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Rapid identification of fungal pathogens in BacT/ALERT, BACTEC, and BBL MGIT media using polymerase chain reaction and DNA sequencing of the internal transcribed spacer regions

Todd M. Pryce[a,*], Silvano Palladino[a], Diane M. Price[a], Dianne J. Gardam[a], Peter B. Campbell[b], Keryn J. Christiansen[a], Ronan J. Murray[a]

[a]*PathWest Laboratory Medicine WA, Department of Microbiology and Infectious Diseases, Royal Perth Hospital, Perth 6847, Western Australia, Australia*
[b]*PathWest Laboratory Medicine WA, Department of Microbiology, Women's and Children's Health Service, Subiaco 6008, Western Australia, Australia*

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Abstract

We report a direct polymerase chain reaction/sequence (d-PCRS)-based method for the rapid identification of clinically significant fungi from 5 different types of commercial broth enrichment media inoculated with clinical specimens. Media including BacT/ALERT FA (BioMérieux, Marcy l'Etoile, France) (n = 87), BACTEC Plus Aerobic/F (Becton Dickinson, Microbiology Systems, Sparks, MD) (n = 16), BACTEC Peds Plus/F (Becton Dickinson) (n = 15), BACTEC Lytic/10 Anaerobic/F (Becton Dickinson) (n = 11) bottles, and BBL MGIT (Becton Dickinson) (n = 11) were inoculated with specimens from 138 patients. A universal DNA extraction method was used combining a novel pretreatment step to remove PCR inhibitors with a column-based DNA extraction kit. Target sequences in the noncoding internal transcribed spacer regions of the rRNA gene were amplified by PCR and sequenced using a rapid (24 h) automated capillary electrophoresis system. Using sequence alignment software, fungi were identified by sequence similarity with sequences derived from isolates identified by upper-level reference laboratories or isolates defined as ex-type strains. We identified *Candida albicans* (n = 14), *Candida parapsilosis* (n = 8), *Candida glabrata* (n = 7), *Candida krusei* (n = 2), *Scedosporium prolificans* (n = 4), and 1 each of *Candida orthopsilosis*, *Candida dubliniensis*, *Candida kefyr*, *Candida tropicalis*, *Candida guilliermondii*, *Saccharomyces cerevisiae*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, *Histoplasma capsulatum*, and *Malassezia pachydermatis* by d-PCRS analysis. All d-PCRS identifications from positive broths were in agreement with the final species identification of the isolates grown from subculture. Earlier identification of fungi using d-PCRS may facilitate prompt and more appropriate antifungal therapy.

Keywords: Fungi; Identification; Blood cultures; PCR; DNA sequencing; Capillary electrophoresis; Internal transcribed spacer regions; ITS; *Candida*; BacT/ALERT; BACTEC; BBL MGIT

1. Introduction

The incidence of invasive fungal infections (IFIs) has increased significantly in the past decade as a direct consequence of increasing patient populations at risk for developing serious fungal infections (Hajjeh et al., 2004; Walsh et al., 2004). Opportunistic fungi other than *Candida albicans* and *Aspergillus fumigatus* are being reported with increased frequency from blood stream infections (BSIs) and IFIs (Pfaller and Diekema, 2004). These include non-*albicans Candida* species, such as *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, and *Candida krusei*, which account for more than 50% of all BSIs caused by *Candida* (Rangel-Frausto et al., 1999). Other fungi increasingly encountered include opportunistic yeast-like fungi such as *Trichosporon* spp., *Rhodotorula* spp., *Geotrichum capitatum*, and filamentous fungi such as *Scedosporium* spp., *Fusarium* spp., *Acremonium* spp., and Mucorales (Pfaller and Diekema, 2004; Walsh et al., 2004). In addition, certain species of fungi are associated with a high degree of mor-

* Corresponding author. Tel.: +61-8-9224-2444; fax: +61-8-9224-1989.
E-mail address: todd.pryce@health.wa.gov.au (T.M. Pryce).

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tality, and some are inherently less susceptible to standard antifungal therapy (Pfaller and Diekema, 2004). The rapid and reliable detection and subsequent identification of fungi from blood and other important clinical specimens remain critical in deciding whether to initiate antifungal therapy and in the choice of agent used.

Continuously monitored automated blood culture systems have improved the detection and isolation of fungi from patients with BSIs compared with manual methods of culture (Nolte et al., 1993; O'Hara et al., 2003). Furthermore, inoculation of blood culture media with other types of fluid or tissue samples has been widely implemented in clinical microbiology laboratories to improve recovery of some fungi (Thomson and Miller, 2003). Regardless of the type of sample and system used, the laboratory handling of positive cultures is usually the same; the presence of fungi is confirmed by microscopy and the broth is subcultured onto solid mycological media for phenotypic identification. However, many fungi are slow growing, and current methods of fungal identification are labor-intensive, may lack specificity, and require a wide range of specialized laboratory media and specialized trained staff (Pfaller and Fromtling, 2003).

Numerous molecular methods have been developed to rapidly identify fungi from solid media (Luo and Mitchell, 2002; Shin et al., 1999) and directly from positive blood culture fluids (Borst et al., 2001; Chang et al., 2001; Li et al., 2003; Shin et al., 1997). Polymerase chain reaction (PCR) methods for identifying fungi directly from blood cultures usually target a few species within the genus *Candida*. Molecular targets for PCR amplification and detection are usually conserved nucleotide sequences within phylogenetically informative genetic regions such as the rRNA gene complex. Ribosomal RNA genes are also targets for sequence-based identification of fungi (Chen et al., 2001; Hall et al., 2003, 2004; Henry et al., 2000; Pryce et al., 2003). PCR amplification using universal primers targeted to conserved regions within the rRNA complex, followed by DNA sequencing of the internal transcribed spacer (ITS) regions, shows promise to identify a broad range of fungi to the species level (Chen et al., 2001; Henry et al., 2000; Iwen et al., 2002; Pryce et al., 2003).

We previously reported a rapid (24 h) sequence-based approach to identify clinically important yeasts and filamentous fungi from the first visible signs of growth on solid media (Pryce et al., 2003). The aim of this investigation was to evaluate a similar approach for the rapid and accurate identification of fungi directly from commercial broth enrichment media. To our knowledge, we report for the first time the use of DNA sequencing for the rapid identification of fungi directly from commercial broth enrichment media. In addition, we report a reliable DNA extraction method to isolate fungal nucleic acids from a range of commercial broth enrichment media commonly used in diagnostic microbiology laboratories.

2. Materials and methods

2.1. Clinical samples and controls

A total of 140 samples were collected from 138 patients from the Royal Perth Hospital and the Princess Margaret Hospital for Children, Perth, Australia. Clinical specimens included blood (n = 121), vitreous fluid (n = 3), synovial fluid (n = 2), peritoneal fluid (n = 3), skin biopsy (n = 1), and a variety of other tissues and fluids (n = 10). Commercial broth enrichment media including BacT/ALERT FA (BioMérieux, Marcy l'Etoile, France), BACTEC Plus Aerobic/F (Becton Dickinson, Microbiology Systems, Sparks, MD), and BACTEC Lytic/10 Anaerobic/F (Becton Dickinson) were inoculated with 5–10 mL of blood, synovial fluid, or peritoneal fluid. BACTEC Peds Plus/F media (Becton Dickinson) were inoculated with 3–5 mL of blood or peritoneal fluid, and BBL MGIT media (Becton Dickinson) were inoculated with approximately 0.5 mL of processed tissue or fluid. Overall, 45 broth cultures were microscopy-positive for fungi. Negative controls included culture-negative blood cultures (n = 80) and positive blood cultures growing different bacterial species (n = 15).

2.2. Culture and phenotypic identification of isolates

All inoculated media were incubated for a minimum of 14 days or until a signal indicating growth was detected by the automated system used. During routine processing, a small aliquot was withdrawn from each bottle when a signal indicating growth was detected or when the culture was reported as negative. From cultures that signaled positive, the aliquot was used for a Gram stain and inoculation onto routine solid media including chocolate agar, anaerobic blood agar, Sabouraud dextrose agar, and CHROMagar *Candida* (CCAN) (Becton Dickinson). Negative bottles were terminally subcultured on Sabouraud dextrose agar and incubated at 30 °C for 14 days. All isolates were initially identified in a blinded fashion (the medical scientist performing the phenotypic identification was not aware of the direct PCR/sequence [d-PCRS] result). Yeasts were identified by germ tube formation, VITEK Yeast Biochemical Card (YBC) (BioMérieux), appearance on CCAN, and microscopic characteristics such as the presence of pseudomycelium, blastoconidia, and chlamydospore production on cornmeal-Tween 80 agar (CMAT) (Hazen and Howell, 2003). A positive urease and brown pigment production on birdseed agar was used to confirm *Cryptococcus neoformans*. Yeasts not identified using YBC were identified using the ID32C system (BioMérieux). Other fungi were identified based on morphologic and physiologic characteristics using standard mycological techniques (de Hoog et al., 2000).

2.3. DNA extraction from commercial broth enrichment media for d-PCRS

A 0.1-mL aliquot of broth enrichment media was added to 1.0 mL of alkali wash solution (0.5 mol/L NaOH,

0.05 mol/L tri-sodium citrate dihydrate) to overcome potential PCR inhibitors (Kulski and Pryce, 1996). Samples were vortexed for 15 s, incubated at room temperature for 5 min, centrifuged at 16000 × *g* for 5 min, and the supernatant was removed. The pellet was resuspended in 1.0 mL of PBS (0.137 mol/L NaCl, 3 mmol/L KCl, 8 mmol/L Na_2HPO_4, 1 mmol/L KH_2PO_4) by gentle pipetting, vortexed for 15 s, and centrifuged at 16000 × *g* for 5 min. The supernatant was discarded, and the pellet containing fungal cells and charcoal/resin was resuspended in 0.2 mL of double-distilled H_2O (ddH_2O) containing 50 U of lyticase (Sigma-Aldrich, Steinheim, Germany). Tubes were incubated at 37 °C for 1 h, then heated to 95 °C for 10 min. Fungal DNA was isolated using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Castle Hill, Sydney, Australia) with the following modification to the manufacturer's method. Before DNA precipitation with isopropanol, tubes were centrifuged at 16000 × *g* for 2 min to deposit the activated charcoal or resin. The supernatant was removed (0.35 mL) and 0.1 mL of isopropanol was added. The samples were transferred to the glass fiber columns and processed following the protocol described by the manufacturer. An elution volume of 0.2 mL was used. Samples were used immediately for PCR or stored at −70 °C until use.

2.4. DNA extraction for PCR/sequence-based identification of the isolates grown from subculture

DNA extraction of isolates grown from subculture was performed as previously described (Pryce et al., 2003), with some modifications. Briefly, a small amount (~1–2 mm^2) of fungal mycelial mass or yeast colony was removed and emulsified in 0.1 mL of ddH_2O containing 50 U of lyticase (Sigma-Aldrich) in a 2.0-mL screw-cap tube. Samples were incubated at 37 °C for 1 h then heated to 95 °C for 10 min. Proteinase K (Roche Diagnostics) was added to a final concentration of 65 μg/mL and incubated at 60 °C for 10 min. Samples were diluted with 60 μL of a solution containing 50% (wt/vol) of Chelex-100 (Bio-Rad Laboratories, Hercules, CA) with ddH_2O. Samples were heated to 95 °C for 10 min to further facilitate lysis. All tubes were centrifuged at 16000 × *g* for 5 min then diluted 1:10 with a solution containing 10% (wt/vol) of Chelex-100 with ddH_2O. Samples were used immediately for PCR or stored at −70 °C until use. A control (*Candida albicans* ATCC 14053) was used as a positive control for DNA extraction.

2.5. PCR amplification and detection

DNA extracts from commercial broth enrichment media and isolates grown from subculture were tested with primers V9D (5′-TTA AGT CCC TGC CCT TTG TA-3′) (de Hoog and Gerrits van den Ende, 1998) and LS266 (5′-GCA TTC CCA AAC AAC TCG ACT C-3′) (Masclaux et al., 1999) in a PCR reaction (PCR-A) as previously described (Pryce et al., 2003). These primers bind to conserved regions, with corresponding positions to *Saccharomyces cerevisiae* 18S (1609–1627) and 28S (287–266), and amplify an 800- to 1300-bp product that encompasses a portion of the 18S and 28S rRNA gene and the entire intervening ITS1, 5.8S, and ITS2 rRNA gene regions. Those samples negative by PCR-A were tested using primers ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) (White et al., 1990) in a second PCR reaction (PCR-B). These primers bind to conserved regions, with corresponding positions to *Saccharomyces cerevisiae* 18S (1769–1787) and 28S (41–60), and amplify a 400- to 900-bp product that encompasses the entire ITS1, 5.8S, and ITS2 rRNA gene regions. Both PCR-A and PCR-B were designed to use the same PCR conditions. Each PCR assay was performed with 5 μL of DNA template in a total reaction volume of 50 μL. The PCR reaction mixture contained 5 μL of 10× PCR buffer (Roche Diagnostics); 3 μL of 25 mmol/L $MgCl_2$; 1.5 μL of 20 μmol/L of each oligonucleotide; 200 μmol/L of each deoxynucleoside triphosphate; dATP, dGTP, dCTP, dTTP (Amersham Biosciences, Sydney, Australia); 2.25 U of FastStart Taq DNA Polymerase (Roche Diagnostics); and 33 μL of sterile distilled H_2O. The PCR was performed in a MyCycler (Bio-Rad Laboratories, Sydney, Australia) with the following program: 95 °C for 9 min, followed by 95 °C for 30 s, 62 °C for 60 s, 72 °C for 2 min for 33 cycles, followed by 72 °C for 5 min. PCR-amplified products were detected by gel electrophoresis using a 2% (wt/vol) agarose gel stained with ethidium bromide.

2.6. PCR amplification controls and assessment of PCR inhibition

DNA extracted from *Candida albicans* ATCC 14053 (1 pg/μL) was used as a positive PCR amplification control for each experiment. In addition, DNA extracts from all negative controls (n = 95) were assessed for the presence of PCR inhibitors. A second PCR master mix was prepared containing DNA from *Candida albicans* ATCC 14053 to reach a final concentration of 1 pg per reaction. A 5-μL aliquot from each DNA extract was added to a PCR tube containing 45 μL of master mix. PCR amplification and detection were performed as previously described. The absence of PCR inhibition was determined by the presence of a PCR product the same molecular weight as the control.

2.7. Sequencing PCR-amplified products

All PCR-amplified products were sequenced at the West Australian Genome Resource Centre at Royal Perth Hospital. ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit Version 3 (Applied Biosystems, Foster City, CA) and the ABI Prism 3730 Genetic Analyzer were used (Applied Biosystems) following protocols supplied by the manufacturer. Primers used for sequencing PCR-A–

amplified products were V9D, LS266, ITS1, and ITS4. Primers used for sequencing PCR-B–amplified products were ITS1 and ITS4. Sequencing controls included PCR products from the DNA extraction control and PCR amplification control (*Candida albicans* ATCC 14053).

2.8. Sequence assembly, assessment of quality, and editing

The electropherograms were visualized and edited using SeqScape Software Version 2.0 (Applied Biosystems). DNA sequencing analysis and interpretation standards were used as a guide (Taylor, 2003).

2.9. Sequence similarity searching of the GenBank database using BLAST and species identification

Sequence search was performed using the BLAST standard nucleotide–nucleotide basic local alignment search tool (Altschul et al., 1997). A test sequence was assigned to a species identification by selecting the GenBank record with the highest bit score with an expect value equal to zero from the list of GenBank entries in the BLAST search, which have been identified as reliable based on previously published criteria (Pryce et al., 2003). Briefly, sequences used as standards for sequence-based identification (reference sequences) were selected based on the following criteria: (i) the sequence should be complete, that is, the sequence should represent the whole region of interest and not just a part thereof; (ii) all sequences used should be derived from cultures obtained from reference collections where possible, preferably cultures nomenclaturally designated as ex-type (the fungal equivalent to bacteriologic-type strains), or alternatively, cultures identified by the use of the most stringent level of traditional mycological techniques based on authoritative monographs and up-to-date literature in an upper-level reference laboratory; and (iii) to the best of the investigator's knowledge, each sequence should be designated with a species name that is nomenclaturally valid and currently recognized as correct at the time of the investigation.

3. Results

3.1. Assessment of the specificity of the PCR method and performance of the DNA extraction methods used

All broth enrichment media determined to be culture-positive for fungi were positive by PCR (n = 45). All isolates grown from subculture were also positive by PCR (n = 46). All positive samples except from patient 5 (*Cryptococcus neoformans*) were successfully amplified using PCR-A (Table 1). The positive sample from patient 5 was successfully amplified using PCR-B. A single PCR product was observed for all PCR-positive samples (data not shown). PCR products were obtained from a single DNA extraction for all broth enrichment media determined to be positive for fungi and from isolates grown from subculture. All broth enrichment media determined to be culture-negative for fungi were negative by PCR, and no PCR inhibition was detected (n = 95).

3.2. d-PCRS and phenotypic identification of culture-positive specimens

For isolates able to be identified using phenotypic methods, d-PCRS identification for all positive specimens was 100% concordant with phenotypic identification of the isolates grown from subculture. However, 1 isolate (*Candida albicans*) was not detected from a mixed culture using d-PCRS (patient 43). This isolate was subsequently identified using phenotypic tests and PCR/sequence (PCRS) of the isolate. Subsequent investigations revealed that *Candida parapsilosis* was the predominating yeast on subculture. In all other positive specimens the d-PCRS result was 100% concordant with the PCRS identification of the isolates grown on solid media (data not shown).

Microscopic characteristics observed on CMAT correlated with the VITEK identification for the majority of isolates. However, for some isolates the VITEK identification was inconclusive and/or the microscopic features on CMAT did not correlate with the species reported by the VITEK system. In these circumstances a definitive phenotypic identification by examination of microscopic features on CMAT was difficult because of many yeasts sharing similar microscopic characteristics. The d-PCRS identification was useful to resolve ambiguous VITEK identifications and guide the selection of the most appropriate phenotypic tests to establish a final phenotypic identification (Table 2).

For 2 isolates the d-PCRS result was the only effective means of identification in our laboratory. One isolate identified by d-PCRS as *Candida orthopsilosis* (patient 3) and phenotypically as *Candida parapsilosis* was unable to be confirmed by further phenotypic tests (Table 2). The final identification of *Candida orthopsilosis* was established by PCRS identification of the isolate. Another isolate (patient 40; *H. capsulatum*) was unable to be identified in a timely fashion by phenotypic tests. The BBL MGIT tube contained clumps of septate hyphae with no distinct features. The fluid was initially subcultured onto solid media for routine identification. However, a more rapid identification was required to determine the clinical significance and selection of appropriate antifungal therapy. Following the d-PCRS result of *H. capsulatum*, subsequent growth on solid media developed microscopic features consistent with *H. capsulatum*. Because of the hazardous nature of this fungus, further phenotypic tests were not performed.

3.3. Time to identification from culture positivity and clinical significance

The time taken to definitively identify fungal isolates from positive fluids using conventional tests ranged from 2 to 13 days (mean, 4.8 days). The time taken to identify fungi from positive fluids using d-PCRS ranged from 24 to

Table 1
Identification of fungi from positive commercial broth enrichment media and sequence analysis

Patient	Sample	Media	Days to positivity	Identification[a]	BLAST results[b]				
					Bit score[c]	Identity[d]	% Similarity	Accession no.	Strain no.
1	B	A	1	*Candida albicans*	1046	535/536	99.8	AF217609	ATCC 28516
22	B	A	1	*Candida albicans*	1046	535/536	99.8	AF217609	ATCC 28516
20	B	A	1	*Candida albicans*	1046	535/538	99.4	AF217609	ATCC 28516
14	B	A	2	*Candida albicans*	1063	536/537	99.8	AF217609	ATCC 28516
21	B	A	2	*Candida albicans*	1063	536/536	100	AF217609	ATCC 28516
24	B	A	2	*Candida albicans*	1063	536/536	100	AF217609	ATCC 28516
8	B	A	2	*Candida albicans*	1063	536/536	100	AF217609	ATCC 28516
29	B	A	3	*Candida albicans*	1063	536/536	100	AF217609	ATCC 28516
2	B	A	3	*Candida albicans*	1063	536/536	100	AF217609	ATCC 28516
16	B	A	3	*Candida albicans*	1063	536/536	100	AF217609	ATCC 28516
39	B	A	3	*Candida albicans*	1049	536/537	99.8	AF217609	ATCC 28516
35	PF	A	1	*Candida albicans*	1049	536/537	99.8	AF217609	ATCC 28516
4	B	A	3	*Candida glabrata*	1199	629/634	99.2	AY198398	CBS 138
26	B	A	4	*Candida glabrata*	1203	622/626	99.4	AY198398	CBS 138
36	B	A	3	*Candida glabrata*	1203	622/626	99.4	AY198398	CBS 138
27	B	A	5	*Candida glabrata*	1199	627/632	99.2	AY198398	CBS 138
33	VF	A	3	*Candida glabrata*	1212	627/632	99.2	AY198398	CBS 138
31	VF	A	3	*Candida glabrata*	1195	621/626	99.2	AY198398	CBS 138
28	B	A	1	*Candida parapsilosis*	1031	520/520	100	AY391843	CBS 604
7	B	A	2	*Candida parapsilosis*	1031	520/520	100	AY391843	CBS 604
38	B	A	3	*Candida parapsilosis*	1031	520/520	100	AY391843	CBS 604
3	B	A	6	*Candida orthopsilosis*	914	461/461	100	AJ698048	ATCC 96139
32	VF	A	2	*Candida dubliniensis*	1001	505/505	100	AB049123	CBS 7987
23	PF	A	1	*Candida kefyr*	1255	633/633	100	AJ401699	CBS 4857
30	B	A	2	*Candida tropicalis*	898	519/523	99.2	AF321539	FEMS Yeast Res.
5	B	A	7	*Cryptococcus neoformans*	952	489/492	99.4	AF444326	CBS 132
6	B	A	2	*Saccharomyces cerevisiae*	1108	559/559	100	Z73326	S288C
13	B	A	4	*Scedosporium prolificans*	954	495/497	99.6	AF022484	CBS 114.90
36	B	A	4	*Scedosporium prolificans*	954	495/497	99.6	AF022484	CBS 114.90
25	B	A	3	*A. fumigatus*	1176	596/597	99.8	AY214446	ATCC 16907
11	B	B	5	*Candida albicans*	1063	536/536	100	AF217609	ATCC 28516
10	B	B	6	*Candida glabrata*	1221	634/639	99.2	AY198398	CBS 138
9	B	B	3	*Candida parapsilosis*	1031	520/520	100	AY391843	CBS 604
19	B	B	4	*Candida krusei*	876	480/483	99.4	AF246989	ATCC 6258
15	B	B	4	*Candida krusei*	926	480/483	99.4	AF246989	ATCC 6258
18	B	D	7	*Candida parapsilosis*	1031	520/520	100	AY391843	CBS 604
12	SF	B	7	*Scedosporium prolificans*	954	495/497	99.6	AF022484	CBS 114.90
12	SF	C	5	*Scedosporium prolificans*	954	495/497	99.6	AF022484	CBS 114.90
34	B	D	2	*Candida albicans*	1033	534/536	99.6	AF217609	ATCC 28516
43	B	D	3	*Candida parapsilosis*[e]	1031	520/520	100	AY391843	CBS 604
17	B	D	7	*Candida parapsilosis*	1031	520/520	100	AY391843	CBS 604
37	PF	D	2	*Candida parapsilosis*	1031	520/520	100	AY391843	CBS 604
40	SB	E	11	*H. capsulatum*	1070	552/556	99.3	AF038353	UAMH 7141
41	B	A	6	*Candida guilliermondii*[f]	1203	607/607	100	AY939792	ATCC 6260
42	B	A	5	*M. pachydermatis*	1039	534/536	99.6	AY743637	CBS 1879

B = venous blood (sample); SF = synovial fluid; PF = peritoneal fluid; VF = vitreous fluid; SB = skin biopsy; A = BacT/Alert FA; B = BACTEC Plus Aerobic/F (media); C = BACTEC Lytic/10 Anaerobic/F = D, BACTEC Peds Plus/F; E = BBL MGIT; ATCC = American Type Culture Collection (Manassas, VA); CBS = Centraal Bureau voor Schimmelcultures (Utrecht, the Netherlands); IFM = Institute for Food Microbiology (Chiba, Japan); UAMH = University of Alberta Microfungus Collection and Herbarium (Edmonton, AB, Canada); FEMS Yeast Res. = isolate recovered from fermented orange (Las Heras-Vazquez et al., 2003); S288C = *Saccharomyces cerevisiae* Genome Sequencing Project.

[a] Based on PCRS and phenotypic identification.

[b] BLAST results based on the criteria outlined in Materials and methods.

[c] NCBI BLAST terminology: normalized value of alignment derived from the raw alignment score (used to compare alignment scores from different searches).

[d] NCBI BLAST terminology: sequence length of the query (test sequence) compared with the subject (GenBank record).

[e] Mixed culture: *Candida parapsilosis* and *Candida albicans* isolated from subculture.

[f] Mixed culture: *Candida guilliermondii* and *Corynebacterium* sp. isolated from subculture.

36 h. The majority of fungi were identified by d-PCRS within 24 h of the initial positive microscopy result. The d-PCRS results for all fungi were obtained before the results of conventional identification, and in some cases, d-PCRS identification resulted in a change in patient management (data not shown).

Table 2
VITEK identification results of fungi requiring additional phenotypic testing after d-PCRS identification

Patient	d-PCRS identification	VITEK identification	VITEK bionumber	Final identification established by:
32	*Candida dubliniensis*	99% *Candida albicans*, <1% *Candida tropicalis*	514455411	Growth at 45 °C, dark-green colonies on CCAN, morphology on CMAT: chlamydospores in groups of 3–4
23	*Candida kefyr*	99% *Saccharomyces cerevisiae*, 1% *Candida kefyr*	500200410	ID32C (99.8% *Candida kefyr*, bionumber: 7220710001), morphology on CMAT
38	*Candida parapsilosis*	75% *Candida albicans*, 14% *Candida parapsilosis*	557457015	Mauve colonies on CCAN, repeat VITEK identification (99% *Candida parapsilosis*, bionumber: 513447011)
29	*Candida albicans*	79% *Candida guilliermondii*, 20% *Candida famata*	577657615	Germ-tube formation, repeat VITEK identification (99% *Candida albicans*, bionumber: 555457411)
41	*Candida guilliermondii*	69% *Candida famata*, 30% *Candida guilliermondii*	575777015	ID32C (low discrimination 92.6% *Candida guilliermondii*, bionumber: 7377352117), morphology on CMAT
3	*Candida orthopsilosis*	80% *Candida parapsilosis*, 15% *Candida tropicalis*	557557015	PCRS identification of the isolate grown from subculture

3.4. BLAST similarity search results and sequence quality assessment

All test sequences from d-PCRS analysis showed >99% similarity when compared with known reference sequences using BLAST. Twenty test sequences demonstrated 100% sequence similarity to the reference sequence used for identification (44%). These included *Candida parapsilosis* (n = 8), *Candida albicans* (n = 7), *Candida orthopsilosis*, *Candida dubliniensis*, *Candida guilliermondii*, *Saccharomyces cerevisiae*, and *Candida kefyr*. Six test sequences demonstrated 99.8% sequence similarity to the reference sequence used for identification (13%). These included *Candida albicans* (n = 5) and *A. fumigatus*. Six test sequences demonstrated 99.6% sequence similarity to the reference sequence used for identification (13%). These included *Scedosporium prolificans* (n = 4), *Candida albicans*, and *M. pachydermatis*. The remainder of test sequences demonstrated 99.2% to 99.4% sequence similarity to the reference sequence used for identification (30%). The sequence test length remained relatively constant for test sequences identified as the same species.

3.5. Cost analysis: conventional versus d-PCRS

The average cost of performing conventional identification of fungi from blood cultures was AU$24.07 per isolate. This comprised reagents (AU$17.75) and labor (AU$6.32). The cost of performing d-PCRS identification (including the initial amplification step and sequencing reactions) was AU$24.54 per isolate. This comprised reagents (AU$18.00) and labor (AU$6.54).

4. Discussion

Given that methods for recovering fungi from blood, fluids, and tissues have improved with continuously monitored blood culture systems (Nolte et al., 1993; O'Hara et al., 2003; Thomson and Miller, 2003), it is reasonable to assume that uncommon fungi are likely to be encountered more frequently in the clinical microbiology laboratory. The routine identification of some fungi can be time consuming. Isolates from blood cultures are recovered by subculture and are identified using a range of culture-based methods. Rapid identification of fungi directly in blood cultures using molecular methods has been reported (Borst et al., 2001; Shin et al., 1999). However, the number of species identified by PCR-based methods is limited to the range of species-specific primers, nucleotide probes, or fluorogenic labels utilized in the assay. Hence, these methods usually target a small number of clinically important species. In contrast, broad-range PCR amplification and DNA sequencing of rRNA genes can potentially identify a wider range of fungi (Hall et al., 2003; Hall et al., 2004; Pryce et al., 2003). Although not as rapid as other molecular methods, a sequencing result can be obtained within 24–48 h from the first visible signs of growth (Hall et al., 2003; Henry et al., 2000; Pryce et al., 2003).

Until now, sequence-based approaches have not been evaluated for the direct identification of fungi in blood cultures. The MicroSeq D2 large-subunit ribosomal DNA sequencing kit (Applied Biosystems) has shown promise for identifying common clinical yeasts and filamentous fungi from solid media (Hall et al., 2003, 2004). However, less common yeasts and many clinically important filamentous fungi are not represented in MicroSeq sequence database, which may result in incorrect identification (Hall et al., 2003, 2004). In addition, the D2 large-subunit ribosomal DNA target may not demonstrate sufficient sequence variation to discriminate some species (Hall et al., 2004; Iwen et al., 2002). Furthermore, the cost of performing a sequence-based identification using this commercial system is high compared with phenotypic methods. Cost analysis comparing phenotypic testing to nucleic acid sequencing showed that the MicroSeq D2 identification was US$ 29.50 higher than the cost of using the API 20C AUX system (Hall et al., 2003).

We previously reported a rapid sequence-based approach for the accurate identification of 27 different species of fungi from the first visible signs of growth on solid media (Pryce et al., 2003). In the present study, we applied a similar approach for the rapid identification of fungi directly in liquid media. Depending on the time of day the culture

signaled positive, we obtained a d-PCRS identification within 24–36 h from the initial detection of fungi by Gram staining. The d-PCRS results agreed with the results obtained by conventional phenotypic methods to the species or genus level (Table 1). However, the d-PCRS identification of mixed cultures may be problematic. Other fungi not detected by d-PCRS may significantly alter patient management. Therefore, assessment of mixed cultures using appropriate culture methods is important. In contrast, the d-PCRS identification of fungi in mixed cultures containing bacteria (patient 41) may be advantageous. In this particular case, a slow-growing *Corynebacterium* sp. contaminated biochemical reactions, resulting in an ambiguous VITEK identification. Furthermore, the d-PCRS identification was useful to resolve ambiguous VITEK identifications and guide the selection of the most appropriate phenotypic tests to establish a final phenotypic identification (Table 2). The d-PCRS result of *Candida orthopsilosis* was of particular interest. The isolate grown from subculture was unable to be reliably identified. *Candida orthopsilosis* is a newly proposed species name to replace *Candida parapsilosis* group II and is morphologically indistinguishable from *Candida parapsilosis* group I and *Candida metapsilosis* (formerly *Candida parapsilosis* group III). There are currently no reliable phenotypic tests to differentiate these species (Tavanti et al., 2005). For other fungi, the d-PCRS method was the only effective means of species identification, particularly when a slow-growing fungus was encountered and only elementary distinctions such as septate hyphae were observed by microscopy. In these situations, nucleic acid sequencing may provide the greatest benefit to the laboratory and the clinician.

Overall, the broad-range primers V9D and LS266 primers worked well (PCR-A). However, these primers did not generate a PCR product from 1 blood culture containing *Cryptococcus neoformans*. Hence, primers ITS1 and ITS4 were used as an alternative (PCR-B) based on previous work in our laboratory (unpublished data). Both PCR methods target the entire ITS1 and ITS2 regions of the rRNA gene complex. These regions have been reported as promising targets for probe-based PCR assays and sequence-based assays for the identification and discrimination of many species of fungi (Lin et al., 2001; Pryce et al., 2003). The ITS1 and ITS2 regions combined are relatively short in length (~300–800 bp) and demonstrate significant sequence variation. In contrast, the 18S and the 28S rRNA genes of fungi are more highly conserved and larger in length (~1800 and 2900 bp, respectively), limiting the usefulness of these regions for species identification by sequencing. In some groups of fungi, species identification using ITS regions may also pose difficulties (Iwen et al., 2002). Within the genus *Fusarium* for example, reevaluation of the phenotypic positioning or the proposed revision of morphologic-based taxonomic schemes may be required. Whether sequence analysis of ITS regions will provide the ultimate sequence-based identification method needs to be established. In the present study and from previous work (Pryce et al., 2003), we have demonstrated the usefulness of the ITS regions as suitable targets for the sequence-based identification of a range of clinically important fungi. Furthermore, we have demonstrated comparable costs between the d-PCRS method and phenotypic methods of identification used in our laboratory, particularly identifications requiring additional or repeat phenotypic tests. From our assessment of total costs, screening positive blood cultures for *Candida albicans* using a rapid and specific real-time PCR assay, followed by d-PCRS identification if *Candida albicans* is not detected, may be a more cost-effective alternative for our laboratory.

Overall, the test sequences from each positive sample showed a high degree of similarity to each reference sequence used as a standard for d-PCRS identification (Table 1). The reference sequences used as standards for d-PCRS identification were selected from GenBank records based on strict criteria. Following these criteria, we were able to overcome some of the major limitations concerning the use of sequences in GenBank for organism identification. These include the use of incomplete sequences or sequences derived from nonreferenced or even misidentified cultures. We identified fungi by using the BLAST algorithm to compare our test sequences with sequences in the GenBank database. The output from BLAST arranges the sequences producing significant alignments by bit score followed by expect value. In our approach, the GenBank record with the highest bit score and expect value equal to zero from the reference sequences used as standards (Table 1) was recorded as the most likely species identification. Care should be taken although, as many species names that appear in the BLAST output may be incorrect, synonyms, or teleomorphs for a particular species.

Broth enrichment media inoculated with blood and other types of clinical samples are not normally considered to be ideal samples for PCR because of inhibitory substances such as sodium polyanetholesulfonate (SPS) (Fredricks and Relman, 1998). Various methods have been reported for the extraction of DNA from a variety of blood culture media (Chang et al., 2001; Fredricks and Relman, 1998, Iwen et al., 2004; Millar et al., 2000). Some of these methods are labor-intensive, and PCR inhibitors have been reported in DNA extractions from BACTEC and BacT/ALERT media using the QIAmp blood kit (Qiagen Corporation, Valencia, CA) (Fredricks and Relman, 1998) and the Roche High Pure PCR Template Preparation Kit (Millar et al., 2000). Steps to remove SPS by washing of inoculated BacT/ALERT medium by centrifugation before QIAmp DNA extraction are ineffective (Fredricks and Relman, 1998). DNA extraction of inoculated BacT/ALERT blood culture fluid based on a simple alkali wash method developed for BACTEC media (Kulski and Pryce, 1996) has been reported as the most sensitive, reproducible, and reliable method in a study evaluating 7 different DNA extraction methods (Millar et al., 2000). We developed a

similar approach to pretreat inoculated BacT/ALERT FA, BACTEC Plus Aerobic/F, Peds Plus/F, Lytic/10 Anaerobic/F, and BBL MGIT media with an alkali wash buffer before DNA extraction with a column-based kit. In addition, we removed charcoal and resins before DNA precipitation with isopropanol. This modification to the Roche high pure PCR preparation method is essential to remove these interfering substances before the DNA elution step. No PCR inhibition was observed in inoculated broth enrichment media positive for fungi. Furthermore, no PCR inhibition was observed in a separate PCR assay assessing the presence of PCR inhibitors in the negative controls. The column-based DNA extraction kit used provided sufficient DNA for PCR from all culture-positive samples and is well suited for routine diagnostic use. Current automated systems of nucleic acid extraction, such as the MagNA Pure LC instrument (Roche Diagnostics), may offer a less labor-intensive approach than column-based methods in the future.

The d-PCRS method described is an accurate and reliable tool for the identification of fungi directly in commercial broth enrichment media. Furthermore, it is possible that the d-PCRS method may be able to identify newly described species of fungi without the need to design new molecular approaches in the future. Moreover, this method may be useful in identifying fungi directly from clinical specimens. It is these types of situations where nucleic acid sequencing may provide the greatest benefit to the clinician. Further investigation is required to assess the clinical impact of rapid d-PCRS identification of fungi in the setting of a controlled clinical trial. Finally, more sequences are being deposited in GenBank and many have been identified by our laboratory as suitable reference sequences for identification of fungi, providing strict criteria are used for their inclusion.

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