</i>	NRRL 116	+
<i>A. janus</i>	NRRL 1787	+
<i>A. japonicus</i>	NRRL 360	+
<i>A. nidulans</i>	NRRL 187	+
<i>A. niger</i>	NRRL 326	+
<i>A. ochraceus</i>	NRRL 398	+
<i>A. oryzae</i>	NRRL 447	+
<i>A. penicillioideis</i>	NRRL 4548	+
<i>A. restrictus</i>	NRRL 154	+
<i>A. tamari</i>	NRRL 4911	+
<i>A. terreus</i>	NRRL 260	+
<i>A. unguis</i>	NRRL 216	+
<i>Candida albicans</i> (SC5314)	MRL 842	-
<i>Candida glabrata</i>	NRRL Y-27785	-
<i>Candida parapsilosis</i>	NRRL Y-12969	-
<i>Cunninghamella echinulata</i>	NRRL 1386	-
<i>Mucor circinelloides</i>	NRRL 1405	-
	NRRL 223	-
<i>Mucor indicus</i>	NRRL 13083	-
	NRRL 555	-
<i>Mucor ramosissimus</i>	NRRL 5904	-
<i>Penicillium chrysogenum</i>	This study	+
<i>Penicillium citrinum</i>	This study	+
<i>Penicillium purpurogenum</i>	This study	+
<i>Rhizomucor pusillus</i>	NRRL 28626	-
<i>Pneumocystis jirovecii</i>	ATCC 50385	-
<i>Rhizomucor pusillus</i>	NRRL 3639	-
<i>Rhizopus microsporus</i>	NRRL 13477	-
	NRRL 3671	-
<i>Rhizopus oryzae</i>	NRRL 395	-

^a Please see the footnotes to Table 2. ATCC, American Type Culture Collection.

general anesthesia, as described previously (5). Antifungal therapy was initiated 24 h postinoculation in treated animals.

Quantitative cultures of lung tissue and BAL fluid. Lung tissue from each rabbit was sampled postmortem and cultured by standard excision of tissue from each lobe. Each fragment was weighed individually, placed in a sterile polyethylene bag (Whirl-Pak bags; Nasco, Fort Atkinson, WI), and homogenized with sterile normal saline (NS) for 30 s per tissue sample (Stomacher 80; Tekmar Corp., Cincinnati, OH). Lung homogenate dilutions (10⁻¹ and 10⁻²) were prepared in sterile NS. Aliquots (100 μl) of homogenates and homogenate dilutions were plated onto SGA plates and incubated at 37°C for the first 24 h and then placed at room temperature for another 24 h. The number of CFU of *Aspergillus* spp. was counted and recorded for each lobe, and the CFU/g was calculated. A finding of one colony of *Aspergillus* spp. was considered positive. Lung tissue specimens were obtained from 20 untreated *Aspergillus*-infected rabbits and 25 treated *Aspergillus*-infected rabbits.

BAL was performed postmortem on each lung preparation, as previously described (19), by the instillation of 10 ml of sterile NS into the clamped trachea

with a sterile 12-ml syringe and subsequent withdrawal. The instillations were repeated twice. The lavage fluid was then centrifuged for 10 min at $400 \times g$. Part of the supernatant was discarded, leaving the pellet and 2 ml of supernatant, which was then vortexed. Aliquots of 100 μ l of BAL fluid and 100 μ l of a dilution (10^{-1}) of this fluid were cultured on 5% SGA plates. Specimens of BAL fluid were obtained from archived samples from previous studies of IPA using *A. fumigatus* (20, 21) and *A. terreus* (28), as well as from newly conducted experiments on IPA for all four species. BAL fluid specimens were obtained from 12 noninfected rabbits, 73 untreated *Aspergillus*-infected rabbits, and 62 treated *Aspergillus*-infected rabbits. Postmortem-collected BAL fluid samples were divided into two portions and used for GM and PCR studies. Aliquots of BAL fluid and 500- to 1,000- μ l tissue homogenate samples were stored at -70°C and subsequently shipped on dry ice to Viracor-IBT Laboratories. The identities of specimens were coded alphanumerically and blinded for the organism and treatment group.

Galactomannan assay. The BAL fluid samples were analyzed for galactomannan concentrations by using the Platelia *Aspergillus* enzyme-linked immunosorbent assay (Bio-Rad Laboratories, France) performed according to the manufacturer's instructions, as previously described for serum galactomannan determination (4, 19). Peroxidase-linked monoclonal rat antibody EB-A2 was used to sensitize the microplate wells and bind the *Aspergillus* galactomannan antigen. The optical density (OD) of each BAL fluid specimen and control was measured by using a microplate spectrophotometer (Multiscan MMC/340) at 450-nm and 620-nm wavelengths. Enzyme immunoassay data were expressed as the BAL fluid galactomannan index (GMI). The GMI for each BAL fluid specimen is equal to the OD of the BAL fluid sample divided by the OD of a threshold serum control (1 ng/ml of galactomannan) provided in the test kit.

Nucleic acid extraction from fungal isolates and BAL fluids. Nucleic acid extraction from BAL fluid was performed using a Roche MagNA Pure automated extraction system (large-volume DNA isolation kit). In a 2-ml microcentrifuge tube, 600 μ l of 710- to 1,180- μm glass beads (Sigma-Aldrich, St. Louis, MO), 200 μ l Roche bacterium/fungus lysis buffer, and 40 μ l Roche proteinase K (Indianapolis, IN) were combined. For nucleic acid extraction from fungal isolates, approximately 1×10^5 germinated conidia were added to the 2-ml microcentrifuge tube as a positive control. For nucleic acid extraction from BAL fluid, the specimen was thawed and 400 μ l was added to the reaction tube. The mixture was bead beaten in a FastPrep24 instrument (MP Biomedicals, Solon, OH) twice at 6.5 m/s for 60 s and then incubated at 56°C for 15 min. After a brief spin to remove drops from the lid, the lysate was transferred from the beads to a MagNA Pure sample cartridge. Using 600 μ l PBS, residual lysate was collected by transferring bead washes into the sample cartridge. An aliquot of 5 μ l of λ -UIC was added to each well of the sample cartridge. The samples were extracted at an elution volume of 100 μ l. An extraction negative control and germinated conidia positive control were included in each extraction run.

Nucleic acid extraction of tissue homogenate. An AllPrep DNA/RNA minikit (Qiagen, Germantown, MD) was utilized for extractions, with some modifications. Five hundred to 1,000 μ l tissue homogenate was pelleted at $15,000 \times g$ for 5 min, and the pellet was resuspended in either 350 or 700 μ l RLT Plus buffer containing β -mercaptoethanol. Three hundred fifty microliters of suspension and 5 μ l of bacteriophage λ -UIC were added to a MagNA Lyser green bead tube. This sample was bead beaten with a Precellys 24 instrument (Bertin Technologies, Montigny-le-Bretonneux, France) at 6,500 rpm for 60 s. The tube was gently centrifuged to collect the suspension, which was then extracted using a Qiagen QIACube instrument and AllPrep DNA/RNA mini kit reagents. The final elution volume was 100 μ l. An extraction negative control and germinated conidia positive control were included in each extraction run.

Real-time PCR. The real-time PCR general formulation mix was 5 μ l of a 6 \times primer-probe mix and 15 μ l of 2 \times ABI Fast mix, which contains a proprietary mix of DNA polymerase, deoxynucleoside triphosphates (dNTPs), buffers, and salts (Applied Biosystems, Foster City, CA), 0.09% bovine serum albumin (Sigma-Aldrich, St. Louis, MO), and 10 μ l of template (extracted nucleic acids, quantitation standards, etc.). An ABI 7500 Fast instrument was used, with initial denaturation at 95°C for 20 s followed by 45 repetitions of a two-step amplification cycle of denaturation at 95°C for 3 s and annealing/extension at 60°C for 30 s. Thermal cycling was followed by cooling down to room temperature. Quantitation standards (serial dilutions of the *Aspergillus* assay target gene cloned into the pCR2.1 plasmid vector [Invitrogen, Carlsbad, CA], ranging from 5×10^1 to 5×10^7 copies per reaction) were run in conjunction with each set of samples. Eluates were analyzed in duplicate real-time PCR wells. Quantitative values used to establish linearity, dynamic range, and lower limits of detection of the assay were derived from wells with crossover thresholds (C_T values) of <40 . The positivity cutoff used in experimental IPA was defined as both wells having C_T values of <40 .

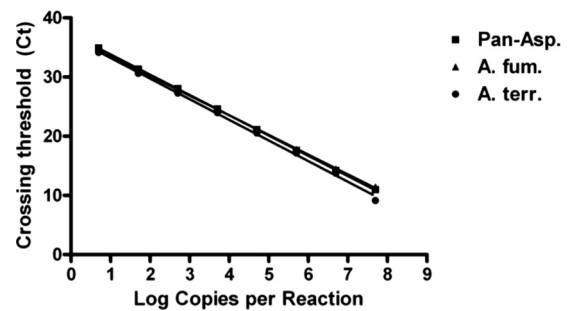


FIG. 2. Real-time PCR linearity and dynamic range. Five to 5×10^7 copies of each assay's target region were introduced into real-time PCR wells and analyzed to determine crossing thresholds. The log copy number detected per reaction was plotted versus the crossing threshold for pan-*Aspergillus* (squares), *A. fumigatus* (triangles), and *A. terreus* (circles).

Statistical analysis. Differences in proportions were analyzed using Fisher's exact test. Differences in means were analyzed utilizing the Mann-Whitney t test (two-tailed). A P value of <0.05 was considered to be statistically significant. Summary data are reported as means \pm standard errors of the means (SEM).

RESULTS

In vitro performance testing of specificity, linearity, dynamic range, and limit of detection. The *Aspergillus* PCR assays were examined for specificity by utilizing a panel of species (Tables 2 and 3). The *Aspergillus* species-specific and genus-specific primers were designed to maximize and minimize 3' homology to the target species and nontarget species, respectively. The species-specific assays were examined using genomic extracts from at least two isolates of *A. fumigatus*, *A. flavus*, *A. niger*, *A. terreus*, and *Penicillium* spp. *Candida albicans* genomic DNA was also tested. The species-specific assays detected only the target species, as summarized in Table 2. The pan-*Aspergillus* real-time PCR assay detected all *Aspergillus* spp. tested (Table 3). However, it also detected all *Penicillium* species tested (*Penicillium citrinum*, *Penicillium chrysogenum*, and *Penicillium purpurogenum*), but it did not detect the five *Candida* species tested (*C. albicans*, *Candida glabrata*, *Candida krusei*, *Candida parapsilosis*, and *Candida tropicalis*) or *Pneumocystis jirovecii*. To assess the potential for cross-reactivity with other molds, the pan-*Aspergillus*, *A. fumigatus*, and *A. terreus* assay oligonucleotides were analyzed using BLAST searches against other major hyaline and pigmented mold pathogens and were determined to be not cross-reactive with the following genera: *Fusarium*, *Scedosporium*, *Pseudallescheria*, *Paecilomyces*, *Bipolaris*, *Curvularia*, *Alternaria*, *Cladosporium*, *Cladophialophora*, and *Phyllophora*. Rare matches were encountered in *Paecilomyces* BLAST searches using the pan-*Aspergillus* assay oligonucleotides as query sequences. *Paecilomyces marquandii*, *Paecilomyces variotii*, and several *Byssoschlamys* sp. (teleomorph of *Paecilomyces*) sequences matched the pan-*Aspergillus* assay sequences, but the matches were infrequent. Most of the available sequences of the suspect *Paecilomyces* spp. did not match the pan-*Aspergillus* assay sequences.

The assays reliably detected as few as 5 copies of purified plasmid standard target and were linear from 5 copies to 5×10^7 copies (Fig. 2). The PCR efficiency was calculated by the following equation: $e = [10^{-(1/\text{slope})}] - 1$, where e and

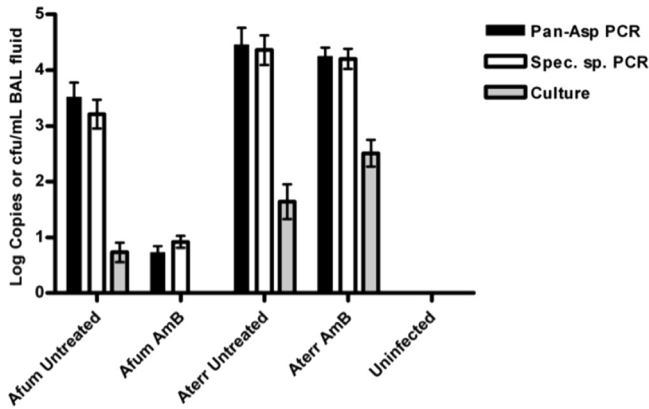


FIG. 4. Detection of rDNA copies in BAL fluid, showing efficacy of amphotericin B (AmB) treatment in an experimental model of invasive pulmonary aspergillosis. Copies/ml and CFU/ml of BAL fluid are shown for real-time PCR assays (pan-*Aspergillus* [black bars] and *A. fumigatus* or *A. terreus* species-specific [white bars] assays) and culture (gray bars), respectively. Utilizing the *A. fumigatus* and pan-*Aspergillus* assays, there was a statistically significant difference in numbers of copies/ml of BAL fluid between the untreated and treated groups for *A. fumigatus* ($P < 0.0001$) but not for *A. terreus* ($P = 0.813$) (two-tailed Mann-Whitney t test).

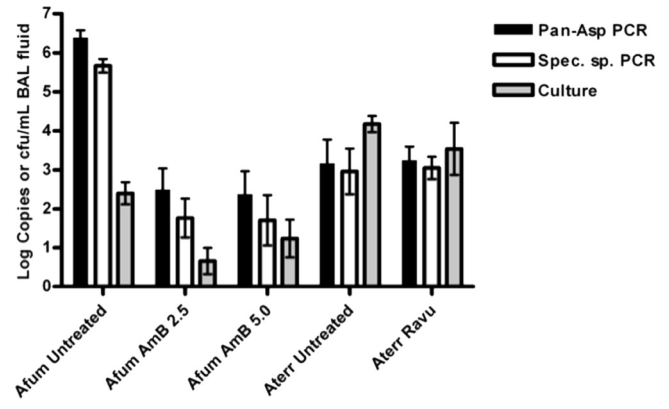


FIG. 5. Detection of rDNA copies in tissue homogenates, showing efficacy of amphotericin B (AmB) treatment (*A. fumigatus*) and ravuconazole (Ravu) treatment (*A. terreus*) in an experimental model of invasive pulmonary aspergillosis. Log copies per tissue sample and log CFU/g tissue are shown for real-time PCR assays (pan-*Aspergillus* [black bars] and *A. fumigatus* or *A. terreus* species-specific [white bars] assays) and culture (gray bars), respectively. Utilizing the two-tailed Mann-Whitney t test, there was a statistically significant difference in numbers of copies per tissue extraction between the untreated and treated groups for *A. fumigatus* ($P < 0.0001$ for amphotericin B at 2.5 mg/kg of body weight/day and $P < 0.0003$ for amphotericin B at 5.0 mg/kg/day) but not for *A. terreus* ($P = 0.8988$).

of the assay for *A. flavus*, we proceeded with fungal tissue burden assessments in experimental models for only *A. fumigatus* and *A. terreus*. Qualitative results of culture and real-time PCR with lung tissue from rabbits with experimental IPA are summarized in Table 5. Pan-*Aspergillus* and species-specific real-time PCRs with tissue yielded higher rates of positivity for detection of IPA caused by *A. fumigatus* than those observed for culture for treated animals, but the differences were not statistically significant. The quantitative results of culture and real-time PCR data are summarized in Fig. 5. The tissue specimens with negative results and the extraction negative controls for the run were required to have threshold crossing cycles of <35 for negative results to be valid and for acceptance of the extraction run, respectively. All such specimens and controls met this criterion. There was a significant difference in copies/ml tissue homogenate between the untreated

and treated groups for *A. fumigatus* but not for *A. terreus* (Fig. 5). With the exception of untreated *A. fumigatus*-infected rabbits, gene target copy detection in the real-time PCR assays correlated with quantitative cultures of pulmonary residual fungal burdens in lung tissue.

DISCUSSION

We developed a set of real-time PCR assays for the detection of *Aspergillus* spp. by utilizing pan-*Aspergillus* primers as well as species-specific primers for *A. fumigatus*, *A. flavus*, *A. niger*, and *A. terreus* and then validated these systems in animal models of invasive pulmonary aspergillosis for each of these species. Infections caused by each of the four species were detected successfully by the pan-*Aspergillus* and species-spe-

TABLE 5. Sensitivities of culture and real-time PCR assays for detection of *Aspergillus* spp. in lung tissue specimens from experimental invasive pulmonary aspergillosis

Species	n	Treatment status ^a	No. (%) of positive specimens		
			Culture	Real-time PCR assay	
				Pan- <i>Aspergillus</i>	Species specific
<i>A. fumigatus</i>	14	Untreated	13 (93)	14 (100)	14 (100)
<i>A. terreus</i>	6	Untreated	6 (100)	6 (100)	6 (100)
Total	20	Untreated	19 (95)	20 (100)	20 (100)
<i>A. fumigatus</i>	11	Treated (AMB at 2.5 mg/kg/day)	3 (27)	6 (55)	7 (64)
	8	Treated (AMB at 5.0 mg/kg/day)	4 (50)	6 (75)	5 (63)
<i>A. terreus</i>	6	Treated (RVC)	5 (83)	6 (100)	6 (100)
Total	25	Treated	12 (48)	18 (72)	18 (72)

^a *A. fumigatus*-infected rabbits were administered liposomal amphotericin B (AMB) at either 2.5 or 5 mg/kg/day for several days prior to assay (6 to 12 days). *A. terreus*-infected rabbits were administered ravuconazole (RVC) at 10 mg/kg/day for several days prior to assay (12 days).

cific real-time PCR assays, using BAL fluid from animals with experimental IPA. Through a series of iterative quality control studies, primer-probe designs, and correlation with the four models of experimental pulmonary aspergillosis, the final repertoire of assays consists of the pan-*Aspergillus* assay as well as the species-specific assays for *A. fumigatus* and *A. terreus*. These studies led to the availability of assays to health care providers in the United States for their application to direct patient care through Viracor-IBT Laboratories.

These laboratory studies also provided the experimental foundation for clinical investigation of the real-time PCR system with archived BAL fluid specimens from a large cohort of lung transplant recipients. The findings of the laboratory animal studies presented here were predictive of the outcome (16). That study analyzed the performance of the pan-*Aspergillus* and *A. fumigatus*- and *A. terreus*-specific real-time PCR assays and included 16 cases of proven or probable IPA and 97 negative controls. The *Aspergillus* PCR system detected 100% of cases of proven or probable IPA, with specificities of 89%, 96%, and 99% for the pan-*Aspergillus*, *A. fumigatus*, and *A. terreus* assays, respectively.

For the purposes of clinical and epidemiological studies, the European Organization for the Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) established criteria for proven and probable IPA (3). The presence of defined thresholds of galactomannan in serum and BAL fluid and of (1 \rightarrow 3)- β -D-glucan in serum is considered an acceptable microbiological criterion for diagnosis of probable IPA. However, diagnostic PCR was not included as a suitable microbiological criterion due to the absence of validation and standardization. The assay reported here has been validated rigorously in laboratory animal model systems and, more recently, in clinical specimens. If standardization in additional centers is achieved, this *Aspergillus* PCR system should be considered for inclusion in the next iteration of EORTC/MSG definitions for diagnosis of invasive fungal infections (3).

The rationale for selection of the four *Aspergillus* species in these studies was predicated upon their clinical importance. *A. fumigatus* constitutes the most common cause of invasive pulmonary and disseminated aspergillosis. *A. flavus* is the second most common cause of invasive aspergillosis. However, in some institutions, *A. flavus* has been the most common cause of invasive disease, particularly in the setting of nosocomial outbreaks (7, 25). *A. niger* is a less common cause of acute invasive disease but is a frequent cause of chronic aspergillosis, especially aspergilloma (23). Finally, *A. terreus*, which has been described as the second most common cause of IPA in some institutions, causes invasive pulmonary and disseminated aspergillosis and is usually resistant to amphotericin B (28).

Although several quantitative, real-time PCR (qPCR) assays have been reported for diagnosis of IPA, many lack specificity (11). Moreover, due to differences in antifungal susceptibility, species-specific identification is important to improve outcomes.

The pan-*Aspergillus* assay is specific for more than 100 *Aspergillus* spp. and does not amplify genomic DNA from *Candida* spp. or members of the Mucorales. Moreover, BLAST searches did not demonstrate significant homology with other medically important pathogenic fungi. However, the primers and probes did produce a reaction with genomic DNAs of

Penicillium spp. While penicillia are rarely the cause of documented invasive disease, they may be a source of laboratory contamination as well as a cause of tracheobronchial colonization, particularly in patients with damaged airways. However, based upon the findings of the real-time PCR study of lung transplant recipients, the clinical significance of this reactivity with genomic DNAs of *Penicillium* spp. was relatively small (16). That study found that among 11 lung transplant recipients colonized by *Penicillium* spp., only one patient's BAL fluid specimen reacted with the pan-*Aspergillus* real-time PCR assay. These data suggest that the hyphal burden of the organism is relatively low or that *Penicillium* is present principally in the form of conidia. The real-time PCR system was comparable in sensitivity to BAL fluid galactomannan assay for treated and untreated rabbits. The two assays may be complementary in a clinical setting by permitting rapid species identification. The real-time PCR system allows a rapid turnaround time that is comparable to that of BAL fluid galactomannan assay, thus permitting simultaneous availability of antigen molecular data to clinicians.

The clinical performance of the assay was determined in a study of 150 lung transplant recipients, including 16 with proven or probable IPA, 26 with *Aspergillus* colonization, 11 with non-*Aspergillus* mold colonization, and 97 negative controls (16). The sensitivities of the pan-*Aspergillus* and *A. fumigatus* assays were 100% and 85%, respectively. The sensitivity of the *A. terreus* assay could not be determined due to too few *A. terreus* cases (the single *A. terreus* case was found to be positive by the assay). The specificities of the pan-*Aspergillus*, *A. fumigatus*, and *A. terreus* assays were 88%, 96%, and 99%, respectively.

For acceptable specificity, the sequence design required that the *A. flavus* reverse primer be short (Table 1). While the *A. flavus* assay performed in acceptable fashion with purified quantitation standards, subsequent testing revealed that it performed poorly with nucleic acids extracted from specimens (Table 4). To test whether the short (15 bp) reverse primer was being absorbed nonspecifically, an alternate, 24-bp reverse primer was designed. Using a genomic template, the new reverse primer rescued the detection to match that of the pan-*Aspergillus* real-time PCR assay. Unfortunately, this new reverse primer reduced the specificity of the assay for *A. flavus* and thus could not be included in a redesigned assay. Problems in designing species-specific molecular *Aspergillus* assays stem from discrepancies (taxonomic problems as well as misidentifications and possible sequencing errors) in the NCBI GenBank public database. Problems in primer and probe design also emerge in *Aspergillus* taxonomy from identifications made using different methods (traditional versus molecular) (1).

One hundred to 10,000 times higher values were measured for numbers of copies detected (via real-time PCR) than numbers of CFU detected (via culture) for analyses of BAL fluid (Fig. 4). Similar results were obtained with analyses of tissue homogenates for the untreated *A. fumigatus*-infected group only (Fig. 5). These differences are owed in part to the presence of many ribosomal DNA (rDNA) gene targets for PCR for every organism detectable by culture. One report of 6 *A. fumigatus* clinical isolates indicated a range of 38 to 91 rDNA copies per genome (9). In addition, many genomes likely correspond to each CFU due to the presence of nonviable and

viable organisms in specimens. Molecular identification of *Aspergillus* spp. is best accomplished by using primers and probes targeting several different gene loci. However, such a multiplexed assay would be complex and expensive for a clinical laboratory.

The real-time PCR system demonstrated greater sensitivity than culture for detection of *A. fumigatus* in BAL fluid (Table 4). Pan-*Aspergillus* PCR was also more sensitive than culture for detection of *A. flavus* in treated animals. The same-day turnaround time (8 to 12 h from specimen receipt) affords a further advantage toward timely initiation of antifungal therapy, as culture usually requires several days to generate a result. While the real-time PCR and galactomannan assay sensitivities generally agreed for BAL fluid, the species-specific real-time PCR assays provide additional information to guide antifungal drug selection. This is relevant to the question of whether *A. terreus*, which is intrinsically resistant to amphotericin B, is the infectious agent. For those medical centers or reference laboratories conducting molecular diagnostic procedures but not galactomannan assays within their facility, the pan-*Aspergillus* and species-specific PCR assays may improve diagnostic yields over those with conventional culture methods.

Determination of the real-time PCR performance using blood specimens is an important task, as collection of blood is less invasive than that of BAL fluid or tissue. We are developing methods toward efficient extraction of *Aspergillus* DNA from blood for this evaluation. Pan-*Aspergillus* and species-specific real-time PCR assays may improve the rapid and accurate identification of IPA in immunocompromised patients. To evaluate the utility of the real-time PCR system presented here, alterations and optimizations are being developed, to be followed by *in vivo* and clinical evaluations.

This report highlights the value of validation of diagnostic assays in well-characterized laboratory animal models, including the persistently neutropenic rabbit models of IPA, in predicting the performance of novel diagnostic methods for patients. Given the paucity of well-characterized clinical samples and the uncontrolled variability of host factors in patients with IPA, the use of a robust panel of samples from well-characterized animal models can be a powerful tool for development of new assays for detection of aspergillosis and other uncommon mycoses.

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