JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 2007, p. 2872–2880 Vol. 45, No. 9
0095-1137/07/$08.00+0 doi:10.1128/JCM.00687-07

Reverse Line Blot Hybridization Assay for Identification of Medically Important Fungi from Culture and Clinical Specimens▽

Xianyu Zeng,[1,2]† Fanrong Kong,[1]† Catriona Halliday,[1] Sharon Chen,[1] Anna Lau,[1] Geoffrey Playford,[3,4] and Tania C. Sorrell[1,3]*

Centre for Infectious Diseases and Microbiology–Public Health (CIDM-PH), Institute of Clinical Pathology and Medical Research, Westmead Hospital, Westmead, Australia[1]; Research Laboratory for Infectious Skin Diseases, Department of Dermatology, Wuhan First Hospital, Wuhan, Hubei Province, People's Republic of China[2]; Westmead Millennium Institute and Department of Medicine, University of Sydney, Sydney, Australia[3]; and Infection Management Services, Princess Alexandra Hospital, Brisbane, Australia[4]

Received 28 March 2007/Returned for modification 7 June 2007/Accepted 5 July 2007

We evaluated a combined panfungal PCR-reverse line blot (RLB) hybridization assay based on internal transcribed spacer 1 (ITS1) and ITS2 region polymorphisms to identify 159 *Candida*, *Cryptococcus neoformans*, and *Aspergillus* isolates (22 species). Its utility to identify fungal pathogens directly from 27 clinical specimens was also determined. ITS sequence analysis was performed to resolve discrepant identifications or where no RLB result was obtained. Species-specific ITS2- and ITS1-based probes correctly identified 155 of 159 isolates (98%) and 149 (93.7%) isolates, respectively. All strains were unambiguously differentiated with the exception of cross-reactivity between the *Candida norvegensis* probe and *Candida haemulonii* DNA product. Species identification of the pathogen was made for all 21 specimens (sensitivity of 100%) where species-specific probes were included in the RLB; however, there was no ITS2 probe-based hybridization signal for two specimens. Results were concordant with the culture results for 18 (85.7%) specimens. The assay was able to provide species identification in the absence of a culture result (two specimens) and to detect mixed infection (one specimen). The results indicate that the RLB assay is capable of reliably detecting yeasts and *Aspergillus* spp. in clinical specimens and that the incorporation of both ITS1- and ITS2-targeted probes is required for optimal sensitivity. The test has potential utility in the early diagnosis of invasive fungal infection, since "fungal" DNA was detected in all 27 specimens. Prior to incorporation of probes to detect other fungal species, ITS sequencing may be performed to achieve species identification.

Invasive fungal infections (IFIs) are an important cause of morbidity and mortality in severely ill and immunocompromised patients. Recent epidemiological trends indicate a significant shift towards species of *Candida* and *Aspergillus* other than *Candida albicans* and *Aspergillus fumigatus* and a diverse range of less common fungal opportunists (27, 29, 35). Given the reduced susceptibility of many of these pathogens to standard antifungal agents (30, 35, 36), timely and accurate identification to the species level is essential in guiding clinical management. Conventional culture-based phenotypic identification methods, however, are slow and prone to misidentification, particularly with less common or unusual species (20, 34). In addition, the databases of commercial yeast identification systems do not contain all potential pathogens (34).

Molecular approaches using PCR-based methods have been developed to provide rapid and accurate detection of fungi. In particular, the internal transcribed spacer (ITS) regions, ITS1 and ITS2, of the fungal ribosomal DNA gene complex, have shown promise as targets for species identification in a variety of formats, including DNA sequencing and DNA probe hybridization (9, 13, 22). Both length and sequence polymorphisms within the ITS region have permitted accurate identification of pathogenic yeasts and molds (5–7, 12, 14, 22, 25, 33). We previously developed a simple method to detect and identify *Candida*, *Cryptococcus*, and *Aspergillus* species using a panfungal nested PCR followed by hybridization with species-specific oligonucleotide probes targeting the ITS2 region in a reverse line blot (RLB) assay (31).

For optimal sensitivity and specificity, combined analysis of both ITS regions (ITS1 and ITS2) has been proposed (5, 22). We have now designed and incorporated into the RLB assay an additional set of probes targeting the ITS1 region and two fungus-specific probes (targeting the 5.8S rRNA region). In the present study, first we tested the ability of the new RLB assay to identify 159 reference and clinical isolates representing 22 fungal species. We then evaluated the RLB assay on clinical specimens obtained from patients with proven systemic mycoses to determine its utility in the early diagnosis of IFI. When the species identification was ambiguous or differed from the result obtained by culture-based methods (isolates and clinical specimens) or when a culture result was not obtained (clinical specimens), DNA sequence analysis of the ITS region was performed to resolve the uncertainty.

MATERIALS AND METHODS

Fungal isolates and clinical specimens. Reference fungal strains were obtained from the American Type Culture Collection (ATCC), the Centraalbureau voor Schimmelcultures (CBS), the Australian Medical Mycology Reference Lab-

* Corresponding author. Mailing address: Centre for Infectious Diseases and Microbiology, Westmead Hospital, Darcy Road, Westmead, New South Wales 2145, Australia. Phone: 61-2-9845 7195. Fax: 61-2-9893 8659. E-mail: tsorrell@mail.usyd.edu.au.

† Xianyu Zeng and Fanrong Kong made similar contributions to the work and are listed as joint first authors.

▽ Published ahead of print on 18 July 2007.

TABLE 1. Species identification of fungal isolates studied by the RLB assay

Strain	Species submitted as	Species identification by RLB with the following probe:	
		ITS1	ITS2
ATCC 90028	*Candida albicans*	*C. albicans*	*C. albicans*
CBS 562	*C. albicans*	*C. albicans*	*C. albicans*
WM01-157	*C. albicans*	*C. albicans*	*C. albicans*
WM01-158	*C. albicans*	*C. albicans*	*C. albicans*
WM01-159	*C. albicans*	*C. albicans*	*C. albicans*
WM01-160	*C. albicans*	*C. albicans*	*C. albicans*
WM01-161	*C. albicans*	*C. albicans*	*C. albicans*
WM01-162	*C. albicans*	*C. albicans*	*C. albicans*
WM01-163	*C. albicans*	*C. albicans*	*C. albicans*
WM01-217b	*C. albicans*	*C. albicans*	*C. albicans*
WM01-217h	*C. albicans*	*C. albicans*	*C. albicans*
WM01-218	*C. albicans*	*C. albicans*	*C. albicans*
CBS 7988	*Candida dubliniensis*	*C. dubliniensis*	*C. dubliniensis*
CBS 7987	*C. dubliniensis*	*C. dubliniensis*	*C. dubliniensis*
WM02-73	*C. dubliniensis*	*C. dubliniensis*	*C. dubliniensis*
WM03-78	*C. dubliniensis*	*C. dubliniensis*	*C. dubliniensis*
WM03-133	*C. dubliniensis*	*C. dubliniensis*	*C. dubliniensis*
WM03-160	*C. dubliniensis*	*C. dubliniensis*	*C. dubliniensis*
WM03-66	*C. dubliniensis*	*C. dubliniensis*	*C. dubliniensis*
WM03-79	*C. dubliniensis*	*C. dubliniensis*	*C. dubliniensis*
WM03-119	*C. dubliniensis*	*C. dubliniensis*	*C. dubliniensis*
WM01-54	*C. dubliniensis*	*C. dubliniensis*	*C. dubliniensis*
ATCC 90030	*Candida glabrata*	*C. glabrata*	*C. glabrata*
WM02-57	*C. glabrata*	*C. glabrata*	*C. glabrata*
WM02-174	*C. glabrata*	*C. glabrata*	*C. glabrata*
CBS 2030	*Candida guilliermondii*	*C. guilliermondii*	*C. guilliermondii*
CBS 2031	*C. guilliermondii*	*C. guilliermondii*	*C. guilliermondii*
CBS 5256	*C. guilliermondii*	*C. guilliermondii*	*C. guilliermondii*
WM02-72	*C. guilliermondii*	*C. guilliermondii*	*C. guilliermondii*
WM02-91	*C. guilliermondii*	*C. guilliermondii*	*C. guilliermondii*
WM02-131	*C. guilliermondii*	*C. guilliermondii*	*C. guilliermondii*
WM02-356	*C. guilliermondii*	*C. guilliermondii*	*C. guilliermondii*
WM02-374	*C. guilliermondii*	*C. guilliermondii*	*C. guilliermondii*
WM02-132	*C. guilliermondii*	*C. guilliermondii*	*C. guilliermondii*
WM02-361	*C. guilliermondii*	*C. guilliermondii*	*C. guilliermondii*
CBS 5149	*Candida haemulonii*	*C. haemulonii* + *C. norvegensis*	*C. haemulonii* + *C. norvegensis*
WM 889	*C. haemulonii*	*C. haemulonii* + *C. norvegensis*	*C. haemulonii* + *C. norvegensis*
WM 890	*C. haemulonii*	*C. haemulonii* + *C. norvegensis*	*C. haemulonii* + *C. norvegensis*
WM 891	*C. haemulonii*	*C. haemulonii* + *C. norvegensis*	*C. haemulonii* + *C. norvegensis*
CBS 834	*Candida kefyr*	*C. kefyr*	*C. kefyr*
CBS 712	*C. kefyr*	*C. kefyr*	*C. kefyr*
ATCC 4135	*C. kefyr*	*C. kefyr*	*C. kefyr*
WM 927	*C. kefyr*	*C. kefyr*	*C. kefyr*
ATCC 6258	*Candida krusei*	*C. krusei*	*C. krusei*
CBS 573	*C. krusei*	*C. krusei*	*C. krusei*
WM02-8	*C. krusei*	*C. krusei*	*C. krusei*
WM02-78	*C. krusei*	*C. krusei*	*C. krusei*
WM03-98	*C. krusei*	*C. krusei*	*C. krusei*
WM03-108	*C. krusei*	*C. krusei*	*C. krusei*
WM03-189	*C. krusei*	*C. krusei*	*C. krusei*
WM03-190	*C. krusei*	*C. krusei*	*C. krusei*
WM03-204	*C. krusei*	*C. krusei*	*C. krusei*
WM1057	*C. krusei*	*C. krusei*	*C. krusei*
WM1044	*C. krusei*	*C. krusei*	*C. krusei*
AS142	*C. krusei*	*C. krusei*	*C. krusei*
CBS 5094	*Candida lusitaniae*	*C. lusitaniae*	*C. lusitaniae*
CBS 5901	*C. lusitaniae*	*C. lusitaniae*	*C. lusitaniae*
CBS 4413	*C. lusitaniae*	*C. lusitaniae*	*C. lusitaniae*
CBS 6936	*C. lusitaniae*	*C. lusitaniae*	*C. lusitaniae*
WM02-92	*C. lusitaniae*	*C. lusitaniae*	*C. lusitaniae*
WM02-348	*C. lusitaniae*	*C. lusitaniae*	*C. lusitaniae*
WM02-9	*C. lusitaniae*	*C. lusitaniae*	*C. lusitaniae*
WM1036	*C. lusitaniae*	*C. lusitaniae*	*C. lusitaniae*
WM1037	*C. lusitaniae*	*C. lusitaniae*	*C. lusitaniae*
WM1038	*C. lusitaniae*	*C. lusitaniae*	*C. lusitaniae*
WM1074	*C. lusitaniae*	*C. lusitaniae*	*C. lusitaniae*

Continued on following page

TABLE 1—*Continued*

Strain	Species submitted as	Species identification by RLB with the following probe:	
		ITS1	ITS2
WM1138	*C. lusitaniae*	*C. lusitaniae*	*C. lusitaniae*
CBS 6403	*Candida norvegensis*	*C. norvegensis*	*C. norvegensis*
CBS 6564	*C. norvegensis*	*C. norvegensis*	*C. norvegensis*
CBS 4239	*Candida norvegica*	*C. norvegica*	*C. norvegica*
ATCC 22019	*Candida parapsilosis*	*C. parapsilosis*	*C. parapsilosis*
CBS 8501	*C. parapsilosis*	*C. parapsilosis*	*C. parapsilosis*
WM02-2	*C. parapsilosis*	*C. parapsilosis*	*C. parapsilosis*
WM02-61b	*C. parapsilosis*	*C. parapsilosis*	*C. parapsilosis*
WM02-75	*C. parapsilosis*	*C. parapsilosis*	*C. parapsilosis*
WM02-89	*C. parapsilosis*	*C. parapsilosis*	*C. parapsilosis*
WM02-95	*C. parapsilosis*	*C. parapsilosis*	*C. parapsilosis*
WM02-130a	*C. parapsilosis*	*C. parapsilosis*	*C. parapsilosis*
WM02-166	*C. parapsilosis*	*C. parapsilosis*	*C. parapsilosis*
WM02-177	*C. parapsilosis*	*C. parapsilosis*	*C. parapsilosis*
WM02-351	*C. parapsilosis*	*C. parapsilosis*	*C. parapsilosis*
WM2 359	*C. parapsilosis*	*C. parapsilosis*	*C. parapsilosis*
WM01-201	*C. parapsilosis*	*C. parapsilosis*	*C. parapsilosis*
WM01-219	*C. parapsilosis*	*C. parapsilosis*	*C. parapsilosis*
WM01-216	*C. parapsilosis*	*C. parapsilosis*	*C. parapsilosis*
CBS 605	*Candida pelliculosa*	*C. pelliculosa*	*C. pelliculosa*
WM 1039	*C. pelliculosa*	*C. pelliculosa*	*C. pelliculosa*
CBS 94	*Candida tropicalis*	*C. tropicalis*	*C. tropicalis*
WM01-202	*C. tropicalis*	*C. tropicalis*	*C. tropicalis*
WM01-203	*C. tropicalis*	*C. tropicalis*	*C. tropicalis*
WM01-215	*C. tropicalis*	*C. tropicalis*	*C. tropicalis*
WM02-86	*C. tropicalis*	*C. tropicalis*	*C. tropicalis*
WM02-87	*C. tropicalis*	*C. tropicalis*	*C. tropicalis*
WM02-181	*C. tropicalis*	*C. tropicalis*	*C. tropicalis*
WM02-183	*C. tropicalis*	*C. tropicalis*	*C. tropicalis*
WM02-355	*C. tropicalis*	*C. tropicalis*	*C. tropicalis*
WM1045	*C. tropicalis*	*C. tropicalis*	*C. tropicalis*
CBS 621	*Candida utilis*	*C. utilis*	*C. utilis*
CBS 1600	*C. utilis*	*C. utilis*	*C. utilis*
CBS 4024T	*Candida viswanathii*	*C. viswanathii*	*C. viswanathii*
CBS 619	*Candida zeylanoides*	*C. zeylanoides*	*C. zeylanoides*
ATCC 204305	*Aspergillus fumigatus*	*A. fumigatus*	*A. fumigatus*
AS41	*A. fumigatus*	*A. fumigatus*	*A. fumigatus*
AS42	*A. fumigatus*	*A. fumigatus*	*A. fumigatus*
AS43	*A. fumigatus*	*A. fumigatus*	*A. fumigatus*
AS44	*A. fumigatus*	*A. fumigatus*	*A. fumigatus*
AS45	*A. fumigatus*	*A. fumigatus*	*A. fumigatus*
AS46	*A. fumigatus*	*A. fumigatus*	*A. fumigatus*
AS48	*A. fumigatus*	*A. fumigatus*	*A. fumigatus*
AS49	*A. fumigatus*	*A. fumigatus*	*A. fumigatus*
AS51	*A. fumigatus*	*A. fumigatus*	*A. fumigatus*
AS52	*A. fumigatus*	*A. fumigatus*	*A. fumigatus*
AS116	*Aspergillus nidulans*	*A. nidulans*	*A. nidulans*
AS60	*A. nidulans*	*A. nidulans*	*A. nidulans*
AS69	*A. nidulans*	*A. nidulans*	*A. nidulans*
AS128	*A. nidulans*	*A. nidulans*	*A. nidulans*
AS53	*A. fumigatus*	*A. fumigatus*	*A. fumigatus*
AS54	*A. fumigatus*	*A. fumigatus*	*A. fumigatus*
AS56	*A. fumigatus*	*A. fumigatus*	*A. fumigatus*
AS57	*A. fumigatus*	*A. fumigatus*	*A. fumigatus*
AS58	*A. fumigatus*	*A. fumigatus*	*A. fumigatus*
AS59	*A. fumigatus*	*A. fumigatus*	*A. fumigatus*
ATCC 204304	*Aspergillus flavus*	*A. flavus*	*A. flavus*
AS119	*A. flavus*	*A. flavus*	*A. flavus*
AS120	*A. flavus*	*A. flavus*	*A. flavus*
AS121	*A. flavus*	*A. flavus*	*A. flavus*
AS123	*A. flavus*	*A. flavus*	*A. flavus*
AS61	*Aspergillus terreus*	*A. terreus*	*A. terreus*
AS62	*A. terreus*	*A. terreus*	*A. terreus*
AS63	*A. terreus*	*A. terreus*	*A. terreus*
AS64	*A. terreus*	*A. terreus*	*A. terreus*
AS75	*A. terreus*	*A. terreus*	*A. terreus*

Continued on following page

TABLE 1—*Continued*

Strain	Species submitted as	Species identification by RLB with the following probe:	
		ITS1	ITS2
AS7	*Aspergillus niger*	NS[a]	*A. niger*
AS31	*A. niger*	NS	*A. niger*
AS32	*A. niger*	*A. niger*	*A. niger*
AS33	*A. niger*	NS	*A. niger*
AS35	*A. niger*	*A. niger*	*A. niger*
AS36	*A. niger*	NS	*A. niger*
AS37	*A. niger*	NS	*A. niger*
AS38	*A. niger*	*A. niger*	*A. niger*
AS39	*A. niger*	NS	*A. niger*
RV 58146	*Cryptococcus neoformans* var. *neoformans*	*C. neoformans* complex	*C. neoformans* complex
JG 02	*C. neoformans* var. *neoformans*	*C. neoformans* complex	*C. neoformans* complex
RV 58146	*C. neoformans* var. *neoformans*	*C. neoformans* complex	*C. neoformans* complex
PR 101	*C. neoformans* var. *neoformans*	*C. neoformans* complex	*C. neoformans* complex
CDCR 461	*Cryptococcus neoformans* var. *grubii*	*C. neoformans* complex	*C. neoformans* complex
KRIMM 2	*C. neoformans* var. *grubii*	*C. neoformans* complex	*C. neoformans* complex
CBS 7816	*C. neoformans* var. *grubii*	*C. neoformans* complex	*C. neoformans* complex
WM01-84	*C. neoformans* var. *grubii*	*C. neoformans* complex	*C. neoformans* complex
WM01-85	*C. neoformans* var. *grubii*	*C. neoformans* complex	*C. neoformans* complex
WM148	*C. neoformans* var. *grubii*	*C. neoformans* complex	*C. neoformans* complex
WM179	*Cryptococcus gattii*	*C. neoformans* complex	*C. neoformans* complex
4A	*C. gattii*	*C. neoformans* complex	*C. neoformans* complex
4B	*C. gattii*	*C. neoformans* complex	*C. neoformans* complex
4C	*C. gattii*	*C. neoformans* complex	*C. neoformans* complex
10A	*C. gattii*	*C. neoformans* complex	*C. neoformans* complex
10B	*C. gattii*	*C. neoformans* complex	*C. neoformans* complex
10B	*C. gattii*	*C. neoformans* complex	*C. neoformans* complex
10C	*C. gattii*	*C. neoformans* complex	*C. neoformans* complex

[a] Abbreviation: NS, hybridized only with fungus-specific probes and no hybridization signal with species-specific probes.

oratory at Royal North Shore Hospital, Sydney, Australia, and the Molecular Mycology Research Laboratory at Westmead Hospital, Westmead, Australia. Clinical isolates were obtained from the Mycology Laboratory at Westmead Hospital. Isolates were identified using standard colonial and microscopic characteristics (for molds) (20, 37) and the VITEK I (bioMerieux Vitek, Hazelwood, MO) and/or ID 32C (bioMerieux, Marcy-l'Etoile, France) commercial systems (for yeasts). Canavanine-glycine bromothymol blue (CGB) agar was used to differentiate between *Cryptococcus neoformans* (*Cryptococcus neoformans* var. *neoformans* and *Cryptococcus neoformans* var. *grubii*) and *Cryptococcus gattii* (19).

A total of 159 (32 reference and 127 clinical) isolates belonging to 22 fungal species were studied; all species were represented by species-specific probes in the RLB assay (Table 1). Isolates comprised 16 *Candida* species (101 strains; *Candida albicans*, *Candida dubliniensis*, *Candida glabrata*, *Candida guilliermondii*, *Candida haemulonii*, *Candida kefyr*, *Candida krusei*, *Candida lusitaniae*, *Candida norvegica*, *Candida norvegensis*, *Candida parapsilosis*, *Candida pelliculosa*, *Candida tropicalis*, *Candida utilis*, *Candida viswanathii*, *Candida zeylanoides*), *C. neoformans* complex (five strains of *C. neoformans* var. *grubii*, four strains of *C. neoformans* var. *neoformans*, and eight strains of *C. gattii*), and five *Aspergillus* species (40 strains; *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus terreus*, and *Aspergillus nidulans*).

Twenty-seven clinical specimens from 24 patients with proven IFI were evaluated (Table 2). Specimens included fresh tissue ($n = 13$), formalin-fixed paraffin-embedded (PE) tissue ($n = 7$), anticoagulated whole blood ($n = 4$), bronchoalveolar lavage (BAL; $n = 2$) fluid, and cerebrospinal fluid (CSF; $n = 1$) samples.

DNA extraction. For fungal isolates, DNA extraction was performed as previously described (31). For clinical specimens, DNA extractions were performed using the MagNA Pure LC instrument (Roche Diagnostics, Mannheim, Germany) in a class II laminar flow cabinet as outlined below.

(i) Blood, BAL fluid, and CSF specimens. The MagNA Pure LC total nucleic acid isolation kit (Roche Diagnostics) was used for extraction of blood, BAL, and CSF specimens with some modifications. Blood samples (500 μl) were lysed with erythrocyte lysis buffer and incubated with sorbitol buffer and lyticase as outlined previously (11). BAL (600-μl) and CSF (100- to 200-μl) samples were centrifuged at 16,100 × *g* for 10 min, and the pellet was resuspended in 200 μl of sorbitol buffer (1 M sorbitol, 100 mM EDTA, and 0.1% 2-mercaptoethanol) (38) and 200 U lyticase (Sigma-Aldrich, Castle Hill, Australia). After incubation at 37°C for 60 min, the spheroplasts were precipitated by centrifugation at 5,400 × *g* for 5 min. The supernatant was discarded, and the pellet was resuspended in 200 μl of distilled H_2O before being transferred to the MagNA Pure sample cartridge for processing on the MagNA Pure LC instrument (Roche Diagnostics).

(ii) Fresh and PE tissue specimens. The MagNA Pure LC DNA isolation kit II (tissue) (Roche Diagnostics) was used to extract DNA from fresh and PE tissue specimens as previously described (21).

Oligonucleotide design. Relevant fungal DNA sequences spanning the fungal ribosomal DNA gene complex (18S, 5.8S, and 28S) and the intervening ITS1 and ITS2 regions were accessed from GenBank and compared using the Pileup and Pretty programs provided by BioManager, ANGIS (http://biomanager.angis.org.au/). Three pairs of panfungal primers amplifying the ITS1 (its1Sb and its2Ab), ITS2 (its3Sb and its4Ab), and ITS1, 5.8S, and ITS2 regions (SR6RL/LR1L) (Table 3) were designed from the multiple-sequence alignment. All primers were 5′-end biotin labeled (Sigma-Aldrich). Two panfungal oligonucleotide probes (its12Ap and its23Sp) were designed to target the 5.8S rRNA region (Table 3). Species-specific fungal probes targeted the ITS1 or ITS2 region. The ITS2 probes have been described previously (31). The ITS1 probes were either modified from published probe sequences (28) or designed de novo (Table 3). All oligonucleotide probes were 5′-hexylamine labeled (Sigma-Aldrich).

Multiplex PCR and nested PCR. (i) Fungal isolates. Multiplex PCR using the primer pairs, its1Sb/its2Ab and its3Sb/its4Ab, was performed to amplify the ITS1 and ITS2 regions. PCRs were performed in 25-μl reaction mixtures containing 0.5 U HotStar *Taq* polymerase and 1× PCR buffer (catalogue no. 203203; QIAGEN, Doncaster, Victoria, Australia), 125 μM of each dATP, dCTP, dGTP, and dTTP (Roche Diagnostics), 0.5 μM of each forward and reverse primer, and 5 μl of DNA template. Amplification was performed on a Mastercycler gradient thermocycler (Eppendorf AG, North Ryde, Australia). The thermal cycling conditions were 95°C for 15 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s, with a final extension step at 72°C for 10 min.

(ii) Clinical specimens. A nested PCR assay was used as follows: the first-round PCR targeted the ITS1, 5.8S, and ITS2 regions using the primer pair SR6RL/LR1L, and the second-round PCR amplified the ITS1 or ITS2 region using primer pairs its1Sb/its2Ab and its3Sb/its4Ab, respectively. The first-round

TABLE 2. Results of culture, histology, RLB, and ITS sequence analysis of clinical specimens from patients with proven IFI

Patient no.	Specimen type	Clinical diagnosis[a]	Identification by culture/histology	Molecular identification by:		
				RLB	ITS1 sequencing (% identity with GenBank sequence)	ITS2 sequencing (% identity with GenBank sequence)
1	Blood	Candidemia	*Candida albicans*	*C. albicans*		
2	Blood	Candidemia	*C. albicans*	*C. albicans*		
3	Blood	Candidemia	*C. albicans*	*C. albicans*[b]	*C. albicans* (99)	No result
4	CSF	IC	*C. albicans*	*C. albicans*		
5	Tissue (vocal cord)	IC	*C. albicans*	*C. albicans*		
6	Blood	Candidemia	*Candida parapsilosis*	*C. parapsilosis*		
7	Tissue (bladder)	IC	*Candida krusei*	*C. krusei*		
8	Tissue (skin)	IC	*Candida glabrata*	*C. glabrata*		
9	BAL	Cryptococcosis	*Cryptococcus neoformans*	*C. neoformans* complex[c]		
10	BAL	Cryptococcosis	*C. neoformans*	*C. neoformans* complex		
11	Tissue (nasal mucosa)	Cryptococcosis	*C. neoformans*	*C. neoformans* complex		
12	PE tissue (nasal cavity)	Cryptococcosis	*C. neoformans*	*C. neoformans* complex[b]	*C. neoformans* complex (99)	No result
13	Tissue (lung)	Cryptococcosis	*C. neoformans*	*C. neoformans* complex		
14	PE tissue (skin)	Cryptococcosis	*C. gattii*	*C. neoformans* complex		
15	Tissue (lung)	IA	*Aspergillus fumigatus*	*A. fumigatus*		
16	Tissue (nasal cavity)	IA	*A. fumigatus*	*A. fumigatus*		
17	PE tissue (nasal cavity)	IA	*A. fumigatus*	*A. fumigatus*		
18	Tissue (sinus)	IFI	Filamentous fungus	*A. fumigatus*		
	PE tissue (sinus)	IFI	Filamentous fungus	*A. fumigatus*		
19	Tissue (nasal mucosa)	IA	*A. fumigatus*	*A. fumigatus* + *A. flavus*[d]	*A. fumigatus* (100)	*A. fumigatus* (100)
20	Tissue (sinus)	IA	*Aspergillus flavus*	*A. flavus*		
21	Tissue (skin)	Dematiaceous IFI	*Exophiala jeanselmei*	NS[e]	*Exophiala spinifera*	No result
22	Tissue (buttock)	Zygomycosis	*Apophysomyces elegans*	NS	*A. elegans* (100)[f]	Not performed
	Tissue (buttock)	Zygomycosis	*A. elegans*	NS	*A. elegans* (96)	No result
23	PE tissue (lung)	Zygomycosis	Filamentous fungus	NS	*Rhizopus microsporus* (99)[f]	Not performed
24	PE tissue (pericardium)	IFI	Fungal hyphae	NS	*Trichosporon asahii* (100)[f]	Not performed
	PE tissue (kidney)	IFI	Fungal hyphae	NS	*T. asahii* (99)[f]	Not performed

[a] Abbreviations: IA, invasive aspergillosis; IC, invasive candidiasis.
[b] Hybridized with the species-specific ITS1 probe only.
[c] The assays did not differentiate between members of the *C. neoformans* complex; however, if necessary, the known limited diversity in the ITS region would be useful in differentiating between species and varieties of *C. neoformans*.
[d] Hybridized with both (ITS1 and ITS2) *A. fumigatus*-specific probes and with both (ITS1 and ITS2) *A. flavus*-specific probes.
[e] NS, hybridization with fungus-specific probes but not with species-specific probes.
[f] As published by Lau et al. (21).

PCR mixture (25 μl) consisted of 1× PCR buffer 1 (0 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, and 0.001% gelatin) (Applied Biosystems, Foster City, CA), 5% glycerol (Sigma-Aldrich), 25 μM each dATP, dCTP, dGTP, and dTTP, 0.5 μM of each forward and reverse primer, 1.25 U of AmpliTaq Gold DNA polymerase, and 10 μl DNA template. The thermal cycling conditions were 95°C for 10 min, followed by 30 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 90 s, and a final extension at 72°C for 6 min. Conditions for the second-round PCR were identical, except that 2 μl of template was used and the amplification involved 35 cycles.

RLB hybridization assay. The RLB assay was performed as previously described (18, 31). Briefly, the amplified PCR product was hybridized with membrane-bound probes at 60°C for 1 h, the membrane was washed twice (10 min each time) at 60°C with prewarmed (to 60°C) 2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7])-0.5% sodium dodecyl sulfate (SDS) and incubated in 15 ml of streptavidin-peroxidase conjugate (Roche Diagnostics) diluted 1:4,000 in 2× SSPE-0.5% SDS for 60 min at 42°C. The membrane was further washed with 2× SSPE-0.5% SDS at 42°C and then at 25°C. If present, bound PCR products were detected by chemiluminescence using ECL detection liquid (Amersham) and visualized by exposure for 7 min to an X-ray film (Hyperfilm; Amersham).

ITS sequencing. ITS sequence analysis was performed for fungal strains and clinical specimens which yielded either a discrepant (compared to culture) or ambiguous species identification result or when no hybridization signal was obtained by the RLB assay. For fungal strains, the ITS1, 5.8S, and ITS2 regions were amplified using the SR6RL and LR1L primer pair as described for multiplex PCR (see above). For clinical specimens, parameters used for PCR were identical to those used for the RLB assay except that in the second amplification, either the primer pair its1Sb/its2Ab or its3Sb/its4Ab was used. Both the ITS1 and ITS2 regions were amplified and analyzed. PCR products were purified using the PCR Product Pre-sequencing kit (USB Corporation, Cleveland, OH) and were sequenced using forward primer and the BigDye Terminator (version 3.1) cycle sequencing kit in the ABI PRISM 3100 genetic analyzer (Applied Biosystems). Sequences were entered into a BLASTn sequence analysis search (2) (accessed via http://biomanager.angis.org.au/).

RESULTS

Fungal isolates. The universal fungal primers SR6RL and LR1L amplified the ITS1, 5.8S, and ITS2 regions for all 159 isolates. Species-specific ITS2 probes correctly identified 155 (98%) isolates by the RLB assay; the corresponding ITS1 probes identified 149 of 159 (93.7%) strains (Table 1). With one exception (see below), the ITS1- and ITS2-specific RLB assays clearly identified major *Candida* species, including *C. zeylanoides* and *C. guilliermondii* (Table 1). The assays did not differentiate between members of the *C. neoformans* complex.

RLB assay results, using both ITS1 and ITS2 species-specific probes, correlated with culture identification for 149 of 159 (93.7%) strains. Isolates that produced inconsistent results or no hybridization signal are listed in Table 4. Four *C. haemulonii* strains hybridized with both (ITS1 and ITS2) *C. haemulonii*-specific and *C. norvegensis*-specific probes; in all instances, ITS sequence analysis yielded sequences with 100% identity to *C. haemulonii* sequences in the GenBank database. In the ITS regions employed for probe design, *C. haemulonii*- and *C. norvegensis*-specific sequences had sequence identities of 35% and 55% for the ITS1 and ITS2 region, respectively. The other discrepant results involved six of the nine *A. niger* strains (AS7,

TABLE 3. Primers and oligonucleotide probes used in this study

Primer or probe	Target	T_m (°C)[a]	GenBank accession no.	Sequence (5′ to 3′)[b]
Primers				
its1Sb	18S	68.4	AF455524	**19**TCCGTAGGGAACCTGCGG**37**
its12Ap	5.8S	64.6	AF455524	**211**CCAAGAGATCCGTTGTTGAAAG**190**
its2Ab	5.8S	69.1	AF455524	**237**CGCTGCGYTCTTCATCGATG**208**
its3Sb	5.8S	62.0	AF455524	**242**GCGATAMGTAATRTGAATTGCAG**264**
its23Sp	5.8S	64.3	AF455524	**271**GTGAATCATCGARTCTTTGAACG**293**
its4Ab	28S	72.9	L28817	**741**GTTGGTTTCTTTTCCTCCGCTTAT TGATATGC**710**
SR6RL	18S	73.5	DQ157694	**529**CAAACYYGGTCAYTTAGAGGAAGWAAAAGTCG TAACAAGG**568**
LR1L	28S	74.6	DQ139803	**46**CTRRGGCAATCCCDGTTGGTTTCTTTTCCT**17**
Probes				
CA	ITS1 of *C. albicans*	68.4	AF455524	**149**TTTATCAACTTGTCACACCAGA**170**
CD	ITS1 of *C. dubliniensis*	67.3	AJ249485	**51**ACATGTGTTTTGTTYTGGACAAACTTG**77**
CG	ITS1 of *C. glabrata*	68.2	AF455515	**22**TGTCTGAGCTCGGAGAGAGACATC**45**
CGU	ITS1 of *C. guilliermondii*	67.0	AB105435	**107**GCTTTGGTTTGGCCTAGAGATAGGT**131**
CH	ITS1 of *C. haemulonii*	62.4	AY500375	**64**GCAACCACCGTTAAGTTCAA**83**
CKF	ITS1 of *C. kefyr*	66.5	AY046214	**118**GAGTTTTCYCTATGAACTACTTCCCTGG**147**
CKR	ITS1 of *C. krusei*	64.8	AB054034	**95**TGTGGAATATAGCATATAGTCGACAAGAG**123**
CLUS	ITS1 of *C. lusitaniae*	62.6	AY493434	**299**TGTCAAACACGTTTACAGCACG**320**
CNOV	ITS1 of *C. norvegica*	64.6	AY936525	**55**TATGCGAGATTGCTTTGGCT**74**
CNS1	ITS1 of *C. norvegensis*	70.4	AY939799	**58**CGTGAGCGCACAACAACAC**77**
CNS2	ITS2 of *C. norvegensis*	69.6	AY939799	**406**GGCCCGCCGAACTTTTTTTT**425**
CP	ITS1 of *C. parapsilosis*	65.1	AF455530	**148**CTGCCAGAGATTAAACTCAACCAA**171**
CPL	ITS1 of *C. pelliculosa*	65.21	AF270936	**118**YGCCCAAAGGTCTAAACACATTT**140**
CT	ITS1 of *C. tropicalis*	64.5	AF287910	**121**CTACCGCCAGAGGTTATAACTAAACC**146**
CU	ITS1 of *C. utilis*	68.5	AF335929	**57**CGGCTCCAACCAATACACAGTG**78**
CV	ITS1 of *C. viswanathii*	69.9	AY139791	**57**GTTTTTTACTGGACAGCTGCTTTGGC**82**
CZ	ITS1 of *C. zeylanoides*	66.7	AF335930	**110**GGTCTGACTTAGAAATGAGTTGGGC**134**
CRY	ITS1 of *Cryptococcus neoformans* complex	64.5	AJ493561	**64**TTCGGCACGTTTTACACAAAC**84**
AFL	ITS1 of *A. flavus*	67.1	AY373848	**174**TCTAGTGAAGTCTGAGTTGATTGTATCGC**202**
AFUM	ITS1 of *A. fumigatus*	64.5	AY373851	**181**GTATGCAGTCTGAGTTGATTATCGTAATC**209**
ANID	ITS1 of *A. nidulans*	70.1	AY452983	**161**CTTCATGCCTGAGAGTGATGCAGTC**185**
ANIG	ITS1 of *A. niger*	63.4	AY213633	**65**CGTGTCTATTATACCCTGTTGCTTC**89**
ATER	ITS1 of *A. terreus*	68.9	AY373871	**182**CTTGCAGTCTGAGTGTGATTCTTTGC**207**

[a] T_m, melting temperature.
[b] Boldface numbers represent the numbered base positions where the primer or probe sequences start or finish (starting at point 1 of the corresponding gene GenBank sequence). Underlined sequences show bases added to modify previously published probes and primers (28).

AS31, AS33, AS36, AS37, and AS39) studied. These isolates hybridized with the ITS2, but not the ITS1, *A. niger*-specific probe (Table 4). Sequence analysis of the ITS1 region for all six strains demonstrated 100% identity to *A. niger* ATCC 16888 sequence (GenBank accession no. AY373852); this sequence showed a single base polymorphism (from A to G) with the GenBank sequence used to design the ITS1-based probe (GenBank accession no. AY213633).

Clinical specimens. The RLB assay detected fungal DNA in all 27 specimens using the panfungal probe. The identification of the fungal pathogen, as determined by phenotypic-based methods, RLB, and/or ITS sequence analysis are summarized in Table 2. RLB analysis assigned a species identification for all 21 specimens for which species-specific probes were available. The sensitivities of the assay with the ITS1- and ITS2-based probes were 100% (21 of 21) and 90.5% (19 of 21), respectively. Hybridization occurred with both probes for all but two specimens (patients 3 and 12) where a hybridization signal was observed only with the ITS1 probe.

Concordant results with culture were obtained for 18 of 21 (85.7%) specimens using both ITS1 and ITS2 species-specific probes. For two specimens (from patient 18), RLB provided a species identification in the absence of a culture result. For the remaining specimen (nasal mucosa; patient 19), *A. fumigatus* was recovered on culture but both *A. fumigatus* and *A. flavus* DNA were detected by RLB (Table 2). ITS sequence analysis detected *A. fumigatus* DNA only (100% sequence identity to *A. fumigatus* sequences in GenBank accession no. AY939790). Examination of *A. fumigatus*- and *A. flavus*-specific ITS sequences in the region targeted by the probes revealed they were dissimilar.

No hybridization signal was observed for six specimens (patients 21 to 24; Table 2). In these instances, ITS1 and ITS2 sequencing identified the pathogen to be a fungus not represented by probes on the RLB (as described previously for specimens from patients 23 and 24 [21] and in this study for specimens from patients 21 and 22). The molecular identification was consistent with culture and/or histological diagnosis for five of six (83.3%) specimens. The single discrepancy involved a skin biopsy specimen (patient 21) where culture-based methods identified the pathogen as *Exophiala jeanselmei* but sequencing revealed results with 99% identity to *Exophiala*

TABLE 4. List of isolates that produced ambiguous species identification or that were not identified by the RLB assay

Strain	Species received as	Species identification by:		
		RLB with ITS1 probe	RLB with ITS2 probe	ITS sequencing (% identity with GenBank sequences)
Reference strains				
CBS 5149	*Candida haemulonii*	*C. haemulonii* + *C. norvegensis*	*C. haemulonii* + *C. norvegensis*	*C. haemulonii* (100)[b]
WM 889	*C. haemulonii*	*C. haemulonii* + *C. norvegensis*	*C. haemulonii* + *C. norvegensis*	*C. haemulonii* (100)[b]
WM 890	*C. haemulonii*	*C. haemulonii* + *C. norvegensis*	*C. haemulonii* + *C. norvegensis*	*C. haemulonii* (100)[b]
WM 891	*C. haemulonii*	*C. haemulonii* + *C. norvegensis*	*C. haemulonii* + *C. norvegensis*	*C. haemulonii* (100)[b]
Clinical isolates				
AS7	*Aspergillus niger*	NS[a]	*A. niger*	*A. niger*[c]
AS31	*A. niger*	NS	*A. niger*	*A. niger*[c]
AS33	*A. niger*	NS	*A. niger*	*A. niger*[c]
AS36	*A. niger*	NS	*A. niger*	*A. niger*[c]
AS37	*A. niger*	NS	*A. niger*	*A. niger*[c]
AS39	*A. niger*	NS	*A. niger*	*A. niger*[c]

[a] Abbreviation: NS, hybridization with fungus-specific probes but not with species-specific probes.
[b] Sequence analysis yielded sequences with 100% identity to *C. haemulonii* sequences in GenBank (accession nos. AY50035 and AJ606467).
[c] Sequence analysis yielded sequences with 100% identity to *A. niger* ATCC 16888 sequence (GenBank accession no. AY373852) but with a single base polymorphism (from A to G) with the GenBank sequence used to design the probe (GenBank accession no. AY213633).

spinifera (88 to 92% identity to *E. jeanselmei*; GenBank accession no. AY163550 and AY163556, respectively).

DISCUSSION

This study demonstrates the feasibility of combining a panfungal nested PCR with probe hybridization technology in a RLB format to identify clinically important fungal pathogens. Important features of this RLB assay, which incorporates both ITS1- and ITS2-targeted probes, include the high sensitivity (98%) for the identification of 22 fungal species and the ability to identify fungal pathogens directly from clinical specimens.

The validity of the RLB assay was confirmed by analysis of a large number of the more common pathogenic yeasts and *Aspergillus* species. With one exception, major *Candida* spp. were unambiguously differentiated. The cross-reactivity between the *C. norvegensis* probe and *C. haemulonii* DNA product is unexplained, as multiple GenBank database ITS sequences in the region employed for probe design between these species are dissimilar. In contrast to previous observations, *C. guilliermondi* was differentiated from *C. zeylanoides* (28, 31). Both *C. neoformans* probes (CNEO [31] and CRY [Table 3]) were designed on conserved sequences and thus hybridized with all members of *C. neoformans* complex. Likewise, previous ITS-based analyses have not provided species identification within *C. neoformans* (7, 15, 22); however, a more recent study found diversity in the ITS region to be useful in differentiating between species and varieties of *C. neoformans* (17). If required, the RLB used in our study can be modified to incorporate probes specific for these species/varieties.

Incorporation of both ITS1 and ITS2 probes into the assay enabled major *Aspergillus* spp. to be identified. The ability of the current assay to distinguish between members of the *A. fumigatus* clade (e.g., *Neosartorya fischeri*, *Aspergillus lentus*) was not tested. This distinction is clinically relevant, as *A. lentulus* and *Neosartorya* spp. have been reported to be less susceptible in vitro to antifungal agents (3). Previous analyses reveal little difference between the ITS sequences of these closely related species (3, 21), suggesting that probes based on alternative gene targets (e.g., the β-tubulin and rodlet A genes) would be required (3).

The results from this study support the notion that the use of both ITS loci for fungal identification provides more informative data than that of either locus alone (5, 7). For fungal isolates, ITS2 sequence polymorphisms were more species specific than their ITS1 counterpart; for example, in *A. niger*, intraspecies ITS1 sequence divergence was higher than in the ITS2 region (Table 4). This has implications for study of potential subtypes of *A. niger* and other *Aspergillus* spp. Conversely, DNA amplified from two clinical specimens hybridized with only the ITS1 probe (patients 3 and 12, Table 2). When these same specimens were subjected to ITS sequence analysis, only the ITS1 (and not ITS2) region was amplified. Similarly, for specimens from patients 21 and 22, DNA sequence analysis was successful for the ITS1, but not the ITS2, region. The reasons for these results are not readily explained. Failure of the ITS2 probe to hybridize to the DNA is unlikely to be due to degradation of fungal DNA, as the ITS1 region was successfully amplified and analyzed. Others have reported yeast identification rates of 96.8% and 99.7% based on ITS1 and ITS2 sequencing, respectively, of fungal cultures (22). The relative utility of ITS1 and ITS2 loci in determining species identification and phylogenetic relationships is the subject of ongoing study.

We successfully applied the RLB assay to identify *Candida*, *Aspergillus*, and *Cryptococcus* spp. in clinical specimens, including blood and PE tissue samples, with high (100% for ITS1-based probes and 90.5% for ITS2-based probes) sensitivity. In particular, the assay was able to provide a diagnosis in the absence of a culture result (patient 18) and was capable of diagnosing possible mixed infection (patient 19). Furthermore, the results indicate that the RLB has potential utility in the early diagnosis of IFI, as it detected the presence of a "fungal pathogen" in all cases of IFI caused by species not represented by probes on the RLB. In one instance (patient 21, Table 2), the pathogen was finally determined to be *E. spinifera* by

ITS sequence analysis but identified morphologically as *E. jeanselmei*. Comparison of GenBank ITS sequences of both these species revealed the two can be easily distinguished (20). Identification of *Exophiala* spp. is problematic due to variable morphological characteristics within this genus (14, 32). It is likely the culture-based identification was incorrect.

While probe hybridization technology has been successfully used for identification of fungal isolates (1, 2, 8, 23, 24), species identification from clinical specimens has remained a challenge. Real-time PCR systems have been developed to meet these demands but are limited by false-negative and false-positive results inherent in PCR and the number of fungal species that can be simultaneously identified (4, 16, 26). We chose a simple nested PCR-RLB format as an alternative to culture for fungal identification given the advantages of low cost, inherent high sensitivity, good specificity (10; this study), and the capacity to simultaneously analyze multiple strains against multiple probes (10). Although only 22 probes (two probes per species) were used in this study, up to 43 may be incorporated (one probe per species), extending the range of pathogens that can be identified. This flexibility and versatility also allow laboratories to readily modify or "customize" the RLB format to meet specific requirements or for use in a particular clinical setting. For example, in the rapid diagnosis of fungemia, species-specific probes representative of causative agents of fungemia other than *Candida* spp., e.g., *Scedosporium prolificans*, may be included.

The identification of uncommon but emerging fungal pathogens continues to pose a challenge. Based on the results of this study, for suspected *Candida*, *Cryptococcus*, or *Aspergillus* isolates, the RLB assay is a reliable alternative to conventional identification methods, especially if there is a need to identify more than one isolate at the same time. Notably, the assay performed well when applied to clinical specimens. We envisage it will be most useful if culture-based identification is not made or when clinical specimens fail to be submitted for culture, as can occur following surgery. In the event a "fungus" is detected on RLB but not identified further due to lack of inclusion of the species-specific probe in the assay, the more expensive and time-consuming ITS sequencing can be performed. ITS sequence analysis to identify fungal pathogens directly from tissue specimens has been successfully implemented in our laboratory, but the turnaround time is significantly increased relative to RLB by our outsourcing of the DNA sequencing (21). Evaluation of larger numbers of specimens is required to position the RLB assay and/or DNA sequence analysis within routine diagnostic algorithms.

ACKNOWLEDGMENTS

Fanrong Kong and Xianyu Zeng made equal contributions to the work and are listed as joint first authors.

We thank Ilya Henner and Maryann Pincevic for their help in sequencing.

This work was funded in part by a National Health and Medical Research Council of Australia Centre of Clinical Research Excellence program grant (264625) to T.C.S.

REFERENCES

1. **Ahmed, A. O., M. M. Mukhtar, M. Kools-Sijmons, A. H. Fahal, S. de Hoog, B. G. van den Ende, E. E. Zijlstra, H. Verbrugh, E. S. Abugroun, A. M. Elhassan, and A. van Belkum.** 1999. Development of a species-specific PCR-restriction fragment length polymorphism analysis procedure for identification of *Madurella mycetomatis*. J. Clin. Microbiol. **37:**3175–3178.
2. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
3. **Balajee, S. A., J. Gribskov, E. Hanley, D. Nickle, and K. A. Marr.** 2005. *Aspergillus lentulus* sp. nov., a new sibling species of *A. fumigatus*. Eukaryot. Cell **4:**625–632.
4. **Bu, R., R. K. Sathiapalan, M. M. Ibrahim, I. Al Mohsen, E. Almodavar, M. I. Gutierrez, and K. Bhatia.** 2005. Monochrome LightCycler PCR assay for detection and quantification of five common species of *Candida* and *Aspergillus*. J. Med. Microbiol. **54:**243–248.
5. **Chen, Y. C., J. D. Eisner, M. M. Kattar, S. L. Rassoulian-Barrett, K. Lafe, U. Bui, A. P. Limaye, and B. T. Cookson.** 2001. Polymorphic internal transcribed spacer region 1 DNA sequences identify medically important yeasts. J. Clin. Microbiol. **39:**4042–4051.
6. **Chen, Y. C., J. D. Eisner, M. M. Kattar, S. L. Rassoulian-Barrett, K. LaFe, S. L. Yarfitz, A. P. Limaye, and B. T. Cookson.** 2000. Identification of medically important yeasts using PCR-based detection of DNA sequence polymorphisms in the internal transcribed spacer 2 region of the rRNA genes. J. Clin. Microbiol. **38:**2302–2310.
7. **Ciardo, D. E., G. Schar, E. C. Bottger, M. Altwegg, and P. P. Bosshard.** 2006. Internal transcribed spacer sequencing versus biochemical profiling for identification of medically important yeasts. J. Clin. Microbiol. **44:**77–84.
8. **de Aguirre, L., S. F. Hurst, J. S. Choi, J. H. Shin, H. P. Hinrikson, and C. J. Morrison.** 2004. Rapid differentiation of *Aspergillus* species from other medically important opportunistic molds and yeasts by PCR-enzyme immunoassay. J. Clin. Microbiol. **42:**3495–3504.
9. **Elie, C. M., T. J. Lott, E. Reiss, and C. J. Morrison.** 1998. Rapid identification of *Candida* species with species-specific DNA probes. J. Clin. Microbiol. **36:**3260–3265.
10. **Gold, B.** 2003. Origin and utility of the reverse dot-blot. Expert Rev. Mol. Diagn. **3:**143–152.
11. **Halliday, C., Q. X. Wu, G. James, and T. Sorrell.** 2005. Development of a nested qualitative real-time PCR assay to detect *Aspergillus* species DNA in clinical specimens. J. Clin. Microbiol. **43:**5366–5368.
12. **Henry, T., P. C. Iwen, and S. H. Hinrichs.** 2000. Identification of *Aspergillus* species using internal transcribed spacer regions 1 and 2. J. Clin. Microbiol. **38:**1510–1515.
13. **Hinrikson, H. P., S. F. Hurst, T. J. Lott, D. W. Warnock, and C. J. Morrison.** 2005. Assessment of ribosomal large-subunit D1-D2, internal transcribed spacer 1, and internal transcribed spacer 2 regions as targets for molecular identification of medically important *Aspergillus* species. J. Clin. Microbiol. **43:**2092–2103.
14. **Hsiao, C. R., L. Huang, J. P. Bouchara, R. Barton, H. C. Li, and T. C. Chang.** 2005. Identification of medically important molds by an oligonucleotide array. J. Clin. Microbiol. **43:**3760–3768.
15. **Huang, A., J. W. Li, Z. Q. Shen, X. W. Wang, and M. Jin.** 2006. High-throughput identification of clinical pathogenic fungi by hybridization to an oligonucleotide microarray. J. Clin. Microbiol. **44:**3299–3305.
16. **Imhof, A., C. Schaer, G. Schoedon, D. J. Schaer, R. B. Walter, A. Schaffner, and M. Schneemann.** 2003. Rapid detection of pathogenic fungi from clinical specimens using LightCycler real-time fluorescence PCR. Eur. J. Clin. Microbiol. Infect. Dis. **22:**558–560.
17. **Katsu, M., S. Kidd, A. Ando, M. L. Moretti-Branchini, Y. Mikami, K. Nishimura, and W. Meyer.** 2004. The internal transcribed spacers and 5.8S rRNA gene show extensive diversity among isolates of the *Cryptococcus neoformans* species complex. FEMS Yeast Res. **4:**377–388.
18. **Kong, F., and G. L. Gilbert.** 2007. Multiplex PCR-based reverse line blot hybridization assay (mPCR/RLB)—a practical epidemiological and diagnostic tool. Nat. Protocols **6:**2668–2680.
19. **Kwon-Chung, K. J., I. Polacheck, and J. E. Bennett.** 1982. Improved diagnostic medium for separation of *Cryptococcus neoformans* var. *neoformans* (serotypes A and D) and *Cryptococcus neoformans* var. *gattii* (serotypes B and C). J. Clin. Microbiol. **15:**535–537.
20. **Larone, D. H.** 2002. Medically important fungi. A guide to identification, 4th ed. ASM Press, Washington, DC.
21. **Lau, A., S. Chen, T. Sorrell, D. Carter, R. Malik, P. Martin, and C. Halliday.** 2007. Development and clinical application of a panfungal PCR assay to detect and identify fungal DNA in tissue specimens. J. Clin. Microbiol. **45:**380–385.
22. **Leaw, S. N., H. C. Chang, H. F. Sun, R. Barton, J. P. Bouchara, and T. C. Chang.** 2006. Identification of medically important yeast species by sequence analysis of the internal transcribed spacer regions. J. Clin. Microbiol. **44:**693–699.
23. **Leinberger, D. M., U. Schumacher, I. B. Autenrieth, and T. T. Bachmann.** 2005. Development of a DNA microarray for detection and identification of fungal pathogens involved in invasive mycoses. J. Clin. Microbiol. **43:**4943–4953.
24. **Lindsley, M. D., S. F. Hurst, N. J. Iqbal, and C. J. Morrison.** 2001. Rapid identification of dimorphic and yeast-like fungal pathogens using specific DNA probes. J. Clin. Microbiol. **39:**3505–3511.
25. **Llorens, A., M. J. Hinojo, R. Mateo, M. T. Gonzalez-Jaen, F. M. Valle-Algarra, A. Logrieco, and M. Jimenez.** 2006. Characterization of *Fusarium*

spp. isolates by PCR-RFLP analysis of the intergenic spacer region of the rRNA gene (rDNA). Int. J. Food Microbiol. **106:**297–306.
26. **Maaroufi, Y., C. Heymans, J. M. De Bruyne, V. Duchateau, H. Rodriguez-Villalobos, M. Aoun, and F. Crokaert.** 2003. Rapid detection of *Candida albicans* in clinical blood samples by using a TaqMan-based PCR assay. J. Clin. Microbiol. **41:**3293–3298.
27. **Marr, K.** 2004. Combination antifungal therapy: where are we now, and where are we going? Oncology (Williston Park) **18:**24–29.
28. **Martin, C., D. Roberts, W. M. van Der, R. Rossau, G. Jannes, T. Smith, and M. Maher.** 2000. Development of a PCR-based line probe assay for identification of fungal pathogens. J. Clin. Microbiol. **38:**3735–3742.
29. **Perfect, J. R.** 2004. Management of invasive mycoses in hematology patients: current approaches. Oncology (Williston Park) **18:**5–14.
30. **Pfaller, M. A., D. J. Diekema, L. Boyken, S. A. Messer, S. Tendolkar, R. J. Hollis, and B. P. Goldstein.** 2005. Effectiveness of anidulafungin in eradicating *Candida* species in invasive candidiasis. Antimicrob. Agents Chemother. **49:**4795–4797.
31. **Playford, E. G., F. Kong, Y. Sun, H. Wang, C. Halliday, and T. C. Sorrell.** 2006. Simultaneous detection and identification of *Candida*, *Aspergillus*, and *Cryptococcus* species by reverse line blot hybridization. J. Clin. Microbiol. **44:**876–880.
32. **Porteous, N. B., A. M. Grooters, S. W. Redding, E. H. Thompson, M. G. Rinaldi, G. S. De Hoog, and D. A. Sutton.** 2003. Identification of *Exophiala mesophila* isolated from treated dental unit waterlines. J. Clin. Microbiol. **41:**3885–3889.
33. **Pryce, T. M., S. Palladino, I. D. Kay, and G. W. Coombs.** 2003. Rapid identification of fungi by sequencing the ITS1 and ITS2 regions using an automated capillary electrophoresis system. Med. Mycol. **41:**369–381.
34. **Reiss, E., T. Obayashi, K. Orle, M. Yoshida, and R. M. Zancope-Oliveira.** 2000. Non-culture based diagnostic tests for mycotic infections. Med. Mycol. **38**(Suppl. 1)**:**147–159.
35. **Walsh, T. J., A. Groll, J. Hiemenz, R. Fleming, E. Roilides, and E. Anaissie.** 2004. Infections due to emerging and uncommon medically important fungal pathogens. Clin. Microbiol. Infect. **10**(Suppl. 1)**:**48–66.
36. **Walsh, T. J., V. Petraitis, R. Petraitiene, A. Field-Ridley, D. Sutton, M. Ghannoum, T. Sein, R. Schaufele, J. Peter, J. Bacher, H. Casler, D. Armstrong, A. Espinel-Ingroff, M. G. Rinaldi, and C. A. Lyman.** 2003. Experimental pulmonary aspergillosis due to *Aspergillus terreus*: pathogenesis and treatment of an emerging fungal pathogen resistant to amphotericin B. J. Infect. Dis. **188:**305–319.
37. **Warren, N., and K. Hazen.** 1999. *Candida*, *Cryptococcus*, and other yeasts of medical importance, p. 1184–1199. *In* R. P. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken (ed.), Manual of clinical microbiology. ASM Press, Washington, DC.
38. **Williamson, E. C., J. P. Leeming, H. M. Palmer, C. G. Steward, D. Warnock, D. I. Marks, and M. R. Millar.** 2000. Diagnosis of invasive aspergillosis in bone marrow transplant recipients by polymerase chain reaction. Br. J. Haematol. **108:**132–139.