Multiplex Tandem PCR: a Novel Platform for Rapid Detection and Identification of Fungal Pathogens from Blood Culture Specimens[▽]

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We describe the first development and evaluation of a rapid multiplex tandem PCR (MT-PCR) assay for the detection and identification of fungi directly from blood culture specimens that have been flagged as positive. The assay uses a short-cycle multiplex amplification, followed by 12 simultaneous PCRs which target the fungal internal transcribed spacer 1 (ITS1) and ITS2 region, elongation factor 1-α (EF1-α), and β-tubulin genes to identify 11 fungal pathogens: *Candida albicans, Candida dubliniensis, Candida glabrata, Candida guilliermondii, Candida krusei, Candida parapsilosis* complex, *Candida tropicalis, Cryptococcus neoformans* complex, *Fusarium solani, Fusarium* species, and *Scedosporium prolificans*. The presence or absence of a fungal target was confirmed by melting curve analysis. Identification by MT-PCR correlated with culture-based identification for 44 (100%) patients. No cross-reactivity was detected in 200 blood culture specimens that contained bacteria or in 30 blood cultures without microorganisms. Fungi were correctly identified in five specimens with bacterial coinfection and in blood culture samples that were seeded with a mixture of yeast cells. The MT-PCR assay was able to provide rapid (<2 h), sensitive, and specific simultaneous detection and identification of fungal pathogens directly from blood culture specimens.

Fungal bloodstream infections (BSIs) are a leading cause of morbidity and mortality in the critically ill and immunosuppressed population (13, 37). *Candida* species represent the fourth most common cause of nosocomial BSIs in the United States (12) and rank as the eighth most common cause of all BSIs in Australia and Europe (9, 15). While *Candida albicans* remains the predominant pathogen, the proportion of invasive candidiasis caused by other species of *Candida*, including *Candida parapsilosis, Candida glabrata, Candida tropicalis, Candida krusei, Candida dubliniensis*, and *Candida guilliermondii*, continues to increase. Together, these species cause 94.9% of all candidemia episodes in Australia (9). *Cryptococcus* species (24) and the molds *Fusarium* sp. (2, 25, 47) and *Scedosporium prolificans* (21, 42, 43) are also important fungal pathogens in blood.

Rapid identification of fungal pathogens in blood is essential for patient management. However, diagnosis is often delayed because current laboratory practices are laborious and slow. The "gold standard" for detection of fungemia is blood culture, which is slow (requiring at least 48 to 96 h to achieve species identification) and insensitive and fails to detect yeasts in up to 65% of patients (6). Furthermore, a period of 5 to 7 days is required to reliably declare a specimen negative (31, 38).

Numerous strategies for more rapid identification of fungal pathogens from blood cultures have been described. These include single-target and multiplex PCR assays which have used electrophoretic migration, DNA sequencing, or fluorescent probe hybridization for fungal identification (7, 8, 22, 30, 34, 39, 40). While these assays have shown promise in diagnosing fungal infections, there are problems that prevent their routine use in clinical laboratories. For example, the use of multiple fluorescent probes is expensive, size differentiation by gel electrophoresis does not provide real-time analysis, and DNA sequence analysis is time-consuming and expensive and cannot detect mixed fungemia. Multiplex tandem PCR (MT-PCR), on the other hand, is a simple, rapid, real-time assay that can provide a sensitive alternative for the simultaneous detection and identification of numerous pathogens (up to 72 targets) directly from clinical specimens (44).

We describe the development and evaluation of a novel, rapid MT-PCR assay for the detection and identification of fungi directly from positive blood cultures. The assay identifies seven *Candida* species (*C. albicans, C. dubliniensis, C. glabrata, C. guilliermondii, C. krusei, C. parapsilosis* complex, and *C. tropicalis*), *Cryptococcus neoformans* complex, *Fusarium solani, Fusarium* sp., and *S. prolificans*. Target species were selected based on their (i) expected frequency of recovery from blood culture, (ii) potential resistance to commonly administered antifungals, and (iii) clinical importance. We targeted sequence-variable regions of the multicopy ribosomal DNA complex (internal transcribed spacer 1 [ITS1] and ITS2) (23) and the elongation factor 1-α (EF1-α) (14) and β-tubulin (17) genes. The MT-PCR assay results were correlated with those obtained by culture.

MATERIALS AND METHODS

Cultured microbial strains. The MT-PCR assay was initially developed and validated with pure cultures of 35 fungi, 13 bacteria, and one parasite (Table 1). Reference fungal and bacterial strains were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Clinical isolates were obtained from

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TABLE 1. Fungal, bacterial, and parasitic strains used to evaluate the MT-PCR assay

Organism	Strain
Candida albicans	ATCC 10231
Candida dubliniensis	ATCC 7988
Candida glabrata	ATCC 90030
Candida guilliermondii	Clinical isolate
Candida famata	Clinical isolate
Candida krusei	ATCC 6258
Candida lusitaniae	ATCC 42720
Candida parapsilosis	ATCC 22019
Candida orthopsilosis	WM01.57
Candida metapsilosis	WM01.56
Candida tropicalis	ATCC 750
Saccharomyces cerevisiae	ATCC 9763
Cryptococcus neoformans	ATCC 90112
Cryptococcus gattii	ATCC 32608
Trichosporon asahii	Clinical isolate
Scedosporium apiospermum	Clinical isolate
Scedosporium prolificans	Clinical isolate
Fusarium dimerum	Clinical isolate
Fusarium moniliforme	Clinical isolate
Fusarium oxysporum	ATCC 48112
Fusarium oxysporum	Clinical isolate
Fusarium solani	Clinical isolate
Fusarium solani	Clinical isolate
Fusarium sp.	Clinical isolate
Fusarium sp.	Clinical isolate
Fusarium sp.	Clinical isolate
Fusarium sp.	Clinical isolate
Aspergillus fumigatus	ATCC 204305
Aspergillus flavus	ATCC 204304
Aspergillus nidulans	Clinical isolate
Aspergillus niger	Clinical isolate
Aspergillus terreus	Clinical isolate
Rhizomucor pusillus	Clinical isolate
Rhizopus microsporus	Clinical isolate
Absidia corymbifera	Clinical isolate
Streptococcus pneumoniae	ATCC 49619
Mycoplasma pneumoniae	Clinical isolate
Chlamydia pneumoniae	Clinical isolate
Klebsiella pneumoniae	ATCC 13883
Legionella pneumophila	Clinical isolate
Legionella longbeachae	Clinical isolate
Haemophilus influenzae	Clinical isolate
Klebsiella pneumoniae	Clinical isolate
Moraxella catarrhalis	Clinical isolate
Pseudomonas aeruginosa	ATCC 27953
Staphylococcus epidermidis	ATCC 12228
Staphylococcus aureus	ATCC 25923
Escherichia coli	ATCC 25922
Toxoplasma gondii	001[a]

[a] Strain 001 was originally obtained from the Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia.

the Mycology and Bacterial Identification Reference Laboratories in the Centre for Infectious Diseases and Microbiology Laboratory Services, Institute of Clinical Pathology and Medical Research, Westmead Hospital (Westmead, New South Wales, Australia). *Toxoplasma gondii* was originally obtained from the Walter and Eliza Hall Institute of Medical Research (Parkville, Victoria, Australia). Fungal isolates were cultured on Sabouraud dextrose agar (Difco, Detroit, MI), and bacteria were cultured on nutrient agar (Oxoid Ltd., Hampshire, England) for 48 h aerobically at 30 and 35°C, respectively. *T. gondii* was propagated in human embryonic lung MRC5 fibroblast cells. Clinical isolates were identified by conventional morphological and physiological methods (11, 28, 35).

Blood culture specimens. Cultures that had been flagged by the automated blood culture systems (Becton-Dickinson BACTEC 9240 Blood Culture System [North Ryde, New South Wales, Australia] and the Baxter Scientific Microscan Walkaway 40 blood culture analyzer [Old Toongabbie, New South Wales, Australia]) in the diagnostic laboratories of three Sydney hospitals (Westmead,

Royal North Shore, and St. Vincent's) and were positive for yeast by Gram staining were investigated. Seventy blood cultures positive for yeast were obtained from 44 patients. Two-milliliter aliquots were frozen at −20°C for DNA extraction. Thirty negative blood culture specimens and 200 specimens containing bacterial species (including *Staphylococcus aureus*, *Staphylococcus epidermidis*, coagulase-negative *Staphylococcus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Streptococcus pneumoniae*, *Proteus mirabilis*, *Salmonella enterica* serovar Typhi, *Bacteroides fragilis*, *Klebsiella oxytoca*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Streptococcus gordonii*, *Streptococcus pyogenes*, *Streptococcus constellatus*, *Streptococcus salivarius*, *Sphingobacterium spiritivorum*, *Corynebacterium jeikeium*, *Propionibacterium acnes*, *Micrococcus* sp., and *Stomatococcus* sp.) were included as negative controls. Broth from a subset of uninoculated blood culture bottles was also tested.

Total nucleic acid extraction. Nucleic acid was extracted in a class II laminar-flow cabinet with the nucliSENS easyMAG instrument (BioMérieux, Baulkham Hills, New South Wales, Australia) as follows.

(i) Microbial cultures. Two-milliliter suspensions of a 0.5 McFarland standard were prepared from pure cultures. For fungal DNA extraction, suspensions were centrifuged at 16,000 × g for 10 min and the supernatant was removed. The pellet was resuspended in 200 μl molecular biology grade water (Sigma-Aldrich, Castle Hill, New South Wales, Australia) containing 50 U lyticase (Sigma-Aldrich) and incubated at 37°C for 1 h. The suspension was transferred to the nucliSENS easyMAG instrument for DNA isolation according to the manufacturer's instructions. DNA was isolated directly from the bacterial and parasite suspensions with the nucliSENS easyMAG instrument. The elution volume selected for all microbial DNA extractions was 110 μl.

(ii) Blood culture specimens. DNA was extracted from blood culture media (100 μl) as previously described (39), with some modifications. Briefly, after washes in alkali wash solution (0.5 M NaOH, 0.05 M trisodium citrate dehydrate) (27) and phosphate-buffered saline (0.137 M NaCl, 3 mM KCl, 8 mM Na$_2$HPO$_4$), the pellet was resuspended in 200 μl molecular biology grade water containing 50 U lyticase and incubated at 37°C for 1 h and at 95°C for 10 min. The suspension was then transferred to the nucliSENS easyMAG instrument for DNA isolation. The elution volume selected was 60 μl.

Primer design. To ensure accuracy and reproducibility and to identify possible intraspecies variation, consensus sequences were generated from available GenBank sequences with BioEdit (18) and CLUSTAL_W (http://www.ebi.ac.uk/Tools/clustalw2/). Consensus sequences from different species were aligned with CLUSTAL_W to identify variable regions suitable for species specific identification. Primers were designed with AusDiagnostics Pty. Ltd. (Alexandria, New South Wales, Australia) software (http://www.ausdiagnostics.com/qilan/AusPrimerDesign .jsp). The ITS1 and ITS2 regions, EF1-α, and β-tubulin genes were used to design specific primers targeting *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. guilliermondii*, *C. krusei*, *C. parapsilosis*, *C. tropicalis*, *C. neoformans*, *F. solani*, *Fusarium* sp., and *S. prolificans*. Primer sequences are not shown due to commercial confidentiality agreements with AusDiagnostics Pty. Ltd. (Table 2). Where possible, inner amplicons were restricted to 70 to 100 bp and outer amplicons were restricted to <200 bp. Primer pairs were rejected if they did not produce the correct-size product on a Bioanalyzer DNA separation chip (Agilent Technologies, Forest Hill, Victoria, Australia). The theoretical specificities of all primer sequences were tested with the Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

MT-PCR amplification and detection. Mastermix reagents and 72-well gene discs containing lyophilized primers were prepared by AusDiagnostics Pty. Ltd.

(i) First-round multiplexed preamplification. PCRs were performed in a 20-μl volume consisting of 10 μl step 1 mastermix (composition is commercial in confidence; Corbett Research, Mortlake, New South Wales, Australia), 7 μl molecular biology grade water, 1 U MangoTaq DNA polymerase (Bioline, Alexandria, New South Wales, Australia) containing 20 U Moloney murine leukemia virus reverse transcriptase (Invitrogen, Mt. Waverly, Victoria, Australia), and 2 μl template DNA. Amplification was performed on a RotorGene thermal cycler (RG6000; Corbett Research). Thermal cycling conditions were 55°C for 2 min and 95°C for 5 min, followed by 10 cycles of 95°C for 10 s, 60°C for 20 s, and 72°C for 20 s.

(ii) Second-round quantification amplification. Four microliters of each multiplexed amplification product was diluted into 150 μl step 2 mastermix (composition commercial in confidence; Corbett Research), 138.4 μl molecular biology grade water, and 7.6 U MangoTaq DNA polymerase. A 20-μl aliquot was then added to the corresponding 12 positions on the gene disc containing the lyophilized inner primers. Amplification was performed on the RG6000 thermal cycler. Thermal cycling conditions were 55°C for 2 min, followed by 30 cycles of 95°C for 1 s, 60°C for 10 s, and 72°C for 10 s. Fluorescence was measured at the end of each 72°C extension step. Following cycling, a melting curve was gener-

TABLE 2. MT-PCR gene targets, amplicon sizes, and expected melting temperatures for species identification[a]

Organism	Target	Size (bp)	Expected melting temp (°C)[b]
Candida albicans	ITS2	70	83.8
Candida dubliniensis	ITS2	90	82
Candida glabrata	ITS2	82	82–85
Candida guilliermondii	ITS2	93	83
Candida krusei	ITS1	99	88.2
Candida parapsilosis	ITS2	81	79.9
Candida tropicalis	ITS1	96	80
Cryptococcus neoformans complex	ITS1	70	81.4
Fusarium solani	EF1-α	77	83.1
Fusarium sp.	ITS2	70	84.8
Scedosporium prolificans	β-Tubulin	76	81
Positive control	Artificial sequence	76	82

[a] Primer sequences are withheld due to commercial confidentiality agreements with AusDiagnostics Pty. Ltd.
[b] The expected melting temperature is compared with the melting temperature determined by each assay to determine the presence or absence of an organism.

ated from 72 to 95°C at 0.5°C intervals. The expected melting temperature of each fungal target was established from pure culture DNA (Table 2). The presence or absence of an organism was determined by analysis software (Aus-Diagnostics) which compared the given melting temperature to the expected melting temperature. An internal positive control was included for each specimen to monitor for PCR inhibition.

Assay detection limit. To determine the MT-PCR detection limit for fungal cells extracted from whole blood, serial dilutions of *C. albicans* (10^4 to 1 CFU/ml) were seeded into blood obtained from healthy donors, in triplicate. Yeast cell numbers were confirmed by plating dilutions onto Sabouraud dextrose agar (Difco) and counting colonies following 2 days of incubation at 30°C. DNA was extracted and subjected to MT-PCR (see above).

RESULTS

Primer specificity. All primers hybridized specifically with their respective DNA targets. Primers for *C. parapsilosis* amplified all members of the *C. parapsilosis* complex (*Candida orthopsilosis* and *Candida metapsilosis*) (46). Primers for *C. glabrata* did not cross-react with *Candida nivariensis* (1), a member of the *C. glabrata* complex. No cross-reactivity to other clinically relevant microorganisms (Table 1) or human DNA was observed.

Detection limit and reproducibility. The detection limit of the assay was tested in triplicate by inoculating serial dilutions of *C. albicans* into blood obtained from healthy individuals. The detection limit was 10 CFU/ml blood, equivalent to 0.33 cell per PCR (Fig. 1).

The reproducibility of the MT-PCR was confirmed with a mixture containing 50 different template DNAs; the identifying melting temperature (Fig. 2 and Table 2) was consistent in six replicates.

Fungal identification from polymicrobial mixtures. To simulate the possibility of polyfungal infection and demonstrate the multiplex capability of MT-PCR, blood cultures seeded with two or more *Candida* species were tested. The results show the specific identification of each *Candida* species (Fig. 3). In the case of polymicrobial samples, no interference with fungal identification was observed in blood cultures seeded with a mixture of fungal and bacterial cells.

Fungal identification from blood culture bottles. Seventy blood culture specimens from 44 patients that were flagged as positive for microbial growth and were found to contain yeast by Gram staining were tested. Species identification by MT-PCR was concordant with culture-based identification for 42 patients for which there was a detection target (Table 3). No MT-PCR result was obtained for patients 25 and 38 due to the absence of targets for *Candida nivariensis*, *Kodamaea ohmeri*, and *C. lambica* in this assay.

For sample 16A, phenotypic tests could only provide pathogen identification to the genus level. MT-PCR, however, determined the organism to be *C. parapsilosis*. This result was confirmed by MT-PCR of the culture isolate. Specimen 36 was positive for yeast by Gram staining; however, no isolate was recovered after 5 days of incubation. MT-PCR, followed by panfungal PCR (29), confirmed the absence of fungal DNA. Specimen 33 contained both *C. parapsilosis* and *C. lusitaniae*; however, only *C. parapsilosis* DNA was detected by MT-PCR due to the absence of a *C. lusitaniae* target in this assay.

Five samples (specimens 10, 19A, 25, 32, and 33) showed that fungal identification was not inhibited in the presence of bacterial coinfection. No cross-reactivity was detected in 200 control blood culture specimens that were positive for bacteria, 30 negative blood cultures, or broth from a subset of uninoculated blood culture bottles.

DISCUSSION

Rapid and accurate fungal identification is critical for early initiation of targeted therapy and improving patient outcomes. Diagnosis, however, is often delayed by the need to obtain a pure culture (24 to 48 h) and by time-consuming conventional tests. The results from this study indicate that the application
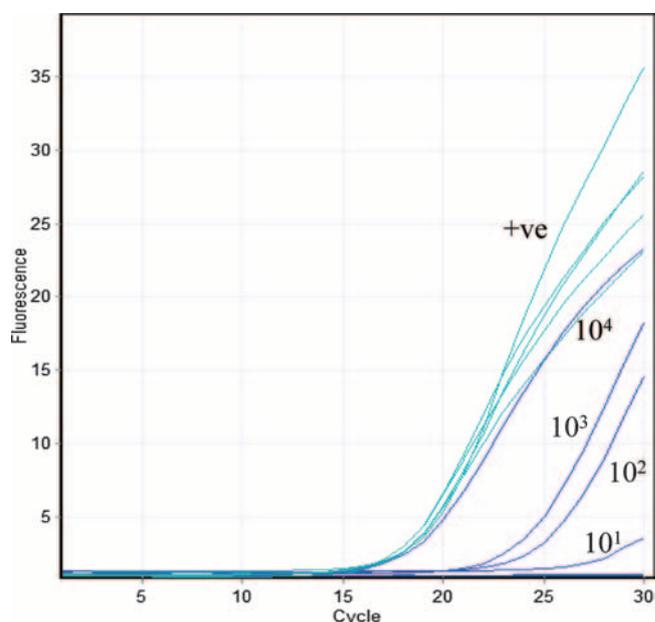

FIG. 1. Cycling curve produced from blood samples seeded with serial dilutions of *C. albicans*, showing the detection limit of MT-PCR (CFU/ml). The positive control (+ve) indicates that the assay was not inhibited.
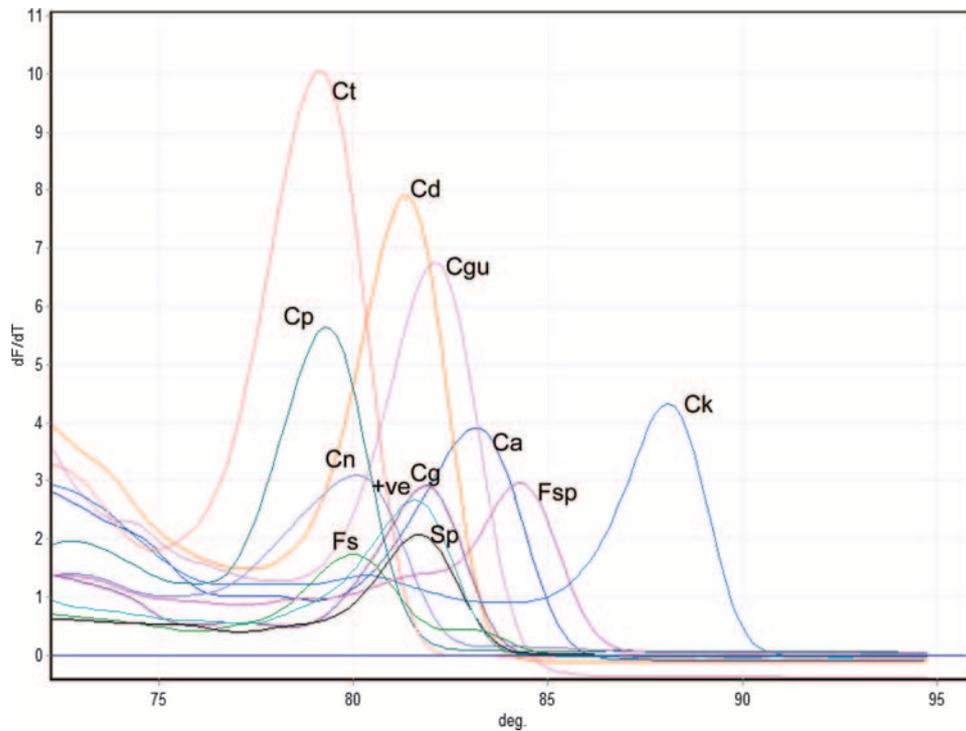
FIG. 2. Melting curves generated from a mixture of 49 different DNA templates (Table 1) illustrating the melting peaks obtained from the 12 MT-PCR targets: *C. albicans* (Ca), *C. dubliniensis* (Cd), *C. glabrata* (Cg), *C. guilliermondii* (Cgu), *C. krusei* (Ck), *C. parapsilosis* (Cp), *C. tropicalis* (Ct), *C. neoformans* (Cn), *F. solani* (Fs), *Fusarium* sp. (Fsp), *S. prolificans* (Sp), and a positive control (+ve). deg, degrees; dF/dT, derivative of fluorescence with respect to temperature.

of a fungal MT-PCR assay can greatly improve routine diagnostics by providing a highly sensitive and practical means to rapidly identify yeasts and molds directly from blood culture specimens.

The clinical applicability of the assay was confirmed by testing 70 blood culture specimens from 44 patients that were positive for yeast by Gram staining. Results concordant between culture and MT-PCR were achieved for 42 patients for which there was a detection target (Table 3). For patients 25 and 38, no MT-PCR result was obtained due to the absence of a detection target for *C. nivariensis*, *K. ohmeri*, and *C. lambica* on the assay. All three organisms are uncommon causes of
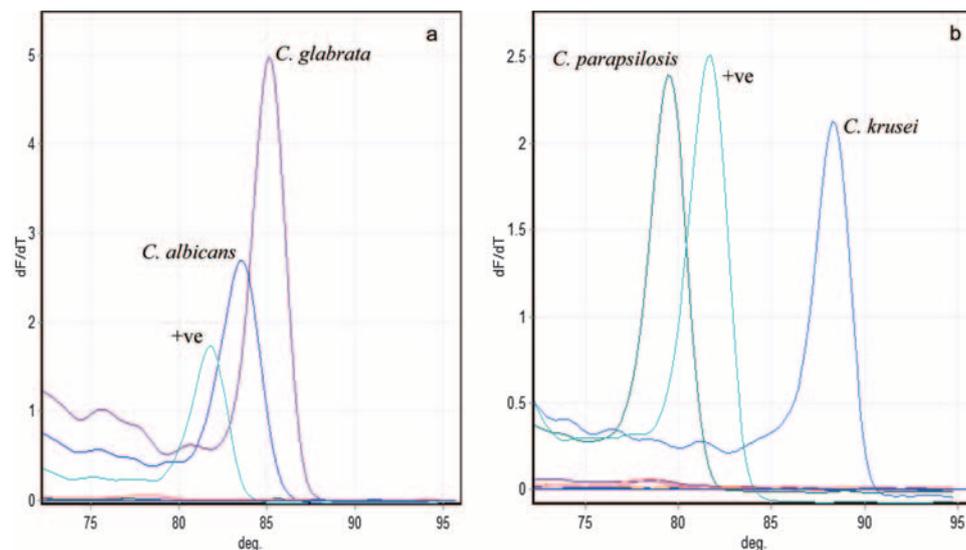

FIG. 3. Melting curves generated from blood cultures seeded with *C. albicans* and *C. glabrata* (a) and with *C. parapsilosis* and *C. krusei* (b). The melting temperatures determined were compared to the expected melting temperatures (Table 2) by automated analysis software to determine the presence or absence of an organism. +ve, positive control; deg, degrees; dF/dT, derivative of fluorescence with respect to temperature.

TABLE 3. Results of culture and MT-PCR analysis of blood culture specimens

Patient	Identification by culture	MT-PCR identification
1A	*Candida parapsilosis*	*C. parapsilosis*
1B	*C. parapsilosis*	*C. parapsilosis*
1C	*C. parapsilosis*	*C. parapsilosis*
1D	*C. parapsilosis*	*C. parapsilosis*
2	*Candida albicans*	*C. albicans*
3	*Candida glabrata*	*C. glabrata*
4	*C. albicans*	*C. albicans*
5A	*C. parapsilosis*	*C. parapsilosis*
5B	*C. parapsilosis*	*C. parapsilosis*
6A	*C. albicans*	*C. albicans*
6B	*C. albicans*	*C. albicans*
7A	*C. glabrata*	*C. glabrata*
7B	*C. glabrata*	*C. glabrata*
7C	*C. glabrata*	*C. glabrata*
7D	*C. parapsilosis*	*C. parapsilosis*
8	*C. albicans*	*C. albicans*
9	*C. parapsilosis*	*C. parapsilosis*
10	*C. albicans, Klebsiella oxytoca*	*C. albicans*
11	*C. glabrata*	*C. glabrata*
12A	*Candida tropicalis*	*C. tropicalis*
12B	*C. tropicalis*	*C. tropicalis*
13	*C. albicans*	*C. albicans*
14	*C. albicans*	*C. albicans*
15	*Cryptococcus neoformans* complex	*C. neoformans* complex
16A	*Candida* species	*C. parapsilosis*
16B	*C. parapsilosis*	*C. parapsilosis*
17	*C. tropicalis*	*C. tropicalis*
18	*C. krusei*	*C. krusei*
19A	*C. parapsilosis, Staphylococcus epidermidis*	*C. parapsilosis*
19B	*C. parapsilosis*	*C. parapsilosis*
20	*C. glabrata*	*C. glabrata*
21	*C. parapsilosis*	*C. parapsilosis*
22	*C. albicans*	*C. albicans*
23	*C. glabrata*	*C. glabrata*
24A	*C. albicans*	*C. albicans*
24B	*C. albicans*	*C. albicans*
25	*Candida nivariensis,[a] S. epidermidis*	None[c]
26A	*C. glabrata*	*C. glabrata*
26B	*C. glabrata*	*C. glabrata*
26C	*C. glabrata*	*C. glabrata*
26D	*C. glabrata*	*C. glabrata*
26E	*C. glabrata*	*C. glabrata*
27	*C. albicans*	*C. albicans*
28	*C. albicans*	*C. albicans*
29	*C. glabrata*	*C. glabrata*
30A	*C. glabrata*	*C. glabrata*
30B	*C. glabrata*	*C. glabrata*
31A	*C. neoformans* complex	*C. neoformans* complex
31B	*C. neoformans* complex	*C. neoformans* complex
32	*C. glabrata, Streptococcus constellatus*	*C. glabrata*
33	*C. parapsilosis, Candida lusitaniae,* coagulase-negative *Staphylococcus*	*C. parapsilosis*
34A	*C. tropicalis*	*C. tropicalis*
34B	*C. tropicalis*	*C. tropicalis*
34C	*C. tropicalis*	*C. tropicalis*
34D	*C. tropicalis*	*C. tropicalis*
35	*C. albicans*	*C. albicans*
36	None[d]	None[e]
37	*C. albicans*	*C. albicans*
38A	*Kodamaea ohmeri[b]*	None[c]
38B	*Candida lambica*	None[c]
39	*C. albicans*	*C. albicans*
40A	*C. parapsilosis*	*C. parapsilosis*
40B	*C. parapsilosis*	*C. parapsilosis*
40C	*C. parapsilosis*	*C. parapsilosis*
40D	*C. parapsilosis*	*C. parapsilosis*
41A	*C. albicans*	*C. albicans*
41B	*C. albicans*	*C. albicans*
42	*C. parapsilosis*	*C. parapsilosis*
43	*C. parapsilosis*	*C. parapsilosis*
44	*C. parapsilosis*	*C. parapsilosis*

[a] This isolate was originally identified as *C. glabrata* by the ID32C yeast identification system (BioMérieux); however, the genetically close and emerging pathogen *C. nivariensis* was identified following PCR amplification and DNA sequencing of the ITS2 region.

[b] This isolate was originally identified as *C. guilliermondii* by the RapID Yeast Plus (Remel Products, Lenexa, KS); however, the genetically close and emerging pathogen *K. ohmeri* was identified following PCR amplification and DNA sequencing of the ITS2 region.

[c] No MT-PCR identification is available due to the absence of a detection target for this organism on this assay.

[d] No growth after 5 days.

[e] No MT-PCR detection.

candidemia. In an Australian survey of candidemic episodes, only one isolate of *C. lambica* was identified from 1,095 cases over a 4-year period (9). Similarly, there have been few reports documenting fungemia due to *K. ohmeri* (4, 19, 20, 33, 41, 45) and *C. nivariensis* (1, 5, 16). Targets for these organisms can be added to the MT-PCR in the future if there are increasing reports of their causing candidemia.

Polycandidal infections were observed in 2.2% of the cases surveyed in the Australian Candidemia Study (9). To simulate such episodes and demonstrate the specific and multiplex capability of the MT-PCR platform, blood cultures were spiked with two or more *Candida* species and each species was identified (Fig. 3a and b). Furthermore, specificity of fungal identification was maintained in the presence of bacterial coinfection (specimens 10, 19A, 25, 32, and 33).

The choice of species targeted and configuration of the assay were determined by the frequency with which various *Candida* species are recovered from blood, likely resistance to commonly administered antifungal drugs (*C. glabrata*, *C. krusei*, *Fusarium* species, and *S. prolificans*), and a high probability of early death (*S. prolificans*). Although no cases of *Fusarium* or *S. prolificans* infection were observed in this study, there have been increasing reports highlighting the significant need for the rapid identification of these molds from blood culture (3, 32, 36, 42). *Aspergillus* species were not included as a target in this assay because they are rarely cultured from blood (10).

The fungal MT-PCR assay has demonstrated high levels of sensitivity (10 cells/ml blood) and specificity. For primer design, it was important to consider the quality of the input sequence, as GenBank sequence submissions are not peer reviewed and it has been estimated that 10 to 20% of the fungal sequences in GenBank have been misidentified (26). Therefore, primers were designed from consensus sequences generated in regions devoid of intraspecies variability.

The "nested" feature of the MT-PCR also contributed to the high level of specificity, as it depended on two different sets of specific primers for both rounds of amplification. This differs from other published assays, which have only provided one level of specificity by combining panfungal amplification and species-specific primers (7), probes (40), or product size differentiation (8, 30) for fungal identification. Manipulation of post-PCR products always carries the potential for contamination. However, MT-PCR is not like a nested PCR assay since the first-step multiplex PCR is not run to completion and serves only as a preamplification process. Only 10 cycles of amplification were used in step 1, and at this stage of a PCR, undetectable amounts of product are made. To date, we have observed no case of cross-contamination.

The fungal MT-PCR offers further advantages over traditional multiplex assays because it is a real-time test and uses automated software analysis to determine the presence or absence of an organism. Unlike classical multiplex assays, where inhibition due to competition between primers is inevitable, MT-PCR incorporates two separate steps for multiplexing and quantification. Competition between primers in the first-step multiplex assay is avoided, as DNA is only amplified for a limited number of cycles (10 cycles), at which point the amount of deoxynucleoside triphosphates and primers is not limiting. Carryover of primers from the first step to the quantification PCR is also negligible since the first-round product is diluted

50-fold (44). This prevents the step 1 primers from taking part in the step 2 reaction and avoids problems with PCR inhibition. The inclusion of an internal positive control for each specimen monitors for PCR inhibition.

The MT-PCR process is simple, and a robot for automated operation (AusDiagnostics Pty. Ltd.) is available so that the platform can be operated by laboratory staff without molecular test experience. Furthermore, this assay utilizes SYBR green technology, so expensive probes are not required. Altogether, the MT-PCR assay is a rapid and cost-effective platform, requiring <4 h for the whole procedure (DNA extraction, MT-PCR, and analysis), and has costs similar to those of current routine laboratory tests.

In conclusion, this study illustrates the potential benefits of using fungal MT-PCR for the sensitive and specific identification of fungal pathogens from blood culture specimens. The assay has the advantage of accurate and simultaneous identification of microbial infections. It is envisaged that the assay will provide a major advantage in the routine diagnostic laboratory by providing faster identification, leading to prompt initiation of targeted therapy and better clinical outcomes. The platform has shown excellent potential for the development of additional fungal MT-PCR assays targeting other organisms for different specimen types. Evaluation of the fungal MT-PCR on other types of clinical specimens is currently under way.

ACKNOWLEDGMENTS

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