JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 2006, p. 876–880 Vol. 44, No. 3
0095-1137/06/$08.00+0 doi:10.1128/JCM.44.3.876–880.2006
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Simultaneous Detection and Identification of *Candida*, *Aspergillus*, and *Cryptococcus* Species by Reverse Line Blot Hybridization

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Received 7 July 2005/Returned for modification 15 September 2005/Accepted 24 October 2005

We report on a reverse line blot (RLB) assay, utilizing fungal species-specific oligonucleotide probes to hybridize with internal transcribed spacer 2 region sequences amplified using a nested panfungal PCR. Reference and clinical strains of 16 *Candida* species (116 strains), *Cryptococcus neoformans* (five strains of *Cryptococcus neoformans* var. *neoformans*, five strains of *Cryptococcus neoformans* var. *grubii*, and six strains of *Cryptococcus gatti*), and five *Aspergillus* species (68 strains) were all correctly identified by the RLB assay. Additional fungal species (16 species and 26 strains) not represented on the assay did not exhibit cross-hybridization with the oligonucleotide probes. In simulated clinical specimens, the sensitivity of the assay for *Candida* spp. and *Aspergillus* spp. was $10^{0.5}$ cells/ml and 10^2 conidia/ml, respectively. This assay allows sensitive and specific simultaneous detection and identification of a broad range of fungal pathogens.

The incidence of invasive fungal infections is increasing in association with increasing populations of immunocompromised and critically ill patients. Given that invasive fungal infections are associated with high crude and attributable mortality rates despite therapy with effective antifungal agents, early diagnosis remains an important, but as yet unresolved, challenge to potentially improve clinical outcomes. Deficiencies with current diagnostic approaches include poor specificity (e.g., clinical and radiological features) and poor sensitivity (e.g., blood cultures, which are rarely positive in invasive aspergillosis and only 40 to 60% sensitive in invasive candidiasis) (22). Furthermore, culture-based phenotypic identification techniques are slow and especially prone to misidentification of fungal pathogens, particularly uncommon species (22). Delays in identification have important clinical implications, given the relative increase in the incidence of non-*C. albicans Candida* spp. and the predictable intrinsic or relative fluconazole resistance associated with certain *Candida* spp. such as *Candida krusei* and *Candida glabrata*.

Molecular microbiological techniques have the potential to achieve rapid, sensitive, and specific detection and identification of fungi from clinical specimens or cultures. Although a variety of targets, primers, and product detection methods has been reported (2, 13), several challenges remain. Given the diversity of human fungal pathogens, assays that amplify or detect only one or a restricted number of fungal species have limited utility. On the other hand, many panfungal assays have no or limited species specificity (2, 13), which is problematic, since species identification is important for therapeutic and other clinical decisions. Although species-specific oligonucleotide probes to identify panfungal PCR product have been reported (2, 13), the challenge of incorporating this approach into simple, inexpensive, and flexible formats that are readily adaptable to the clinical microbiology laboratory remains.

To address these requirements, we describe the development of a simple method to simultaneously detect and identify clinically relevant fungal pathogens using a panfungal nested PCR followed by hybridization with species-specific oligonucleotide probes in a reverse line blot (RLB) assay.

MATERIALS AND METHODS

Fungal strains. Reference fungal strains were obtained from the American Type Culture Collection, the Centraalbureau voor Schimmelcultures, and the Molecular Mycology Research Laboratory at Westmead Hospital and clinical isolates from the Mycology Laboratory at Westmead Hospital (Table 1). All strains were characterized by colonial and microscopic morphology and physiological testing using the VITEK I (bioMérieux Vitek, Hazelwood, Missouri) and/or ID 32C (bioMérieux, Marcy-l'Etoile, France) commercial system. Clinical strains were further characterized by sequencing of the internal transcribed spacer region 1 (ITS1), 5.8S rRNA, and ITS2 regions.

Oligonucleotide design. Relevant fungal DNA sequences spanning the 5.8S rRNA, ITS2, and 28S rRNA regions were accessed from GenBank and compared using the Pileup and Pretty programs in the Multiple Sequence Analysis program group provided in the Australia National Genomic Information Service (ANGIS, 3rd version; accessed through http://biomanager.angis.org.au/).

Two pairs of primers, based on the universal fungal primers ITS3 and ITS4 (32) were designed for the nested amplification of the ITS2 region (Table 2). The inner primer pair, designated ITS3B and ITS4B, were 5′-end biotin labeled (Sigma-Aldrich, Castle Hill, Australia).

Oligonucleotide probes targeting ITS2 sequences of *Candida* species were modified (to increase their melting temperatures) from those described previously (5), and those of *Aspergillus* species and *Cryptococcus neoformans* were designed (Table 2). All oligonucleotide probes were 5′ end hexylamine labeled (Sigma-Aldrich).

DNA extraction. Isolated yeast colonies or a small amount of fungal mycelial mass was obtained from growth on Sabouraud's dextrose agar incubated at 30°C for 48 to 72 h and suspended in saline. DNA was extracted either directly or, for

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TABLE 1. Reference and clinical strains used in the RLB assay

Organism	No. of reference strains (identifier[s])	No. of clinical strains
Candida species		
C. albicans	3 (ATCC 90028, CBS 562)	14
C. dubliniensis	1 (CBS 7988)	9
C. glabrata	2 (ATCC 90030, CBS 138)	8
C. guilliermondii	3 (CBS 2030, CBS 2031, CBS 5256)	10
C. haemulonii	1 (CBS 5149)	3
C. kefyr	3 (ATCC 4135, CBS 834, CBS 712)	1
C. krusei	1 (CBS 6258)	9
C. lusitaniae	5 (CBS 5305, CBS 5901, CBS 5094, CBS 4413, CBS 6936)	9
C. norvegica	1 (CBS 4239)	0
C. parapsilosis (group I)	1 (ATCC 22019)	14
C. pelliculosa	2 (CBS 605 CBS 5759)	0
C. rugosa	0	1
C. tropicalis	0	14
C. utilis	2 (CBS 621 CBS 1600)	0
C. viswanathii	1 (CBS 4024)	1
C. zeylanoides	1 (CBS 619)	1
C. famata	1 (CBS 940)	0
C. holmii	1 (CBS 135)	0
C. lipolytica	3 (CBS 6124, CBS 599, MMRL 210B)	0
C. norvegenisis	2 (CBS 6564 CBS 6403)	0
C. pulcherrima	0	1
Cryptococcus species[a]		
C. neoformans var. *grubii*	5 (GI (VNI), LA 404 (VNII), JG 02 (VNII), RV 58146 (VNII), PR 101 (VNII))	0
C. neoformans var. *neoformans*[a]	5 (CDCR 461 (VNIV) KRIMM 2 (VNIV) CBS 7816 (VNIV) WM 01.84 (VNIV) WM 01.85 (VNIV))	0
C. gattii	6 (4A (VGI) 4B (VGI) 4C (VGI) 10A (VGI) 10B (VGI) 4A (VGI))	0
Other yeast species		
Saccharomyces cerevisiae	1 (CBS 1907)	1
Aspergillus species		
A. flavus	1 (ATCC 204304)	12
A. fumigatus	1 (ATCC 204305)	12
A. nidulans	0	10
A. niger	0	18
A. terreus	0	14
A. candidus	0	1
Other mold species		
Scedosporium prolificans	0	2
S. apiospermum	0	2
Penicillium citrinum	0	1
P. chrysogenum	1 (AMMRL 42.29)	0
P. brevicompactum	1 (AMMRL 42.26)	0
P. aurantiogriseum	1 (AMMRL 42.28)	0
Eurotium amstelodamii	1 (AS107)	0
Trichophyton mentagrophytes	0	5
Trichophyon rubrum	0	3
Microsporum gypseum	0	1
Total	56	176

[a] See reference 21 for designations of molecular types of *C. neoformans.*

assessment of assay sensitivity, from dilutions of yeast cells or fungal mycelia in EDTA-blood.

All reagents were obtained from Sigma-Aldrich or from BDH Laboratory Supplies (Dorset, England). DNA extraction was performed in a class II laminar flow cabinet using the GenElute mammalian genomic DNA kit (Sigma-Aldrich) with some modifications. Briefly, 500 μl of EDTA blood was mixed with 1.5 ml of erythrocyte lysis buffer (0.155 M NH_4Cl, 0.01 M NH_4HCO_3, and 0.1 mM EDTA [pH 7.4]) (26) and incubated for 10 min at −20°C. Following centrifugation (6,600 rpm for 10 min), the pellet was resuspended in 200 μl of sorbitol buffer (1 M sorbitol, 100 mM EDTA, 0.1% 2-mercaptoethanol) (33) with 200 U of lyticase (Sigma-Aldrich) and incubated at 37°C for 60 min. Spheroplasts were centrifuged (7,600 rpm for 5 min) and resuspended in 180 μl of lysis solution T and 20 μl of proteinase K (Sigma-Aldrich), and then incubated at 55°C for 60 min. The DNA was then extracted according to the manufacturer's instructions in a final elution volume of 120 μl. DNA was stored at −20°C before use.

ITS2 nested PCR conditions. The first-round PCR mixture (20 μl) consisted of 125 μM each dATP, aCTP, dGTP, and dTTP (Roche Diagnostics, Castle Hill, Australia), 1× buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% Tween 20, 0.01% [wt/vol] gelatin, and 0.01% Niaproof 4), 2.5 μM each outer primer (ITS3outer and ITS4outer), 0.5 U *Taq* DNA polymerase (Promega, Annandale, Australia), and 5 μl extracted genomic DNA. Amplification was performed on an Eppendorf Mastercycler gradient thermocycler (Eppendorf AG, North Ryde, Australia) for 30 cycles of denaturation at 94°C for 10 seconds, annealing at 60°C for 10 seconds, and elongation at 74°C for 20 seconds. The second-round PCR mixture (40 μl) consisted of 125 μM each deoxynucleotide, 1× buffer (as above), 2.5 μM each inner primer (ITS3B and ITS4B), 1 U *Taq* DNA polymerase, and 2 μl of first-round product. The reaction conditions were the same as for the first round except for an annealing temperature of 65°C.

Positive and negative controls were processed in parallel with each sample to detect possible false-negative results and PCR contamination. The potential for contamination was minimized by the use of dedicated equipment in separate laboratory areas for each assay step as well as other standard measures (16).

Appropriately sized DNA bands (350 to 400 base pairs) were visualized following electrophoresis of 8 microliters of amplification product using a 2% agarose gel and stained with 0.5 g/liter ethidium bromide.

Reverse line blot hybridization assay. The RLB hybridization assay was modified (31), based on a previously described method (29). In brief, the nylon membrane-bound oligonucleotide probes were incubated with the PCR products. If present, the relevant PCR product hybridized with the probe and was detected by chemiluminescence. The same membrane was able to be reused on at least nine occasions following the stripping of bound PCR products without loss of signal. The melting temperatures of the probes ranged between 60 and 80°C (Table 1) and the optimal hybridization temperature was determined to be 60°C. Serial dilutions of yeast cells and fungal mycelia were prepared in molecular biology-grade water and added to whole blood to achieve final concentrations ranging from 10^5 to 10^0 cells/ml. These were then extracted (as above) and assayed to determine the sensitivity of the RLB assay.

ITS1 and ITS2 sequencing. The ITS1 and ITS2 regions of all clinical fungal isolates were sequenced. In brief, a 250-μl PCR mixture consisting of 5 μl of extracted genomic DNA, 2.5 mM each of dATP, dCTP, dGTP, and dTTP (Roche Diagnostics, Germany), 1× GeneAmp PCR buffer (Applied Biosystems, Melbourne, Australia), 5% glycerol, 10 μM of forward (ITS1) and reverse (ITS4) primers, and 1.25 U *Taq* DNA polymerase (Applied Biosystems) was amplified on an Eppendorf Mastercycler (94°C for 2 min, 30 cycles of 94°C for 15 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, followed by 72°C for 6 min). DNA was purified using a commercial kit (GFX PCR DNA and gel band purification kit, Amersham Biosciences, Castle Hill, Australia) according to the instructions. The sequencing reactions were run using a POP6 polymer and 50-cm capillary array on the ABI 3100 PRISM genetic analyzer (Applied Biosystems). Sequences were edited using Chromas software, version 2.23, and compared with sequences in GenBank using the FASTA nucleotide sequence search tool provided through ANGIS.

RESULTS

Fifty-five reference strains and 177 clinical strains comprising 40 fungal species were tested (Table 1). The universal primers ITS3 and ITS4 amplified all strains. Results of identification by RLB and ITS1-2 sequencing were concordant for all fungal strains represented on the RLB assay: 16 *Candida* species (116 strains), *Cryptococcus neoformans* (five strains of *Cryptococcus neoformans* var. *neoformans*, five strains of *Cryptococcus neoformans* var. *grubii*, and six strains of *Cryptococcus gatti*), and five *Aspergillus* species (68 strains). Predictable nonspecific hybridization occurred between *Candida zeylanoides* DNA and the *Candida guilliermondii* oligonucleotide probe, as previously described (5) but no other nonspecific hybridization occurred (Fig. 1 and 2). DNA from one specimen, putatively *Candida glabrata*, hybridized with both the *Candida glabrata* and *Candida parapsilosis* probes but failed to yield an accurate

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TABLE 2. Oligonucleotide primers and probes

Primer or probe	Target	T_m (°C)[a]	GenBank accession no.	Sequence (5′ to 3′)[b]
Primers				
ITS1	18S rRNA universal fungal 5′ primer	68.4	AF455531	**19**TCC GTA GGT GAA CCT GCG G**37**
ITS3 outer	5.8S rRNA universal fungal 5′ outer primer	76.62	AF455531	**200**CGG ATC TCT TGG C/TTC/T C/TG/CA/G CAT CGA TGA AGA ACG**232**
ITS3B[c]	5.8S rRNA universal fungal 5′ inner primer	73.0	AF455531	**210**GGC/T TC/TC/T G/CA/GC ATC GAT GAA GAA CGC AGC**236**
ITS4B[c]	28S rRNA universal fungal 3′ inner primer	72.9	L28817	**741**GTT GGT TTC TTT TCC TCC GCT TAT TGA TAT GC**710**
ITS4 outer	28S rRNA universal fungal 3′ outer primer	79.96	L28817	**751**GGC AAT CCC TGT TGG TTT CTT TTC CTC CGC TTA TTG**716**
Probes[d]				
CA	ITS2 region of *C. albicans*	74.3	AF455531	**456**ATT GCT TGC GGC GGT AAC GTC C**477**
DB	ITS2 region of *C. dubliniensis*	79.5	AJ249485	**443**AAG GCG GTC TCT GGC GTC GCC C**465**
CGE	ITS2 region of *C. glabrata*	62.2	AF167993	**715**TTT ACC AAC TCG GTG TTG ATC T**736**
GU	ITS2 region of *C. guilliermondii*	69.9	AF455495	**510**CCC GGC CTT ACA ACA ACC AAA C**531**
CH	ITS2 region of *C. haemulonii*	64.5	U70501	**19**CCG TTG GTG GAT TTG TTT CTA A**40**
KF	ITS2 region of *C. kefyr*	64.1	AJ401701	**557**GAG ACT CAT AGG TGT TAT AAA GAC TCG C**584**
CK	ITS2 region of *C. krusei*	65.5	AF246989	**406**GGC CGA GCG AAC TAG ACT TTT**427**
LU	ITS2 region of *C. lusitaniae*	71.2	AF009215	**279**CTC CGA AAT ATC AAC CGC GCT G**300**
NC	ITS2 region of *C. norvegica*	75.6	U70508	**119**ACG AGC GTC TGC TGG CTC CAC A**140**
CP	ITS2 region of *C. parapsilosis*	61.4	AF455530	**483**ACA AAC TCC AAA ACT TCT TCC A**504**
PL	ITS2 region of *C. pelliculosa*	60.4	AF321543	**502**ATC AGC TAG GCA GGT TTA GAA G**523**
CR	ITS2 region of *C. rugosa*	62.9	AF218971	**189**CAG TTA AGC TTG TTA CAG ACT CAC G**213**
CT	ITS2 region of *C. tropicalis*	65.0	L47112	**426**AAC GCT TAT TTT GCT AGT GGC C**447**
CU2	ITS2 region of *C. utilis*	65.5	AF458091	**430**CCA ACT CGT TAT TTT CCA GAC AGA C**454**
VS	ITS2 region of *C. viswanathii*	65.6	AY139792	**148**CTA CCA AAA CGC TTG TGC AGT C**169**
CZ	ITS2 region of *C. zeylanoides*	61.3	AF218976	**256**TCG TTG ACC AGT ATA GTA TTT GTT TAT TAC**285**
CNEO	ITS2 region of *C. neoformans*	64.9	AJ493561	**426** TAT GGG GTA GTC TTC GGC TTG **446**
AFUM	ITS2 region of *A. fumigatus*	65.29	AY660923	**140** AGC CGA CAC CCA ACT TTA TTT TT **162**
AFL	ITS2 region of *A. flavus*	66.77	AF454111	**147** ACG CAA ATC AAT CTT TTT CCA GG **169**
ANID	ITS2 region of *A. nidulans*	65.1	AY660924	**142** GGC GTC TCC AAC CTT ATT TTT CT **164**
ANIG	ITS2 region of *A. niger*	67.88	AF454120	**141** GCC GAC GTT TTC CAA CCA TT **160**
ATER	ITS2 region of *A. terreus*	66.58	AF454134	**148** GCA TTT ATT TGC AAC TTG TTT TTT TCC **174**

[a] Melting temperatures as provided by the oligonucleotide synthesizer (Sigma Aldrich, Castle Hill, Australia).
[b] Boldface numbers represent the numbered base positions at which the primer or probe sequence starts and finishes (starting at point 1 of the corresponding GenBank sequence). Underlined sequences show bases added to modify previously published primers or probes.
[c] Inner primers were 5′ biotin labeled.
[d] All probes were 5′ hexylamine labeled.

identification by ITS1-2 sequencing: further examination of cultures on chromogenic agar (CHROMagar Candida, Dutec Diagnostics, Croydon, Australia) demonstrated two colonial morphologies, which on phenotypic testing revealed a mixed culture, confirming the RLB result. On ITS sequencing, all *Candida parapsilosis* strains were determined to represent those from *Candida parapsilosis* group I (27). DNA from fungal species not represented on the RLB assay (16 species and 26 strains) and all negative controls failed to hybridize with any of the probes.

The RLB assay reliably yielded positive results from whole-blood samples containing $10^{0.5}$ yeasts/ml for *Candida* spp. (and on at least 50% of occasions with 10^0/ml), and 10^2 conidia/ml for *Aspergillus* spp. The sensitivity of a band on the RLB assay was generally 10-fold higher than visualization under UV light following gel electrophoresis and ethidium bromide staining.

DISCUSSION

This RLB assay is able to detect and identify a diverse range of clinically relevant fungal pathogens, including major *Candida*, *Aspergillus*, and *Cryptococcus* species, by hybridizing the PCR product amplified using panfungal primers with membrane-bound species-specific oligonucleotide probes. Given the diverse range of potential human fungal pathogens and the importance of species identification for therapeutic and other clinical decisions, the competing requirements for molecular assays include broad-range fungal detection and species-specific identification. Although multiplex assays using species-specific primers are able to identify species, their utility is limited by the number of primers able to be incorporated.

Panfungal assays, on the other hand, are potentially able to detect all fungal pathogens, but require additional techniques for their identification. This has been achieved using PCR product sequencing (9, 11, 14), although this remains relatively costly, time-consuming, and potentially inaccurate in the presence of mixed fungal species. Other methods, such as differences in PCR product sizes following electrophoresis, restriction fragment length polymorphism analysis, or single-stranded conformational polymorphism analysis (2, 13), have been used but are not sequence specific and may not be readily adapted for use in the clinical microbiology laboratory.

The use of a panfungal PCR followed by hybridization with species-specific probes is a practical solution to these limitations. Detection of oligonucleotide probe hybridization has been reported using microtitration plate-based enzyme immunoassay (5, 17, 30), Southern or slot blotting (4, 6, 23, 28), and fluorogenic probes (8, 19, 24). We chose the RLB format, given the advantages of relative simplicity, low cost, ready availability of the materials and the methods, and ability to simultaneously analyze multiple specimens against multiple probes (7). This approach has been reported previously using a proprietary line probe assay

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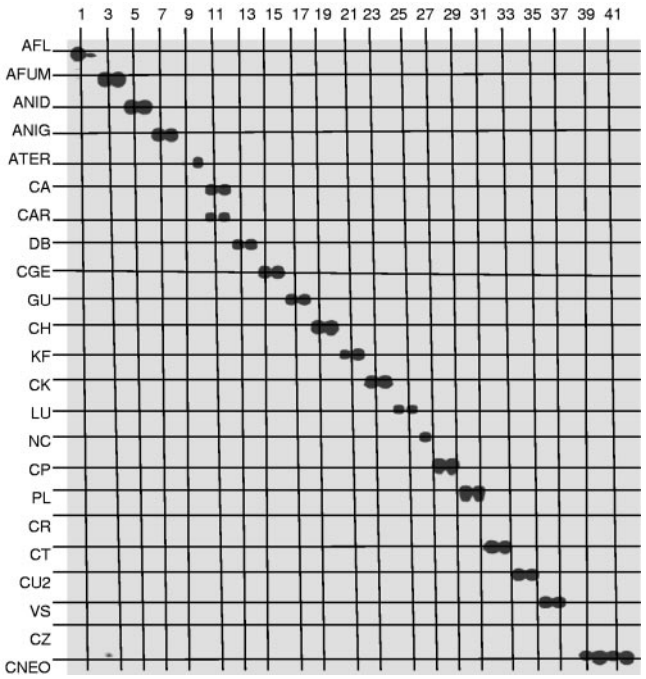

FIG. 1. RLB. Positions of species-specific probes are shown on the left-hand side (abbreviations, Table 2). The ITS2 PCR amplicons are shown in lanes 1 to 42 and include two strains of each species except for one strain of *Candida norvegica* (lane 27), one strain of *Candida famata* (lane 38: no hybridization) and four strains of *Cryptococcus neoformans* (lanes 39 to 42).

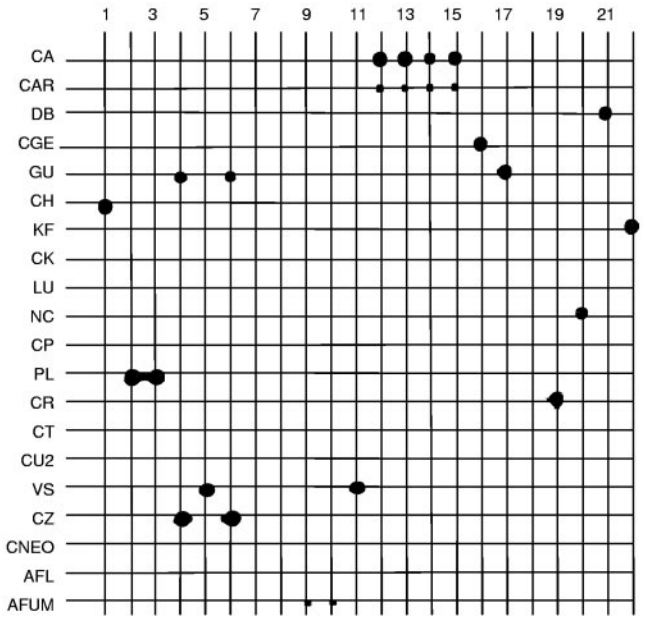

FIG. 2. RLB. Positions of species-specific probes are shown on the left-hand side (abbreviations, Table 2). The ITS2 PCR amplicons are shown in lanes 1 to 22 and include *Candida haemuloni* (lane 1), *Candida pelliculosa* (lanes 2 and 3), *Candida zeylanoides* (lanes 4 and 6), *Candida viswanathii* (lanes 5 and 11), *Candida famata* (lane 7), *Candida lipolytica* (lane 8), *A. fumigatus* (lanes 9 and 10), *Candida albicans* (lanes 12 to 15), *Candida glabrata* (lane 16), *Candida guilliermondii* (lane 17), *Penicillium citrinum* (lane 18), *Candida rugosa* (lane 19), *Candida norvegica* (lane 20), *Candida dubliniensis* (lane 21), and *Candida kefyr* (lane 22). Hybridization between *Candida zeylanoides* DNA (lanes 4 and 6) and both the CZ and GU probes.

with ITS1 probes for eight yeast and three *Aspergillus* species (20). Our assay extends this to 17 yeast species and five *Aspergillus* species. Additional advantages of our method include the capacity to assay up to 43 specimens in a single run and for the membrane to be reused up to nine times without loss of signal. Furthermore, although 23 species-specific probes were used in the present assay, up to 43 can be included on the same membrane, providing the flexibility and capacity to simultaneously detect and identify other fungal or nonfungal microbial pathogens, antimicrobial resistance genes, or microbial virulence genes. Finally, this membrane-based format can be adapted to a DNA microarray format.

On the basis of ITS sequences, all *Candida parapsilosis* strains included in this study were from group I. The ability of the *Candida parapsilosis* probe (CP) to hybridize with *Candida parapsilosis* groups II and II (recently proposed as new species *Candida orthopsilosis* and *Candida metapsilosis* respectively) (27) remains uncertain, although it exhibited 22 of 22, 17 of 22, and 20 of 22 sequence homology with the ITS2 region of *Candida parapsilosis* groups I, II, and III, respectively. The *Cryptococcus neoformans* probe (CNEO) was designed on conserved sequences and thus hybridized with *Cryptococcus neoformans* var. *neoformans*, *Cryptococcus neoformans* var. *grubii*, and *Cryptococcus gattii*.

Although a single amplification round of the panfungal PCR was sufficient for DNA extracted directly from cultures, the nested format maximizes detection limits for DNA extracted directly from clinical specimens. In this regard, the sensitivity of this assay appears promising, as PCR product from DNA extracted from 500-μl aliquots of simulated clinical specimens containing $10^{0.5}$ *Candida* cells/ml of whole blood was reliably detected and identified. Further, from more than half of specimens, 10^0 *Candida* cells/ml were detected. This level of sensitivity will be clinically useful for *Candida* species, given the postulated fungal loads in invasive candidiasis of <1 CFU/ml in approximately 36% of candidemic cases and 1 to 10 CFU/ml in another 28% (1) and is comparable to the limits of detection reported previously using panfungal assays and oligonucleotide probes (4, 10, 20, 25, 28). The sensitivity for *Aspergillus* species was 10^2 conidia/ml, similar to that reported elsewhere (3).

Although not encountered in this study, environmental and carryover contamination remains a potential problem associated with nested panfungal PCR assays. However, as the hybridization of PCR product with species-specific probes is detected visually, the possibility of contamination may be suggested by hybridization with the same probe across multiple samples. Mixed fungal species infections are suggested by hybridization with multiple probes in individual samples.

The multicopy ribosomal gene complex is a useful target for this assay for reasons of sensitivity, high sequence conservation of its 18S, 5.8S, and 28S regions (for panfungal primers), and high variability of its intervening ITS regions (for species-specific probes) with high interspecies and low intraspecies heterogeneity (12, 15, 18). The probes in the present assay were highly specific and differentiated all species unambiguously, apart from the cross-reactivity between the *Candida*

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guilliermondii probe and *Candida zeylanoides* DNA product noted previously (5).

The major limitation of this assay and other molecular assays is time. A full working day is required for DNA extraction (relatively fixed for all assays, approximately 3 h), nested PCR (approximately 3 h), and product detection and identification using the RLB assay (approximately 3 h). We minimized the time required for the PCR by redesigning the panfungal primers ITS3 and ITS4 to allow high annealing temperatures and short cycle times. Although amplification times can be somewhat reduced by real-time PCR techniques, species identification is limited by the number of fluorogenic probes that are able to be incorporated.

We will now evaluate this assay on clinical specimens, particularly whole blood, to determine its utility in the early diagnosis of invasive fungal infections compared with traditional culture-based techniques.

In conclusion, this study demonstrates the accuracy and potential utility of a panfungal molecular assay for the simultaneous detection and identification of a diverse range of clinically important fungal pathogens.

ACKNOWLEDGMENTS

The assistance and advice of Peter Jelfs are gratefully acknowledged. We are grateful to Wieland Meyer for provision of some of the isolates used in this study.

This work was supported in part by an Infrastructure (Stream 3) grant to the Centre for Infectious Diseases and Microbiology (CIDM) Public Health from the New South Wales Department of Health.

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