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Rapid Identification of Yeasts Commonly Found in Positive Blood Cultures by Amplification of the Internal Transcribed Spacer Regions 1 and 2

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Abstract A multiplex PCR method using one universal and eight species-specific primers was developed to rapidly identify eight yeast species found in positive blood cultures. The species-specific primers were designed from the internal transcribed spacer regions 1 and 2 of the rRNA gene, whereas the universal primer was located at the 26S rRNA gene. The eight species were *Candida albicans*, *Candida glabrata*, *Candida guilliermondii*, *Candida krusei*, *Candida lusitanae*, *Candida parapsilosis*, *Candida tropicalis*, and *Cryptococcus neoformans*. The PCR products (116 to 630 bp) were different in length and could be effectively separated and recognized by polyacrylamide gel electrophoresis. By testing 234 positive blood cultures (237 isolates), 234 (98.7%) isolates of the above eight species were correctly identified by the multiplex PCR. The present method is simple to perform and can be completed within 6 h.

Introduction

Over the past decade, the incidence rates of fungemia have risen markedly, with *Candida* spp. now reported as the fourth most common cause of nosocomial blood-stream infections [1]. Collectively, *Candida albicans*, *Candida glabrata*, *Candida guilliermondii*, *Candida krusei*, *Candida lusitanae*, *Candida parapsilosis*, *Candida tropicalis*, and *Cryptococcus neoformans* may account for approximately 99% of all fungal isolates found in blood [2].

Rex et al. [3] reported that fluconazole, which has a low level of toxicity, is as effective as amphotericin B for treatment of candidemia in patients without neutropenia. However, *Candida glabrata* and *Candida krusei* are innately more resistant to fluconazole [2]. It was noted previously that between 1952 and 1992 the rate of *Candida glabrata* isolation from blood cultures was 8% [1]; however, this figure increased to 17–23% in a recent survey [2]. Therefore, a simple and rapid method for differentiating *Candida* spp. in blood cultures is useful for targeted drug therapy.

The identification of non-*albicans* *Candida* spp. in positive blood culture bottles by conventional methods normally takes 2–3 days. Several molecular techniques have been developed to identify yeasts causing fungemia [4, 5, 6, 7, 8]. The purpose of our study was to evaluate a multiplex PCR method using one universal and eight species-specific primers to rapidly identify eight yeast species commonly found in positive blood cultures.

Materials and Methods

A total of 248 pure cultures of the eight target yeasts isolated from clinical specimens were used to test the sensitivity of the multiplex PCR method described below. The clinical isolates were identified to the species level using conventional methods based on cultural and biochemical characteristics [9]; these isolates included *Candida albicans* (n=94), *Candida glabrata* (n=26), *Candida guilliermondii* (n=8), *Candida krusei* (n=16), *Candida lusitanae* (n=10), *Candida parapsilosis* (n=34), *Candida tropicalis* (n=38), and *Cryptococcus neoformans* (n=22). In addition, 168 reference strains (71 species

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representing 36 genera) of other yeasts and molds obtained from several culture collection centers were used to determine the specificity of the test. These non-target microorganisms included species of the following genera: *Aspergillus*, *Candida*, *Cryptococcus*, *Microsporium*, *Pichia*, *Saccharomyces*, *Trichosporon*, *Trichophyton* and others. Yeast DNA was extracted from pure cultures using the method described previously [7], whereas mold DNA was purified according to the method of Turenne et al. [8].

When positive blood culture bottles were detected by the BACTEC NR9240 instrument (Becton Dickinson Microbiology Systems, USA) during the day, Gram stain smears from positive bottles were prepared to check for the presence of yeasts. An aliquot (0.2 ml) from each positive broth containing yeast was immediately used for DNA extraction according to the method described previously [7]. A total of 234 positive blood cultures (237 yeast isolates) were analyzed using the multiplex PCR. Yeasts in positive bottles were isolated on subculture plates, identified to the species level using conventional methods based on cultural and biochemical characteristics [9], and the results were used as a reference to evaluate the performance of the multiplex PCR.

For the multiplex PCR, the eight species-specific forward primers were designed from the internal transcribed spacer (ITS)1 and ITS2 sequences available in the GenBank database. The eight species-specific primers (species, sequence, and accession number) were as follows: CL (*Candida lusitanae*, 5'-GTT AGG CGT TGC TCC GAA AT-3', AY207078); CP (*Candida parapsilosis*, 5'-GGC GGA GTA TAA ACT AAT GGA TAG-3', AY207072); CT (*Candida tropicalis*, 5'-AAG AAT TTA ACG TGG AAA CTT A-3', AY207080); CGU (*Candida guilliermondii*, 5'-GTA TTG GCA TGG GTA GTA CTG-3', AY207076); CA (*Candida albicans*, 5'-TCA ACT TGT CAC ACC AGA TTA TT-3', AY207067); CK (*Candida krusei*, 5'-GAT TTA GTA CTA CAC TGC GTG A-3', AY207070); CN (*Cryptococcus neoformans*, 5'-TGG ACT TTG GTC CAT TTA TCT AC-3', AY207081); and CGL (*Candida glabrata*, 5'-CAC GAC TCG ACA CTT TCT AAT T-3', AY207068). ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') was used as a universal reverse primer [10]. The locations of the eight species-specific forward primers and the universal reverse primer are shown in Fig. 1.

Primer concentrations used in the multiplex PCR were as follows: CL (0.12 μ M), CP (0.2 μ M), CT (0.2 μ M), CGU (0.12 μ M), CA (0.06 μ M), CK (0.3 μ M), CN (0.2 μ M), CGL (0.06 μ M), and ITS4 (0.12 μ M). All other reagents and their concentrations used for multiplex PCR were the same as those used previously [7], except this time bovine serum albumin (0.2%, w/v) was included. A positive culture broth containing *Candida albicans* was kept frozen and an aliquot of this broth was used as a positive control. A negative control was performed by substituting the template DNA with sterilized water in the PCR reaction mixture in each test run. DNA was also extracted from the blood of two healthy individuals

using the Blood and Tissue Genomic DNA Extraction Miniprep kit (Viogene, Taiwan) according to the procedures recommended by the manufacturer. PCR products were analyzed using polyacrylamide gel (10%) electrophoresis as described previously [11]. After electrophoresis, gels were stained with ethidium bromide. To determine the exact length of each PCR product, the PCR products were cycle sequenced in both directions using a model 377 sequencing system (Applied Biosystems, Taiwan) with the BigDye Terminator Cycle Sequencing kit (Applied Biosystems).

For positive blood cultures, test sensitivity was defined as the number of strains correctly identified (true positives) divided by the number of total strains isolated from positive cultures. Test specificity was defined as the number of non-target yeast strains that did not produce PCR product (true negatives) divided by the total number of these strains isolated [12]. Blood yeasts were identified by the conventional method [9] and used as a reference to evaluate the performance of the multiplex PCR. The detection limit of the multiplex PCR was determined by inoculating a blood bottle with a strain of *Candida albicans* (final concentration 10^3 cfu/ml); the bottle was incubated in the BACTEC NR9240 machine to yield a "positive" bottle. The positive broth was serially diluted 10-fold with the blood culture broth and 0.2 ml of the diluted samples was processed for multiplex PCR. The cell numbers (cfu/ml) of the diluted positive culture were determined by the plate count method [7].

Results and Discussion

In the multiplex PCR, *Candida lusitanae* produced the smallest amplicon, whereas *Candida glabrata* produced the largest one. The lengths of the PCR products were as follows: *Candida lusitanae* (116 bp), *Candida parapsilosis* (126 bp), *Candida tropicalis* (149 bp), *Candida guilliermondii* (185 bp), *Candida albicans* (402 bp), *Candida krusei* (475 bp), *Cryptococcus neoformans* (516 bp), and *Candida glabrata* (632 bp). The PCR products could be easily separated and recognized by polyacrylamide gel (10%) electrophoresis (data not shown).

A total of 248 pure clinical isolates of the eight target yeasts were tested using the multiplex PCR method. All isolates were correctly identified and a sensitivity of 100% was obtained. Moreover, 168 strains (71 species representing 36 genera) of other yeasts and molds were also analyzed. Among them, only *Kluyveromyces yarrowii* (2 strains) and *Trichosporon pullulans* (1 strain) produced PCR products with lengths of 115 and 200 bp, respectively. Therefore, the test specificity of the multiplex PCR for pure cultures was 98.3% (165/168). The remaining 69 species (165 strains) were PCR-negative, which might be due to the fact that the eight forward primers used in the PCR were species-specific.

A total of 234 positive blood cultures (237 isolates) containing yeasts were analyzed using the multiplex PCR for species identification. Among these positive cultures, there were three mixed cultures, with each mixed culture containing two different yeasts. Among the 237 isolates, 234 belonged to the group of eight target yeast species and were correctly identified by the multiplex PCR, resulting in a test sensitivity of 98.7% (234/237) (Table 1). The remaining three minor isolates that produced no PCR product were *Candida pelliculosa*, *Rhodotorula rubra*,

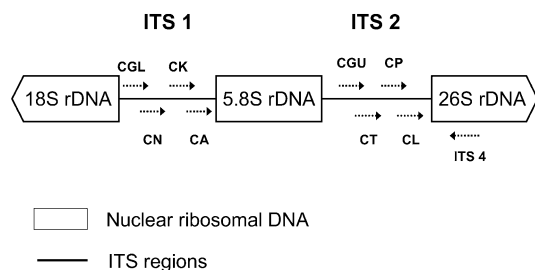


Fig. 1 Genomic organization of 18S rDNA, ITS1, 5.8S rDNA, ITS2, and 26S rDNA. Boxes indicate coding regions. Arrows labeled with CGL, CK, CN, CA, CGU, CP, CT, and CL represent forward species-specific primers for *Candida glabrata*, *Candida krusei*, *Cryptococcus neoformans*, *Candida albicans*, *Candida guilliermondii*, *Candida parapsilosis*, *Candida tropicalis*, and *Candida lusitanae*, respectively. ITS4 was a universal reverse primer

Table 1 Results of multiplex PCR for the identification of yeasts in 234 positive blood cultures (237 isolates)

Yeast	No. of isolates	No. of isolates identified	No. of isolates not identified	Percent sensitivity	Percent specificity
<i>Candida albicans</i>	121	121	0	100	
<i>Candida tropicalis</i>	50	50	0	100	
<i>Candida glabrata</i>	29	29	0	100	
<i>Candida parapsilosis</i>	20	20	0	100	
<i>Cryptococcus neoformans</i>	6	6	0	100	
<i>Candida krusei</i>	4	4	0	100	
<i>Candida guilliermondii</i>	3	3	0	100	
<i>Candida lusitanae</i>	1	1	0	100	
Other species	3	0	3 ^a	0	100
Total	237	234	3	98.7	100

^aThe three isolates not identified by PCR belonged to the species *Candida pelliculosa*, *Rhodotorula rubra*, and an unidentified *Candida* sp.

and an unidentified *Candida* sp. These three minor species were not identified (not misidentified); this might be due to the fact that the forward primers were species-specific and could not anneal to the template DNAs from the non-target species. Therefore, the test specificity of the multiplex PCR was 100% (3/3). The detection limit of the multiplex PCR was approximately 60 cfu/ml, as determined using a blood bottle seeded with a strain of *Candida albicans*. Human DNA isolated from whole blood samples of two healthy individuals also produced no PCR product.

Mixed yeast cultures in positive bottles could be identified simultaneously by the multiplex PCR, as revealed by the observation of more than one relevant PCR product on polyacrylamide gel. Two of three mixed cultures contained one strain of *Candida albicans* and one strain of *Candida parapsilosis*, whereas the third one was a mixed culture of *Candida albicans* and *Candida glabrata*.

To the best of our knowledge, this study is the first to identify as many as eight yeast species causing fungemia using a multiplex PCR set in a single reaction. These yeasts included seven species of *Candida* and *Cryptococcus neoformans*. When a yeast-positive blood culture was found, the present method was able to reduce the routine identification time from approximately 2–3 days to about 6 h. Compared with other methods of molecular diagnosis [4, 5, 6], the present method is relatively simple to perform, time-saving, and able to detect approximately 99% of yeast isolates in blood.

The present method was tested in three laboratories and was found to be reproducible. The efficacy of the present method relies on several factors. First, the cell concentration of yeasts in positive blood cultures normally exceeds 10⁵ cfu/ml [13]. Second, yeast rDNA has a high copy number (40–80 copies per haploid genome) [10]. Third, most fungemia are caused by a limited number of fungal species, and fourth, the ITS sequences within a species are highly conserved and they vary in length and sequence identity among different species [14].

In conclusion, the present method is highly sensitive and specific and is simpler to perform than any previously

reported molecular method for the identification of blood yeasts. From the time a positive bottle is found, the multiplex PCR method can be completed within 6 h.

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References

1. Pfaller MA, Jones RN, Messer SA, Edmond MB, Wenzel RP (1998) National surveillance of nosocomial blood stream infection due to species of *Candida* other than *Candida albicans*: frequency of occurrence and antifungal susceptibility in the SCOPE Program. *Diagn Microbiol Infect Dis* 30:121–129
2. Pfaller MA, Diekema DJ, Jones RN, Messer SA, Hollis RJ, the SENTRY Participants Group (2002) Trends in antifungal susceptibility of *Candida* spp. isolated from pediatric and adult patients with bloodstream infections: SENTRY antimicrobial surveillance program, 1997 to 2000. *J Clin Microbiol* 40:852–856
3. Rex JH, Bennett JE, Sugar AM, Pappas PG, Horst CM van der, Edwards JE, Washburn RG, Scheld WM, Karchmer AW, Dine AP, Levenstein MJ, Webb CD (1994) A randomized trial comparing fluconazole with amphotericin B for the treatment of candidemia in patients without neutropenia. *N Engl J Med* 331:1325–1330
4. Ahmad S, Khan Z, Mustafa AS, Khan ZU (2002) Seminested PCR for diagnosis of candidemia: comparison with culture, antigen detection, and biochemical methods for species identification. *J Clin Microbiol* 40:2483–2489
5. Morace G, Pagano L, Sanguinetti M, Posteraro B, Mele L, Equitani F, D'Amore G, Leone G, Fadda G (1999) PCR-restriction enzyme analysis for detection of *Candida* DNA in blood from febrile patients with hematological malignancies. *J Clin Microbiol* 37:1871–1875
6. Hee SJ, Nolte FS, Morrison CJ (1997) Rapid identification of *Candida* species in blood cultures by a clinically useful PCR method. *J Clin Microbiol* 35:1454–1459
7. Chang HC, Leaw SN, Huang AH, Wu TL, Chang TC (2001) Rapid identification of yeasts in positive blood cultures by a multiplex PCR method. *J Clin Microbiol* 39:3466–3471
8. Turenne CY, Sanche SE, Hoban DJ, Karlowitsky JA, Kabani AM (1999) Rapid identification of fungi by using the ITS2 genetic region and an automated fluorescent capillary electrophoresis system. *J Clin Microbiol* 37:1846–1851
9. Warren NG, Hazen KC (1999) *Candida*, *Cryptococcus*, and other yeasts of medical importance. In: Murray PR, Baron EJ, Pfaller MA, Tenover FC, Tenover RH (eds) *Manual of clinical*

- microbiology, 7th edn. American Society for Microbiology, Washington, DC, pp 1184–1199
10. White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gefland DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic Press, San Diego, pp 315–322
 11. Chrambach A, Rodbard D (1971) Polyacrylamide gel electrophoresis. *Science* 172:440–451
 12. Mahon CR, Manuselis G (2000) Textbook of diagnostic microbiology, 2nd edn. W.B. Saunders Company, London, pp 122–123
 13. Chang HC, Chang JJ, Huang AH, Chang TC (2000) Evaluation of a capacitance method for direct antifungal susceptibility testing of yeasts in positive blood cultures. *J Clin Microbiol* 38:971–976
 14. Iwen PC, Hinrichs SH, Rupp ME (2002) Utilization of the internal transcribed spacer regions as molecular targets to detect and identify human fungal pathogens. *Med Mycol* 40:87–109