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Molecular Probes for Diagnosis of Fungal Infections

GURPREET S. SANDHU,[1] BRUCE C. KLINE,[1]* LESLIE STOCKMAN,[2] AND GLENN D. ROBERTS[2]

Department of Biochemistry and Molecular Biology,[1] Division of Clinical Microbiology,[2] Mayo Clinic and Foundation, Rochester, Minnesota 55905

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We have developed 21 specific nucleic acid probes which target the large subunit rRNA genes from *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus glaucus*, *Aspergillus niger*, *Aspergillus terreus*, *Blastomyces dermatitidis*, *Candida albicans*, *Candida (Torulopsis) glabrata*, *Candida guilliermondii*, *Candida kefyr*, *Candida krusei*, *Candida lusitaniae*, *Candida parapsilosis*, *Candida tropicalis*, *Coccidioides immitis*, *Cryptococcus neoformans* var. *gattii*, *Cryptococcus neoformans* var. *neoformans*, *Filobasidiella neoformans* var. *bacillispora*, *Filobasidiella neoformans* var. *neoformans*, *Histoplasma capsulatum*, *Pseudallescheria boydii*, and *Sporothrix schenckii*. A section of the 28S rRNA gene from approximately 100 fungi, representing about 50 species of pathogens and commonly encountered saprophytes, was sequenced to develop universal PCR primers and species-specific oligonucleotide probes. Each step in the process of detection and identification was standardized to a common set of conditions applicable without modification to all fungi of interest and all types of clinical specimens. These steps consist of DNA extraction by boiling specimens in an alkaline guanidine-phenol-Tris reagent, amplification of a variable region of the 28S rRNA gene with universal primers, and amplicon identification by probe hybridization or DNA sequencing performed under conditions identical for all fungi. The results obtained by testing a panel of fungal isolates and a variety of clinical specimens indicate a high level of specificity.

The increasing incidence of AIDS and the development of new treatments for hematologic malignancies and organ transplants has lead to a steady increase in the number of immunocompromised patients. Immunosuppression is occasionally accompanied by an insidious onset of fungal infections, with some cases being nosocomial. Fungi are traditionally identified by morphology and metabolic characteristics and may require days to weeks to isolate on culture. Identification may sometimes be difficult, and this impedes the selection of antimicrobial agents in cases in which species identification of the organism is necessary. Therefore, it is desirable to have a rapid and accurate method of identification.

Several groups have developed tests (1–3, 5–7, 9–11, 13–15, 17, 18) based on PCR (16). In each instance, only a few species of fungi were used in the development, and despite the variety of strategies described, most have shown limited applicability for routine use in a clinical laboratory. The objective of our study was to develop a common protocol for detecting and identifying a variety of medically important fungi.

In the present report, we describe the development of a diagnostic strategy based on the utilization of highly specific oligonucleotide probes. Approximately 50 fungi representing common pathogens and saprophytes were selected, and a significant portion of the 28S rRNA genes from each organism was sequenced. This sequence information was used to design a single pair of universal PCR primers capable of amplifying a variable region of the 28S rRNA gene from all fungi sequenced by us. An analysis of the variable region from these fungi led to the synthesis of 21 highly specific oligonucleotide probes. These include 17 probes specific for *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus glaucus*, *Aspergillus niger*, *Aspergillus terreus*, *Blastomyces dermatitidis*, *Candida albicans*, *Candida (Torulopsis) glabrata*, *Candida guilliermondii*, *Candida kefyr*, *Candida krusei*, *Candida lusitaniae*, *Candida parapsilosis*, *Candida tropicalis*, *Coccidioides immitis*, *Histoplasma capsulatum*, and *Pseudallescheria boydii*, two probes specific for *Sporothrix schenckii*, and two probes capable of detecting all four serovars of *Cryptococcus neoformans* (teleomorph, *Filobasidiella neoformans*). A universal DNA extraction method that works equally well on culture isolates and all types of clinical specimens was developed to enable the rapid processing of a large number of specimens. Thus, our complete strategy consists of a rapid universal DNA extraction followed by PCR amplification with universal primers for 28S rDNA and amplicon identification by hybridization with species-specific probes used under identical annealing and washing conditions. The results of retrospective testing on a limited panel of culture isolates and clinical specimens are described.

MATERIALS AND METHODS

Fungi used in study. All fungi used in this study were obtained from the Mayo Clinical Mycology laboratory collection (Table 1).

Universal DNA isolation. A 200-μl volume of sputum, broncho-alveolar lavage fluid, cerebrospinal fluid, urine, blood (diluted with an equal volume of sterile water), or other body fluids was used. Swabs from infected sources were immersed in 300 μl of sterile distilled water and expressed out against the side of the tube, and the liquid was used for DNA extraction. Solid tissues were homogenized in sterile water, while paraffin-fixed tissue sections were scraped off slides by using a sterile scalpel blade and were resuspended in 200 μl of sterile distilled water. Pure cultures were prepared by resuspending a loopful of organisms in 200 μl of sterile water.

Sarstedt (Newton, N.C.) polypropylene screw-cap tubes (1.5-ml volume) with o-ring seals were used for the DNA extractions. A 200-μl volume of specimen was added to 500 μl of GPT reagent (6 M guanidine thiocyanate dissolved in 50 mM Tris [pH 8.3] and mixed with an equal volume of phenol buffered in Tris [pH 8.0]). The contents were mixed on a Vortex Genie II (Scientific Industries, Bohemia, N.Y.), and the tubes were placed in a boiling water bath for 15 min. The tubes were spun briefly (5 s) in a microcentrifuge (HBI, Saddle Brook, N.J.), and 250 μl of chloroform-isoamyl alcohol (24:1 by volume) was added and mixed as described above. The liquid phases were separated by a 10-min centrifugation at 14,000 rpm in a microcentrifuge, and 450 μl of the aqueous (upper) phase was transferred to a fresh tube. The aqueous phase was mixed with 500 μl of 100% isopropanol, and the mixture was placed at −20°C for a minimum of 1 h (it could be left overnight). At the end of this period, the tubes were spun in a microcentrifuge at 14,000 rpm for 15 min, and the supernatant was removed without disturbing the nucleic acid pellet. Traces of GPT reagent were removed by the

* Corresponding author. Mailing address: Department of Biochemistry and Molecular Biology, Mayo Clinic and Foundation, Rochester, MN 55905. Phone: (507) 284-2992. Fax: (507) 284-3383. Electronic mail address: kline.bruce@mayo.edu.

TABLE 1. Fungi and probes used in this study

Name	Accession no(s).[a]	Isolate no(s).	Probe[b]
Aspergillus flavus	U26856 and U26861	3-66 and 2-66	AGACTCGCCT CCAG
Aspergillus fumigatus	U26871	f4	CTCGGAATGT ATCA
Aspergillus glaucus	U26872	f2	GTGTCATGCG GCCA
Aspergillus niger	U26862 and U26874	2-9 and f9	CCCTGGAATG TAGT
Aspergillus terreus	U26877	f1	GCTTCGGCCC GGTG
Blastomyces dermatitidis	U26882	f32	ACTCCCCCAC GGG
Candida albicans	U26888	f22	CCTCTGACGA TGCT
Candida glabrata	U26889	f18	CTTGGGACTC TCGC
Candida guilliermondii	U26859 and U26890	2-21 and f21	ATATTTTGTG AGCC
Candida kefyr	U26892	f15	TTCGGCTTTC GCTG
Candida krusei	U26893	f16	GGGATTGCGC ACCG
Candida lusitaniae	U26860 and U26895	2-12 and f12	GCCTCCATCC CTTT
Candida parapsilosis	U26898	f23	ATAAGTGCAA AGAA
Candida tropicalis	U26900	f17	AGAATTGCGT TGGA
Coccidioides immitis	U26891	g6	TCTGGCGGTT GGTT
Histoplasma capsulatum	U26909 and U26907	f31 and g7	CAATCCCCCG CGGC
Pseudallescheria boydii	U26910	f75	GCGATGGGAA TGTG
Sporothrix schenckii	U26852 and U26866	2-25 and 3-25	CGGACCACCC GGCG no. 1[c] CGGCGGCATG CCCC no. 2
Cryptococcus neoformans var. *neoformans* serotype A	U26897	f14	CTCCTGTCGC ATAC no. 1[d] AGTTCTGATC GGTG no. 2
Crytococcus neoformans var. *gattii* serotype B	U26896	g19	
Filobasidiella neoformans var. *bacillispora* serotype C	U26902	g22	
Filobasidiella neoformans var. *neoformans* serotype D	U26903	g17	
Acremonium species	U26869	f35	
Aspergillus clavatus	U26870	f62	
Aspergillus nidulans	U26873 and U26876	f3 and f10	
Aspergillus ochraceus	U26875	f6	
Aspergillus unguis	U26878	f5	
Aspergillus ustus	U26879	f7	
Beauveria species	U26880	f51	
Bipolaris species	U26881	f88	
Blastoschizomyces capitatus	U26863	g55	
Chrysosporium species	U26883	f28	
Cladosporium species	U26886	f101	
Cryptococcus laurentii	U26894	g14	
Cryptococcus terreus	U26899	g21	
Curvularia species	U26887	f85	
Filobasidium capsuligenum	U26901	g15	
Filobasidium uniguttulatum	U26904	g16	
Fusarium species	U26848, U26857, and U26867	2-50, 3-50, and c8	
Geotrichum species	U26858 and U26905	g56 and f36	
Malbranchea species	U26849	g57	
Mucor species	U26850, U26864, and U26908	2-39, c9, and f39	
Paecilomyces species	U26909	f141	
Penicillium species	U26851 and U26865	2-54 and 3-54	
Rhizopus species	U26868, U26885, and U26911	4-53, c170, and f53	
Saccharomyces cerevisiae	U26912	f11	
Scopulariopsis brevicaulis	U26853	2-34	
Scopulariopsis brumptii	U26854	2-89	
Trichosporon beigelii	U26855, U26884, and U26913	2-20, c131, and f20	

[a] GenBank accession numbers for the partial 28S rRNA gene sequences.
[b] Sequences of 21 oligonucleotide probes are listed.
[c] Two probes listed are specific for *S. schenckii*.
[d] Two probes listed detect all four serovars of *C. neoformans* (*F. neoformans*).

addition of 500 μl of ice-cold 70% ethanol, gentle inversion of the tube twice, and then centrifugation in a microcentrifuge at 14,000 rpm for 5 min. The ethanol was removed, and the pellet was dried under a vacuum for 10 min. The DNA-containing pellet was resuspended in 25 μl of sterile deionized water, and 5 μl was used in a 50-μl PCR amplification mixture.

Identification of oligonucleotide sequences. Conserved and variable regions of the 28S genes were identified by aligning published sequences for *C. neoformans*, *C. albicans*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe* 28S genes (GenBank accession numbers L14068, L28817, X70659, J01355, Z19136, and Z19578). A region of sequence variability was found clustered between coordinates 200 and 700 from the 5′ end of these genes (the numbers are comparable to the primary sequence of the *S. cerevisiae* 28S rRNA gene [GenBank accession number J01355]). As a starting point, primers P1 and P2 (ATCAATAAGC GGAGGAAAAG and CTCTGGCTTC ACCCTATTC, respectively) were designed to amplify this variable region from all four fungi, but not the human 28S gene (GenBank accession number M11167). P1 corresponds to coordinates 45 to 64, and P2 corresponds to coordinates 825 to 843 of the reference *S. cerevisiae* gene. Primers P1 and P2 were used in a PCR to amplify this variable region from a majority of fungi listed in Table 1. The amplified rDNA was sequenced across both strands by using primers P1, P2, P3, P4, P5, and P6 (P3, AGACCGATAG

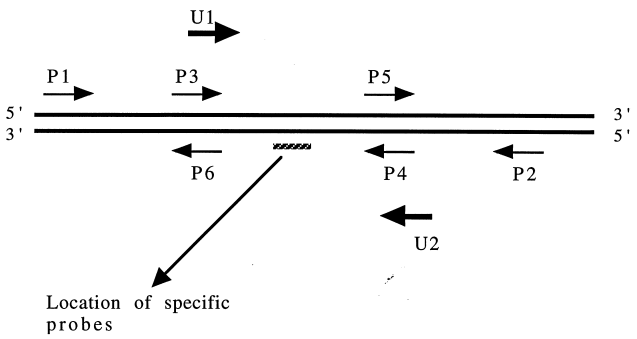

FIG. 1. Locations of primers and probes. The double line represents the 5′ 1 kb of fungal 28S rRNA gene sequence. The relative locations of primers P1, P2, P3, P4, P5, P6, U1, and U2 and the species-specific probes are depicted. Primer P1 corresponds to coordinates 45 to 64, and primer P2 corresponds to coordinates 825 to 843 of a reference *S. cerevisiae* 28S gene (GenBank accession number J01355). When used in a PCR, they yield a 799-bp amplicon. Primer U1 corresponds to coordinates 403 to 422, and primer U2 corresponds to coordinates 645 to 662. They yield a 260-bp amplicon when used in a PCR.

CGAACAAGTA; P4, ACTCCTTGGT CCGTGTTTCA; P5, TGAAACACGG ACCAAGGAGT; P6, ACTCTACTTG TGCGCTATCG G). The relative locations of these primers are depicted in Fig. 1.

The resulting 28S sequences were aligned, and two conserved regions that flanked a highly variable region were identified. These conserved sites were used to develop universal primers for the PCR amplification of a small segment of fungal 28S rDNA. The primer U1 (GTGAAATTGT TGAAAGGGAA) corresponds to coordinates 403 to 422, and the primer U2 (GACTCCTTGG TCCGT GTT) corresponds to coordinates 645 to 662 of the reference *S. cerevisiae* 28S gene. Primers U1 and U2 were used to amplify and sequence independent isolates of all fungi listed in Table 1. Both strands of amplified DNA were sequenced to eliminate sequencing artifacts and to ensure accuracy of data generated.

More than 100 sequences were generated, and these were aligned to create a comparative database. This alignment of variable regions representing about 50 fungal species was used to develop oligonucleotide probes that would be specific for only one species in our database. The two probes for *C. neoformans* (*F. neoformans*) were designed to detect all four serovars. The sequences for the 21 species-specific probes are listed in Table 1.

Hot start PCR amplification. All PCR amplifications were carried out as hot start reactions in a 50-μl reaction volume with Perkin-Elmer (Norwalk, Conn.) 0.5-ml thin-wall polypropylene tubes and a Perkin-Elmer thermal cycler. The reagents added to the tube initially were 2.5 μl of 10× PCR buffer (100 mM Tris [pH 8.3], 500 mM KCl, 15 mM $MgCl_2$), 5.0 μl of 50% glycerol–1 mM cresol red, 8.0 μl of deoxynucleoside triphosphate mix (1.25 mM each dATP, dGTP, dTTP, and dCTP), 10 pmol of each nucleic acid primer, and sterile water to make up a volume of 25 μl. A wax bead (Ampliwax Gem-100; Perkin-Elmer) was added, and the tubes were heated to 77°C for 1 min and cooled to room temperature to form a wax barrier. A 2.5-μl volume of 10× PCR buffer, 5.0 μl of 50% glycerol–1 mM cresol red, 0.2 μl of *Taq* polymerase (5 U of AmpliTaq per μl [Perkin-Elmer]), and 12.3 μl of sterile water were added to the tube along with 5.0 μl of solution from the DNA isolation procedure described above. PCR amplification conditions used for primers P1 and P2 were 50 cycles carried out at 94°C for 30 s, 40°C for 1 min, and 72°C for 2 min. PCR amplification conditions used for primers U1 and U2 were 50 cycles, carried out at 94°C for 30 s, 50°C for 1 min, and 72°C for 2 min. All PCRs included appropriate controls to exclude the possibility of DNA contamination.

Automated sequencing and analysis of amplified DNA. Amplified DNA was purified by electrophoretic separation on a low-melting-point agarose gel and then by a phenol (buffered in Tris [pH 8.0]) extraction, a phenol-chloroform-isoamyl alcohol (25:24:1 by volume) extraction, and three ether extractions. The DNA was precipitated with 100% isopropanol and lyophilized. Semiautomated fluorescent sequencing was carried out by using an ABI 373A sequencer (ABI, Foster City, Calif.). Data analysis was carried out with the ABI Seqed and/or Sequence Navigator programs. GenBank searches and sequence alignment were done with the University of Wisconsin Genetics Computer Group (Madison, Wis.) program.

Sensitivity testing of PCR primers. The minimal amount of fungal DNA that would be successfully detected in a PCR amplification with primers U1 and U2 was determined. Purified genomic DNA obtained from *S. cerevisiae* was quantitated by using a spectrophotometer, and 10-fold serial dilutions starting at 2×10^6 genomes per reaction were subjected to a hot start PCR with primers U1 and U2. Upon completion of PCR, an aliquot from each tube was analyzed by electrophoresis in a 5% polyacrylamide minigel in 1× Tris-borate-EDTA buffer. The gel was stained with ethidium bromide and was photographed under short-wave UV light. The presence of a 260-bp band was considered evidence of successful amplification of 28S rDNA.

Specificity testing of probes against a fungal panel. All 21 species-specific probes were individually tested for hybridization specificity against a test panel consisting of a variety of known fungi. Primers U1 and U2 were used in a PCR to amplify DNA from the fungi listed in Fig. 2, and the amplified DNA was bound to a membrane as follows. A 20-μl volume of each amplicon from a 50-μl PCR mixture was denatured in 1 N NaOH and slot blotted onto a positively charged polysulfone-based membrane (BioTrace HP; Gelman Sciences, Ann Arbor, Mich.) equilibrated in 0.5 N NaOH. The membrane was baked in a vacuum oven at 80°C for 30 min and placed in hybridization buffer (100 ml of hybridization buffer was made with 1 g of nonfat milk powder, 6 g of NaH_2PO_4, 7 g of sodium dodecyl sulfate, and 200 μl of 0.5 M EDTA and adjusted to pH 7.2 with NaOH) at 40°C for 3 h.

The membrane was now probed, in turn, with each one of the species-specific probes. A 50-pmol amount of a species-specific probe was end labeled with radioactive phosphorus by using [^{32}P]ATP and T4 polynucleotide kinase. This radiolabeled probe was added to 70 ml of hybridization buffer, and the membrane was probed at 40°C overnight. Upon completion of hybridization, the unbound probe was removed by washing the membrane in 2× SSC (0.3 M sodium chloride, 0.03 M sodium citrate [pH 7.0]) at room temperature for 2 min and then by washing in 2× SSC at 40°C for 20 min. The membrane was then placed on X-ray film for approximately 1 h.

The same membrane was sequentially probed with all species-specific probes listed in Table 1. The previously hybridized probe was removed by washing the membrane with 0.5 N NaOH at 40°C for 15 to 20 min. Subsequent membrane blocking was done for at least 30 min at 40°C, and probe hybridization was carried out for a minimum of 3 h at 40°C. Posthybridization wash conditions remained the same for all probes.

RESULTS

Sequence analysis and probe design. Primers used for the preliminary amplification and sequencing of the fungal 28S genes were developed as described in Materials and Methods. An analysis of the data obtained by multiple gene alignment indicated a region of sufficient variability centered around coordinate 525 (reference *S. cerevisiae* gene). Primers U1 and U2 were developed to amplify this region and were found to work on all species listed in Table 1 (data not shown). Primers U1 and U2 were subsequently used to sequence these amplicons, and the resultant sequences were deposited in GenBank (see Table 1 for accession numbers). Independent isolates of many fungal species had an identical sequence, and in these cases the sequence obtained from only one representative isolate was deposited in GenBank. In cases in which differences were observed among independent isolates of the same species, all additional sequences were deposited in GenBank.

Primers U1 and U2 were also checked for their potential ability to hybridize to and amplify (in a PCR) 23S sequences from bacteria by searching for hybridization sites among the 539 bacterial 23S genes listed in GenBank. No suitable annealing sites were found, and under stringent conditions these two primers appear to be incapable of amplifying bacterial 23S rRNA genes.

The fungal 28S sequences in our database were analyzed for suitable sites for defining species-specific probes. All species of interest had regions that differed from the sequences of other closely related organisms by 2 or more bases over a stretch of about 15 bases. These regions were further analyzed for thermal profiles and secondary structure. The best possible balance of desirable features was obtained to generate 14-base probes specific for the organisms of interest (13 bases for *B. dermatitidis*). Twenty-one species-specific probes capable of identifying the first 22 fungi listed in Table 1 were identified. Two probes identify all four serovars of *C. neoformans* (*F. neoformans*), while another two detect *S. schenckii*.

All species-specific probes were also analyzed for additional target hybridization sites by searching GenBank for related sequences. At the time at which this search was carried out, 28S sequences for *C. albicans* and *C. neoformans* were present in GenBank, and as expected, the probes for *C. albicans* and

	A	B	C
1	A. terreus	C. kefyr	Rhizopus sp.
2	A. glaucus	T. beigelii	Penicillium sp.
3	A. nidulans	C. guilliermondii	A. clavatus
4	A. fumigatus	C. albicans	A. flavus
5	A. unguis	C. parapsilosis	P. boydii
6	A. ochraceus	S. schenckii	Curvularia sp.
7	A. ustus	Chrysosporium sp.	Bipolaris sp.
8	A. niger	H. capsulatum	Cladosporium sp.
9	Aspergillus sp.	B. dermatitidis	A. fumigatus
10	S. cerevisiae	S. brevicaulis	C. albicans
11	C. lusitaniae	Acremonium sp.	C. immitis
12	C. neoformans	Geotrichum sp.	H. capsulatum
13	C. krusei	Mucor sp.	B. dermatitidis
14	C. tropicalis	Fusarium sp.	C. neoformans
15	C. glabrata	Beauveria sp.	Empty

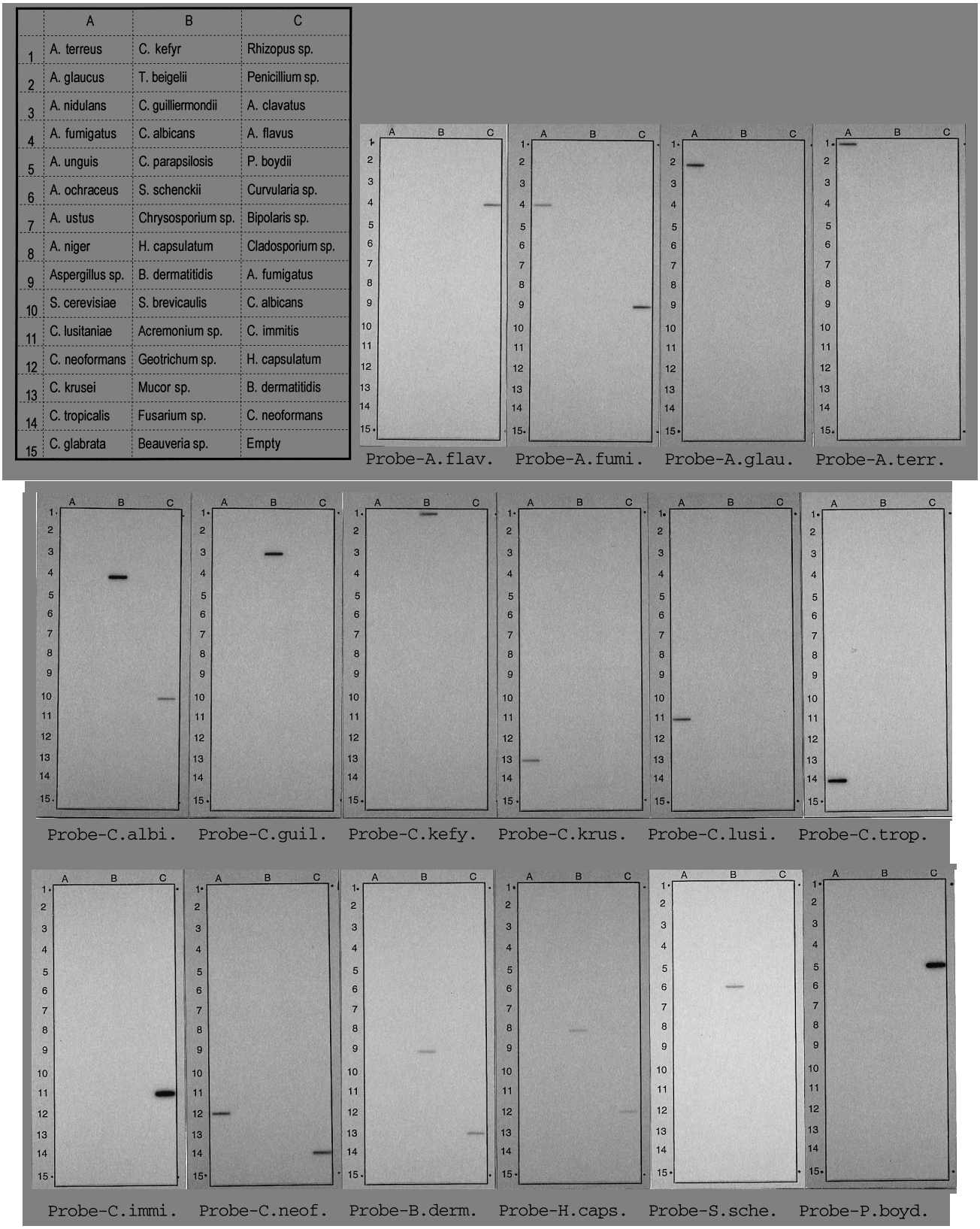

FIG. 2. Testing probes for species specificity. The grid identifies the locations of slot blotted 28S rDNA from various fungi. The 16 photographs show the species-specific hybridization of the probes listed below each panel. The signal intensity for all probes may easily be increased by prolonging the exposure time to 3 or 4 h. The difference in the intensities of the two bands present in the *C. albicans* panel is due to unequal amounts of target DNA in each slot.

TABLE 2. GenBank sequences matching species-specific probes

Probe	Organism and gene (accession no.)
B. dermatitidis	*Streptomyces verticillus* bleomycin acetyltransferases (L26955)
	Giardia muris upstream of rRNA genes (X65063 and S53320)
	Aspergillus nidulans uric acid-xanthine permease (X71807)
	Homo sapiens T-cell surface glycoprotein (X16996)
	Homo sapiens (MIC2, M16279, M22557, J03841, and M22556)
C. neoformans no. 2	*Escherichia coli* 0111 *cld* (Z17241)
A. glaucus	*Pseudomonas denitrificans cob* genes (M62866)
A. terreus	Human cytomegalovirus genome (X17403)
	Homo sapiens GABA receptor (L08485)
C. (T.) glabrata	*Homo sapiens* class 1 major histocompatibility complex (X03664 and X03665)
C. krusei	*Pseudomonas syringae* (penicillin-binding protein (L28837)
C. lusitaniae	Chicken AK1, (D00251)
	Mouse IL10 (M84340)
C. parapsilosis	*Polytomella agilis* β-2 tubulin (M33373)
	Tobacco chloroplast genome (Z00044 and S54304)

C. neoformans correctly identified the 28S sequences from these two organisms. Ten other probes also matched DNA sequences from a variety of genes not related to the 28S gene (Table 2). This was expected because short stretches of sequence identity can often be found for any query sequence in unrelated genes from the same or a different organism. In all cases, sequences that matched a probe sequence were not located within a 28S rRNA gene. Since none of these unrelated genes have priming sites for primers U1 and U2, amplified DNA from these non-28S genes should not be present to hybridize with the species-specific probes. The only way in which these unrelated sequences would result in a false-positive result would be for these sequences to be transferred from the cell lysates in concentrations typical of those obtained by PCR. The possibility of this occurring seems unlikely.

Sensitivity testing of PCR primers. PCR primers designed for diagnostic use should have the ability to amplify their target DNA from limited amounts of genomic DNA. The sensitivity of our primers U1 and U2 was evaluated by amplifying 28S rDNA from known amounts of serially diluted genomic DNA obtained from *S. cerevisiae*. Primers U1 and U2 successfully amplified the appropriate region of the 28S rRNA gene from the equivalent of as little as 0.2 genomes (data not shown).

Specificity testing of probes against a panel of fungi. The 21 specific probes were tested for their ability to identify their correct target organism. All probes listed in Table 1 were tested against DNA amplified from a panel of fungi (Fig. 2), and all were found to hybridize only to DNA amplified from the correct target organisms. No false-positive results were observed, indicating a specificity of 100% for all 21 probes within the selected test panel of organisms. Figure 2 shows 16 species-specific probes hybridized to amplified 28S rDNA. The results in Fig. 2 are typical for the remaining 5 probes (data not shown). Thus, each of the 21 probes will hybridize only to and detect its intended target among the approximately 50 fungi used in our study. Additionally, both probes for *C. neoformans* correctly identified all four serovars of *C. neoformans* and *F. neoformans* (data not shown).

TABLE 3. Testing of clinical specimens[a]

Specimen (source)	Fungus present[b]	Probes[c]			
		A. fumigatus	*C. albicans*	*C. immitis*	*C. neoformans* no. 1
U035 (sputum)	*A. flavus*				
U069 (pleura)	*A. fumigatus*	+			
U070 (bronchial wash)	*A. flavus*				
M019 (bronchial wash)	*A. fumigatus*	+			
M020 (sputum)	Mixed fungi		+		
X35254 (sputum)	*C. albicans*		+		
M20910 (sputum)	*A. fumigatus*	+			
M055 (sputum)	*C. albicans*		+		
M056 (abdomen)	*C. albicans*		○		
M057 (drainage tube)	*C. albicans*		○		
M059 (ind. sputum[d])	*C. albicans*		+		
M060 (ind. sputum)	Mixed fungi				
M083 (bronchial wash)	*C. albicans*		+		
M084 (sputum)	*A. fumigatus*	○			
M085 (throat)	*C. albicans*		○		
A001 (sputum)	*A. fumigatus*	○			
A002 (leg)	*B. dermatitidis*				
A003 (leg)	*B. dermatitidis*				
A005 (disc)	*A. fumigatus*	+			
A037 (disc)	*A. fumigatus*	+			
A039 (trachea)	*C. albicans*		+		
A040 (trachea)	*C. albicans*		+		
A102 (empyema)	*A. fumigatus*	+			
Y004 (sputum)	*C. albicans*		+		
Y016 (ind. sputum)	*C. immitis*			+	
Y028 (sputum)	*C. immitis*			+	
J003 (chest)	*Aspergillus* sp.				
J045 (bronchial wash)	*C. albicans*		+		
J046 (ethmoid)	Yeast				
J047 (chest)	*A. fumigatus*	+			
J048 (sputum)	*C. albicans*		+		
J073 (lung)	*Aspergillus* sp.				
J074 (lung)	*A. fumigatus*	+			
U017 (lung)	*A. fumigatus*	+			
U033 (sputum)	Mixed fungi				
U071 (sputum)	*C. albicans*		+		
U072 (BA lavage)	*S. schenckii*				
U073 (knee)	*H. capsulatum*				
U074 (mandible)	*C. neoformans*				+
U075 (CSF)	*C. neoformans*				+
U076 (knee)	*H. capsulatum*				
U077 (soft tissue)	*H. capsulatum*				
U051 (buccal)	*A. fumigatus*	+			
Y055 (sputum)	Mixed fungi				

[a] Clinical specimens were subjected to universal DNA extraction, PCR amplification with universal 28S primers U1 and U2, and specific hybridization with probes for *A. fumigatus*, *C. albicans*, *C. immitis*, and *C. neoformans* (probe no. 1).
[b] Fungi cultured in clinical laboratory. Specimens labeled mixed fungi contained two or more of *Aspergillus* sp., *Candida* sp., *Cladosporium* sp., *Penicillium* sp., *Scopulariopsis* sp., or *Trichophyton* sp. M020 contained *Candida* sp., *Aspergillus* sp., and *Scopulariopsis* sp.
[c] +, positive; ○, missed.
[d] ind. sputum, induced sputum.

Analysis of clinical specimens. A limited retrospective study was carried out to determine the applicability of our complete detection strategy of universal DNA extraction, universal 28S PCR, and specific probing. A group of 44 clinical specimens representing diverse specimen types was obtained (Table 3 lists sources of clinical specimens). Prior smear and culture results had indicated that all specimens contained at least one species of fungus. DNA extraction and PCR amplification (primers U1 and U2) were carried out as described, and gel electrophoresis on a 5-μl aliquot of the PCR mix confirmed successful ampli-

TABLE 4. Testing of clinical isolates of fungi[a]

Culture isolate	No. tested	No. hybridizing with the following probes:							
		A. fumigatus	*A. terreus*	*B. dermatitidis*	*C. glabrata*	*C. immitis*	*C. neoformans*	*H. capsulatum*	*S. schenckii*
A. fumigatus	9	9							
A. terreus	4		3[b]						
B. dermatitidis	2			2					
C. (T.) glabrata	18				18				
C. immitis	16					16			
C. neoformans	10						10		
H. capsulatum	12							12	
S. schenckii	5								5
Other species	40								

[a] Blank spaces represent failure of a probe to hybridize to amplified DNA from organism listed.
[b] With the exception of one isolate of *A. terreus*, all probes correctly identified their respective target organisms.

fication. The amplicons were denatured, fixed to a membrane, and probed for the presence of *A. fumigatus*, *C. albicans*, *C. immitis*, and *C. neoformans*. As seen by the results in Table 3, no false-positive results were observed, indicating that within the specimens tested, these 4 probes had a specificity of 100%. Ten of 12 specimens containing *A. fumigatus* and 11 of 14 specimens containing *C. albicans* were identified. Additionally, the 2 specimens containing *C. immitis* as well as the two containing *C. neoformans* were correctly identified. This indicated a detection sensitivity ranging from approximately 80 to 100% within this limited although diverse group of specimens.

Analysis of culture isolates. Primers U1 and U2 were used to amplify DNA from 116 fungal isolates cultured from clinical specimens. The amplified DNA was bound to a membrane and sequentially hybridized with probes for *A. fumigatus*, *A. terreus*, *B. dermatitidis*, *C. (T.) glabrata*, *C. immitis*, *C. neoformans* (probe no. 2), *H. capsulatum*, and *S. schenckii* (probe no. 2). The aim of this limited test was to establish that our probes had the ability to correctly identify their target fungi and to show that the species-specific variable regions targeted by our probes remained constant among isolates obtained from a variety of clinical specimens. Other fungi present in this test panel but not specifically probed included *Mucor* sp., *C. albicans*, *Fusarium* sp., *A. flavus*, *A. niger*, *Rhizopus* sp., *P. boydii*, and *Trichosporon beigelii*. The results are shown in Table 4. Within this panel of 116 fungi, the probes for *A. fumigatus*, *B. dermatitidis*, *C. (T.) glabrata*, *C. immitis*, *C. neoformans* probe no. 2, *H. capsulatum*, and *S. schenckii* probe no. 2 correctly detected all homologous target isolates. The probe for *A. terreus* detected DNA amplified from three of four isolates. The one isolate missed was not investigated further. None of the probes showed any false-positive results, indicating an effective specificity of 100%.

DISCUSSION

The development of molecular diagnostic tests for mycotic infections requires information from an extensive sequence database. When this study was initiated, 28S ribosomal sequences of only two pathogenic fungi, *C. albicans* and *C. neoformans*, were available in GenBank. The 28S ribosomal gene was selected as a detection target because its large size was expected to provide adequate species-specific differences to distinguish closely related organisms. Additionally, ribosomal genes in fungi are present at as many as 100 or more copies per genome (12), and this would provide good detection sensitivity. This reasoning was validated when primers U1 and U2 successfully amplified 28S rDNA from the equivalent of 0.2 genomes of *S. cerevisiae* (data not shown).

Our universal lysis and DNA extraction procedure is similar to the RNA extraction protocol described by Chomczynski and Sacchi (4). Their original method used acidic guanidinium thiocyanate-phenol-chloroform to preferentially extract RNA from cells and tissues at room temperature. Instead, we used boiling lysis in alkaline guanidine thiocyanate-phenol-Tris reagent to preferentially extract DNA from a variety of clinical specimens. Boiling in the presence of alkaline GPT reagent disrupts cells and breaks up chromosomes into small fragments of DNA that are less likely to be trapped in tissue debris. Our protocol also renders specimens noninfectious and provides high-quality DNA that is devoid of PCR-inhibiting substances. Additionally, this procedure is rapid, technically simple, broadly applicable, and inexpensive.

While our universal primers were tested only against the organisms listed in Table 1, a computer search indicated broad applicability for the amplification of fungal DNA. Primer U1 matched about 150 fungal 28S sequences in GenBank and no prokaryotic 23S or nonfungal eukaryotic 28S sequences. Primer U2 recognized about 200 sequences consisting of not only fungal but also higher-order 28S eukaryotic sequences (including that from *Homo sapiens*). U2 did not match any prokaryotic 23S sequences. When used in a PCR, primers U1 and U2 selectively amplify fungal sequences.

Clinical specimens taken from the respiratory and gastrointestinal tracts of healthy individuals almost always contain mixed fungal flora. Therefore, any set of universal fungal primers is potentially compromised, since nearly every sample may potentially yield an amplicon. Probing, as in slot blotting, is an effective way of recognizing a specific amplicon in a mixture of amplicons with similar sizes, as long as specificity is high. Specificity would be lost with large probes; therefore we designed small probes that are unstable in the absence of a complete match.

Our initial studies showed that all 21 probes were specific for their intended targets when tested against DNA amplified from a panel of pathogenic and saprophytic fungi (Fig. 2). Species specificity was also confirmed when eight different probes correctly identified their homologous target DNA from a panel of 116 fungi isolated in the clinical laboratory (Table 4). The solitary specimen missed by the *A. terreus* probe may represent a misclassification or a species-sequence variation; however, this was not investigated further. Finally, the slot blot analysis results obtained by testing four probes against a di-

verse collection of 44 clinical specimens (Table 3) showed no false-positive results.

These data clearly support the use of oligonucleotide probes as diagnostic tools for fungal infections. All probes examined indicated that sequence variability among isolates of the same species, especially in the well-characterized pathogens, was unlikely to be a significant concern. While there is no established demarcation, sequence variation among ribosomal genes isolated from organisms belonging to the same species is usually limited to less than 2%. Therefore, the probability of encountering a variation at a 14-base hybridization site of a specific probe, while not impossible, is unlikely. Our present work examines a limited set of organisms, and a much larger study is required to address the issue of encountering sequence variability at the hybridization site of a short probe. Some saprophytic fungi may be poorly characterized by classical techniques, and as phylogenetic knowledge expands, some will likely be reclassified. Opportunistic infections by saprophytic fungi are those most likely to be missed by molecular probes based on sequence data obtained from a limited number of isolates.

The database described here represents one of the largest collections of medically important fungal 28S sequences. A database comparable in size has been generated by Leclerc et al. (8) for analyzing the phylogenetic relationships among fungi. Both databases contain partial 28S sequences for *S. schenckii*, *P. boydii*, *B. dermatitidis*, *H. capsulatum*, and *Chrysosporium* sp. However, Leclerc et al. have not released their sequence data, and we are unable to compare the sequences.

In summary, reducing the DNA extraction and amplification steps to a common protocol and developing a standardized set of hybridization conditions for all probes completed our objective of developing an assay with clinical applicability. This universal fungal detection and identification protocol is a simple yet effective diagnostic tool capable of being streamlined to integrate into a mycology laboratory. The use of common reagent and reaction conditions also makes parts of the strategy amenable to automation. The use of radiolabeled probes in a routine laboratory is not the most desirable situation, and we are exploring the use of nonradioactive signal moieties and faster solution hybridization assays to expedite amplicon analysis. Our comprehensive panel of oligonucleotide probes is being tested as part of a larger prospective clinical study. It should also be noted that the lysis and subsequent detection and identification strategy may also be used for the detection of other organisms.

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