

Experimental Study / Deneysel Çalışma

Identification of *Candida* Species from Blood Cultures with Fluorescent In Situ Hybridization (FISH), Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) and Conventional Methods

Kan Kültürü Örneklerinden Candida Türlerinin Floresan In Situ Hibridizasyon (FISH), Polimeraz Zincir Reaksiyonu-Restriksiyon Fragment Uzunluk Polimorfizmi (PCR-RFLP) ve Konvansiyonel Yöntemlerle İdentifikasyonu

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Objectives: Rapid and accurate identification of *Candida* species from blood cultures is crucial to ensure effective antifungal therapy and to reduce the morbidity and mortality associated with bloodstream fungal infections. In this study, we aimed to identify *Candida* spp. from blood culture samples with fluorescent in situ hybridization (FISH), polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and conventional methods.

Materials and Methods: A total of 50 yeast positive samples out of 325 blood culture positive samples and 50 blood culture negative samples were examined by FISH, PCR-RFLP and conventional methods to identify *Candida* spp.

Results: All three methods generally were compatible for identification of single-species *Candida* spp. ($p<0.001$). But, FISH and PCR-RFLP for the identification of multi-species *Candida* spp. were found more compatible than conventional methods ($p<0.001$). Besides, FISH is cheaper and quicker than the other two methods in the identification of *Candida* spp. from blood culture positive samples. The rates of multi-species candidemia with FISH, PCR-RFLP and conventional methods were 20%, 6% and 4%, respectively.

Conclusion: Both PCR-RFLP and FISH methods might be preferred for the rapid identification of *Candida* spp. from blood culture positive samples. However, FISH is a more suitable method for the detection of multi-species candidemia.

Key words: *Candida* spp.; FISH; PCR-RFLP; Candidemia; identification.

Amaç: Kan kültürlerinden *Candida* türlerinin doğru ve hızlı identifikasyonu fungal kan dolaşımı infeksiyonlarla ilgili mortalite ve morbiditeyi azaltmak ve etkili antifungal tedavi sağlamak için önemlidir. Bu çalışmada, kan kültürü örneklerinden *Candida* türlerinin floresan in situ hibridizasyon (FISH), polimeraz zincir reaksiyonu-restriksiyon fragment uzunluk polimorfizmi (PCR-RFLP) ve konvansiyonel yöntemlerle identifikasyonu amaçlandı.

Gereçler ve Yöntemler: *Candida* türlerinin identifikasyonu için 325 kan kültürü pozitif örnekten maya pozitif olan 50 örnek ile kan kültürü negatif 50 örnek FISH, PCR-RFLP ve konvansiyonel yöntemlerle incelendi

Bulgular: Her üç yöntemde tek tür *Candida* identifikasyonunda uyumlu idi ($p<0.001$). Ancak birden fazla *Candida* türlerinin identifikasyonunda FISH ve PCR-RFLP, konvansiyonel yöntemlere göre daha duyarlı bulundu ($p<0.001$). Ayrıca, FISH kan kültürü pozitif örneklerden *Candida* türlerinin identifikasyonunda diğer iki yöntemle göre daha hızlı ve ucuzdu. Birden fazla türle oluşan kandidemi oranı FISH, PCR-RFLP ve konvansiyonel yöntemle sırasıyla %20, %6 ve %4 olarak bulundu.

Sonuç: Kan kültürü pozitif örneklerden *Candida* türlerinin hızlı identifikasyonunda PCR-RFLP ve FISH yöntemi tercih edilebilir. Ancak FISH birden fazla türle oluşan kandidemilerin belirlenmesinde daha uygun bir yöntemdir.

Anahtar sözcükler: *Candida* spp., FISH, PCR-RFLP, kandidemi, identifikasyon.

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Candidemia is an important cause of morbidity and mortality in hospitalized patients.^[1,3] *Candida* species are the fourth most commonly isolated organism in bloodstream infections in hospitals.^[4,5] *Candida albicans* is the predominant cause of invasive candidiasis and accounting for over half of all cases in the USA. *Candida albicans* and other *Candida* species constitute 70% to 80% of invasive bloodstream fungal infections.^[2,6] Most *Candida* species, including *Candida albicans*, are generally susceptible to systemic antifungal agents, such as fluconazole and amphotericin B.^[1,7] In recent years, some studies have reported an increase in candidemia due to non-*albicans Candida* species, which is the threat of increased mortality and antifungal drug resistance.^[7,8] Non-*albicans Candida* spp. account for 60% of the episodes of candidemia to be related to the use of antifungal prophylaxis.^[1,9]

To reduce mortality due to bloodstream fungal infections and to ensure effective antifungal therapy, early detection and identification of *Candida* species are crucial. Therefore, rapid and accurate methods of identifying *Candida* spp. in blood cultures are critical.^[2,10,11]

Current conventional methods for the identification of *Candida* spp. from positive blood cultures are based on subculture to appropriate fungal medium, including Sabouraud dextrose agar, or CHROMagar *Candida* medium, and isolated yeast colonies are identified by the phenotypic (germ tube formation, carbohydrate assimilation [e.g., API 20C, ID 32C], and chlamyospore formation on cornmeal agar). However, current methods used on isolated *Candida* colonies from positive blood culture bottles can take one to three days for identification at the species level.^[12]

More recently, molecular techniques have found a broad application in the detection and identification of microorganisms in clinical samples without cultivation.^[10,13,14] Molecular techniques such as DNA sequence analysis, real-time polymerase chain reaction (RT-PCR), and multiplex PCR have enabled the detection and rapid identification of pathogenic fungi, including *Candida* species in clinical samples.^[14-16] However, most of these tests are still time-consuming and too expensive for routine use.

In recent years, molecular methods with discriminatory power have been required for the accurate, rapid, straightforward and inexpensive identification of *Candida* at the species level. Various PCR-based methods have been reported for the identification of *Candida* spp. from clinical samples including whole-blood culture. It has been shown that PCR-restriction fragment length polymorphism (RFLP) is a simple and easy method for medical mycology laboratories to use in the identification of *Candida* spp.^[17,18] Fluorescent in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes has also become a highly valuable tool for the identifica-

tion of microorganisms without cultivation.^[10,19] This method has been reported to be a reasonable and rapid method in the detection and identification of pathogens. Moreover, this method is cheaper and easier to perform than the other molecular methods in the microbiology laboratory. Pathogenic microorganisms have been detected rapidly and identified from positive blood culture bottles by using FISH. It has been reported that sensitivity and specificity of this test are 100%.^[11,20,21] There have been various probes to be used in FISH method such as rRNA-targeted oligonucleotide probes and peptide nucleic acid (PNA) probes.^[22,23] Moreover, there has been commercial SeaFast sepsis kit, including yeast to be used for FISH.^[24] It has been reported that the sensitivity and specificity of PNA probes and rRNA-targeted oligonucleotide probes were 100%.^[20,21] The commercial kit is also more expensive than others.

The aim of this study was to identify *Candida* spp. from blood culture positive samples with FISH, PCR-RFLP and conventional methods and to compare the practicability, cost, and identification time of three methods.

MATERIALS AND METHODS

The identification of *Candida* spp. with conventional methods was performed in the Department of Microbiology, Medical Faculty of Mersin University, PCR-RFLP and FISH analyses were conducted in the Department of Medical Genetics, Medical Faculty of Erciyes University.

Reference Strains

Candida albicans ATCC 64454, *Candida glabrata* ATCC 90030, *Candida parapsilosis* ATCC 90018, *Candida tropicalis* ATCC 2014 and *Candida krusei* ATCC 6258 were used as reference strains. In brief, yeast reference strains for FISH were grown in Sabouraud dextrose broth at 35°C and harvested while exponential growth phase. The yeast cells were fixed with 4% paraformaldehyde and then stored at -20°C as previously described.^[19] DNA isolation of reference strains were also performed according to the MagNA Pure LC DNA isolation Kit III (Bacteria, Fungi) instructions. Then, RFLP-PCR was applied as mentioned below.

Blood Cultures and Conventional Methods

325 (13%) out of a total 2492 blood cultures were positive and *Candida* spp. was isolated in 50 (15.4%) of 325 consecutive blood culture positive samples between January 2004 and August 2004 at Medical Microbiology laboratory. All the positive blood cultures belonged to different patients and there were not any occurrences of more than one positive blood culture from the same patients. A total of 50 negative blood cultures were also taken in this study. Blood samples from patients with suspected septicemia were spiked into BACTEC

plus Aerobic/F and BACTEC Peds Plus/F (Becton Dickinson, Sparks, MD, USA) medium and incubated in BACTEC 9050 (BD Diagnostic Systems, UK) blood culture system until the system indicated positive signals. Blood culture suspension was taken aseptically from positive blood culture bottles with a syringe and Gram stain for yeast from positive blood culture bottles was performed. The aliquot of yeast positive blood cultures was divided into three for the identification of *Candida* spp. with conventional methods, FISH and PCR-RFLP. Two of these blood culture samples taken for FISH and PCR-RFLP were stored at -20°C until use. Another blood culture was inoculated onto Sabouraud dextrose agar (Merck, Germany) and CHROMagar *Candida* (Becton Dickinson, France) simultaneously for the identification with conventional culture methods. Subsequently, *Candida* spp. was identified by standard laboratory methods such as germ tube formation, chlamyospore formation on cornmeal agar and API ID 32C (bioMérieux, France) test. Clinically relevant yeasts are conventionally identified by a combination of these phenotypic tests.

FISH

FISH was performed as previously described.^[19,20] An eight-field microscope slide was used for each sample. A total of $5\ \mu\text{l}$ from the yeast culture and blood culture samples was directly pipetted to each well onto an eight-field microscope slide and dried for 20 min at 45°C . The slides were dehydrated in 50%, 80% and 100% ethanol for 3 min each. In this study, RNA-targeted oligonucleotide probes, including one all yeast probe and four different specific probes, selected for *Candida* spp. from probeBase website were used.^[22] Fluorescein isothiocyanate (FITC) and CY3-labelled oligonucleotide probes were synthesized by Microsynth (Switzerland) and were used PF2 for all yeast (CTC TGG CTT CAC CCT ATT C), Caal for *C. albicans* (GCC AAG GCT TAT ACT CGC T), Cagl for *C. glabrata* (CCG CCA AGC CAC AAG GAC T), Cpara for *C. parapsilosis* (CCT GGT TCG CCA AAA AGG C), Ckrus for *C. krusei* (GAT TCT CGG CCC CAT GGG) and non Eub for the control probe (ACT CCT ACG GGA GGC AGC). First, the probes for each sample were arranged CY3 labeled-PF2, FITC or CY3 labeled-Caal, CY3 labeled-Cagl, FITC or CY3 labeled-Cpara, and CY3 or FITC labeled non Eub, respectively. Reference strains were used as positive control strain in the study. One microliter universal or specific probe (50 ng), and $9\ \mu\text{l}$ hybridization buffer (20 % formamide, 5 M NaCl, 1 M Tris pH=7, 10% SDS, H_2O) were added into each well and incubated in a moist chamber at 46°C for 1.5 h. In the mixed samples, $1\ \mu\text{l}$ Caal (CY3), $1\ \mu\text{l}$ Cpara (FITC) probes and $8\ \mu\text{l}$ hybridization buffer were applied into same well-slide in the repeated studies. The slides were washed in washing buffer (5 M NaCl, 1 M Tris pH=7, 10% SDS, H_2O) at 46°C for 15-20 min and $10\ \mu\text{l}$ of 0.001% DAPI

staining was added to each well. They were then incubated at room temperature for 7 min. After the slides were air-dried, Citifluor (Citifluor Ltd., London) was added onto the slides as a mounting medium. Finally, the slides were examined under a microscope (NIKON E 1000) equipped with a standard filter set and imaging analysis system (Applying Image) using software program (Mac probe Version 4.2.3).

PCR-RFLP

DNA isolation from *Candida* reference strains and blood culture samples was performed according to the manufacturer's instructions (MagNA Pure LC DNA isolation kit III; Bacteria, Fungi, Roche, Germany) using the MagNA pure LC system (Roche Diagnostics; Germany). The PCR-RFLP method was applied as described.^[18] Fungus-specific universal primer pairs ITS1 (TCC GTA GGT GAA CCT GCG G) and ITS4 (TCC TCC GCT TAT TGA TAT GC) (Molbio, Germany) were applied for PCR. All reactions were performed in a total volume $50\ \mu\text{l}$. One microliter of template DNA, forward (ITS1) and reverse (ITS4) primer at $0.2\ \mu\text{M}$, deoxynucleotide triphosphate (dNTP) at $0.1\ \text{mM}$, $5\ \mu\text{l}$ of 10X PCR buffer and 2.5 U of Taq DNA polymerase were used for each reaction. The first denaturation step was carried out at 94°C for 5 min and followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 45 s, extension at 72°C for 1 min and final extension at 72°C for 7 min. Fifteen microliters of amplified PCR product was analyzed by agarose 2% gel electrophoresis in TBE buffer stained ethidium bromide ($0.5\ \mu\text{g}\ \text{ml}^{-1}$). Re-amplification was applied to negative or slight band samples on gel electrophoresis after first-round amplification. A total of $5\ \mu\text{l}$ amplified PCR products were used for re-amplification and the applications stated above were repeated.

MspI enzyme (Fermentas, EU) was used for species level identification of *Candida* spp. A $10\ \mu\text{l}$ aliquot of PCR product with 10 U MspI in a final reaction volume of $25\ \mu\text{l}$ was incubated at 37°C for 2 h. Restriction fragments were analyzed by agarose 3% gel electrophoresis in TBE buffer at 80 V for 45 min and stained with ethidium bromide. The results were evaluated according to the sizes of PCR products for *Candida* spp. before and after the digestion with MspI enzyme. Sizes of ITS1-ITS4 PCR products were evaluated as 535 bp (297, 238 bp after MspI) for *C. albicans*, 871 bp (557, 314 bp after MspI) for *C. glabrata*, 524 bp (340, 184 bp after MspI) for *C. tropicalis*, 510 bp (261, 249 bp after MspI) for *C. krusei*, 520 bp (520 bp after MspI) for *C. parapsilosis*.^[18]

Statistical Analysis

Statistical analyses were performed using SPSS software (SPSS 11.5 for Windows). To determine the consistency of FISH, PCR-RFLP and conventional methods for single species and multi-species *Candida* spp., kappa statistics was used.

RESULTS

Identification of *Candida* spp. from blood culture with conventional methods

Fifty blood culture negative samples were negative with conventional methods. Fifty yeasts were isolated from positive blood cultures with conventional methods (Table 1). The results of yeasts identified by a combination of the phenotypic tests were similar. Of the 50 yeasts isolated, *Candida parapsilosis* was identified in 22, *Candida albicans* in 15, *Candida tropicalis* in 7, *Candida glabrata* in 2, *C. albicans* plus *C. parapsilosis* in one, *Debaryomyces carsonii* in one, *Kloeckera japonica* in one and *C. parapsilosis* plus *Rhodotorula mucilaginosa* in one. Two samples detected as *C. glabrata* and *D. carsonii* were misidentified in the first isolation. Yet, these samples were identified as *C. parapsilosis* by another person in the second isolation and also confirmed with FISH and PCR-RFLP methods. *K. japonica* was identified by phenotypic tests such as chlamydospore formation on cornmeal agar, germ tube formation, CHROMagar *Candida* and API 32, *C. albicans*. Also, *D. carsonii* and *R. mucilaginosa* were detected by chlamydospore formation on cornmeal agar, germ tube formation, CHROMagar *Candida*. Two or more *Candida* spp. identified from a single set or different sets of blood cultures were considered to have multi-species candidemia. The rate of multi-species candidemia was found to be 4% (2 of 50 positive samples) with culture methods. Of the blood samples identified as *Candida* spp., 12 samples belonged to internal medicine unit and 11 samples belonged to intensive care unit, and 8 samples to pediatrics unit. *Candida parapsilosis* was found in most of the blood samples as the main aetiological agent in these three units.

FISH Results

All yeast probe, *Candida* specific probes and non-Eub probe were tested by reference strains using FISH

method. In the blood samples, only one blood sample identified as *K. japonica* with conventional methods was not determined with all yeast probe and *Candida* specific probes because morphological structure of the yeast cell deteriorated and evaluated as negative. *Candida* spp. was detected in 49 of the 50 (98%) positive blood culture samples with all yeast probe by FISH (Fig. 1) (Table 1).

The 12 *C. albicans*, 21 *C. parapsilosis*, one *C. glabrata*, one *C. albicans* plus *C. parapsilosis* determined with conventional methods were accurately identified by FISH with *Candida* specific probes. In three samples, *C. albicans* plus *C. parapsilosis* was also observed. *Candida tropicalis* (n=7) was not determined with specific probe because there was no specific probe in the present study. Yet, these samples were confirmed by conventional methods and PCR-RFLP. In 5 of 7 samples identified as *C. tropicalis* with conventional methods, small numbers of *C. parapsilosis* was also observed. *Candida albicans* (n=3), and *C. parapsilosis* (n=1) determined with conventional methods were identified as *C. albicans* plus *C. parapsilosis* (n=4) with FISH. In one blood sample, *C. parapsilosis* plus *R. mucilaginosa* were determined with conventional methods, whereas the only *C. parapsilosis* was identified in this sample with FISH. On the other hand, *D. carsonii* (n=1) and *C. glabrata* (n=1) misidentified with conventional methods were determined as *C. parapsilosis* with FISH. These samples were then confirmed as *C. parapsilosis* by repeating culture methods. All the samples were negative with non-Eub probe. Also, all the blood culture negative samples were negative with FISH.

In the present study, multi-species candidemia was found in 10 of 50 (20%) blood culture positive samples with FISH. In most of the samples with multi-species candidemia, small numbers of *C. parapsilosis* were observed as the other species. Moreover, especially Cpara probe did not have any similarity to sequences of

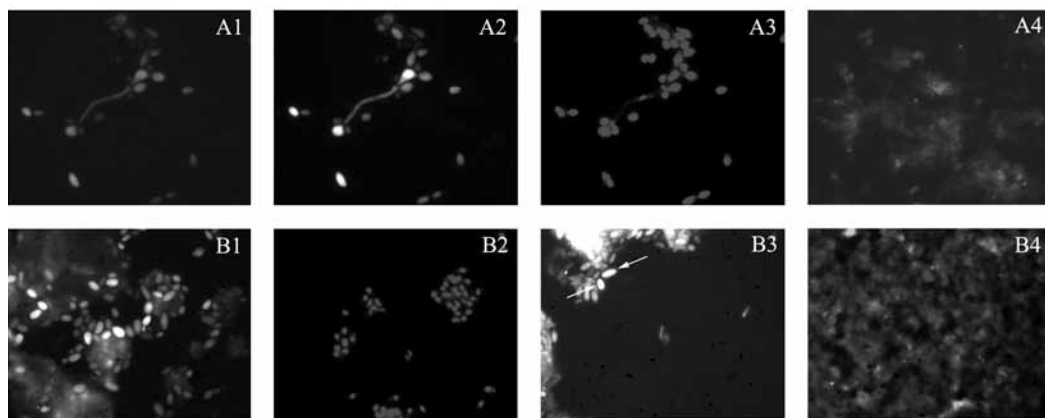


Fig. 1. The images with fluorescence microscopy of *Candida albicans* and *Candida parapsilosis* with CY3 and FITC-labelled oligonucleotid probes. A. 1. *C. albicans* with CY3-labelled PF2 probe (red signal; for all yeast), 2. *C. albicans* with FITC-labelled Caal probe (green signal; specific for *C. albicans* probe) 3. *C. albicans* stained with DAPI (blue signal; for DNA). 4. Negative. B. 1. *C. parapsilosis* with FITC-labelled Cpara probe (green; for *C. parapsilosis*), 2. *C. parapsilosis* stained with DAPI (blue signal; for DNA). 3. Mixture of *C. albicans* with CY3-labelled Caal probe (red signal) and *C. parapsilosis* with FITC-labelled Cpara probe (green signal). 4. Negative.

Table 1. Results of identification of yeast from positive blood cultures by FISH, PCR-RFLP and conventional methods

Sample unit	Conventional methods	FISH (Yeast probe/specific probe)	PCR-RFLP
1. Neurosurgery	<i>C. tropicalis</i>	Positive/ND	<i>C. tropicalis</i>
2. Pediatrics unit	<i>C. tropicalis</i>	Positive/ND	Negative
3. Plastic surgery	<i>C. parapsilosis</i>	Positive/ <i>C. parapsilosis</i>	<i>C. parapsilosis</i>
4. Internal medicine	<i>C. albicans</i>	Positive/ <i>C. albicans</i>	<i>C. albicans</i>
5. Pediatrics unit	<i>C. albicans</i>	Positive/ <i>C. albicans</i>	<i>C. albicans</i>
6. Intensive care	<i>C. albicans</i>	Positive/ <i>C. albicans</i>	<i>C. albicans</i>
7. Internal medicine	<i>C. parapsilosis</i>	Positive/ <i>C. parapsilosis</i>	<i>C. parapsilosis</i>
8. Internal medicine	<i>C. albicans</i>	Positive/ <i>C. albicans</i>	<i>C. albicans</i>
9. Internal medicine	<i>C. parapsilosis</i>	Positive/ <i>C. parapsilosis</i>	<i>C. parapsilosis</i>
10. Intensive care	<i>C. tropicalis</i>	Positive/ <i>C. parapsilosis</i> d+ND	<i>C. tropicalis</i> + <i>C. parapsilosis</i>
11. Internal medicine	<i>C. glabrata</i> a/ <i>C. parapsilosis</i> b	Positive/ <i>C. parapsilosis</i>	<i>C. parapsilosis</i>
12. Neurology	<i>C. parapsilosis</i>	Positive/ <i>C. parapsilosis</i>	<i>C. parapsilosis</i>
13. Plastic surgery	<i>C. parapsilosis</i>	Positive/ <i>C. parapsilosis</i>	<i>C. parapsilosis</i>
14. Urology	<i>C. albicans</i>	Positive/ <i>C. albicans</i>	<i>C. albicans</i>
15. General surgery	<i>C. parapsilosis</i>	Positive/ <i>C. parapsilosis</i>	<i>C. parapsilosis</i>
16. Pediatrics unit	<i>C. albicans</i>	Positive/ <i>C. albicans</i> + <i>C. parapsilosis</i> d	<i>C. albicans</i>
17. Chest diseases	<i>C. parapsilosis</i>	Positive/ <i>C. parapsilosis</i>	<i>C. parapsilosis</i>
18. Internal medicine	<i>C. parapsilosis</i>	Positive/ <i>C. parapsilosis</i>	<i>C. parapsilosis</i>
19. Pediatrics unit	<i>C. albicans</i>	Positive/ <i>C. albicans</i> + <i>C. parapsilosis</i> d	<i>C. albicans</i>
20. Internal medicine	<i>C. albicans</i>	Positive/ <i>C. albicans</i> + <i>C. parapsilosis</i> d	<i>C. albicans</i>
21. Internal medicine	<i>C. parapsilosis</i>	Positive/ <i>C. parapsilosis</i>	<i>C. parapsilosis</i>
22. Neurology	<i>Kloeckera japonica</i>	Negative	<i>C. tropicalis</i>
23. Internal medicine	<i>C. albicans</i>	Positive/ <i>C. albicans</i>	<i>C. albicans</i>
24. Pediatrics unit	<i>C. tropicalis</i>	Positive/ <i>C. parapsilosis</i> d+ND	<i>C. tropicalis</i> + <i>C. parapsilosis</i>
25. Pediatrics unit	<i>C. parapsilosis</i>	Positive/ <i>C. parapsilosis</i>	<i>C. parapsilosis</i>
26. Intensive care	<i>C. parapsilosis</i>	Positive/ <i>C. parapsilosis</i>	<i>C. parapsilosis</i>
27. Neurology	<i>C. tropicalis</i>	Positive/ <i>C. parapsilosis</i> d+ND	<i>C. tropicalis</i> + <i>C. parapsilosis</i>
28. General surgery	<i>D. carsonii</i> a/ <i>C. parapsilosis</i> b	Positive/ <i>C. parapsilosis</i>	<i>C. parapsilosis</i>
29. Thoracic Surgery	<i>C. albicans</i>	Positive/ <i>C. albicans</i>	<i>C. albicans</i>
30. Neurosurgery	<i>C. albicans</i>	Positive/ <i>C. albicans</i>	<i>C. albicans</i>
31. Infectious diseases	<i>C. tropicalis</i>	Positive/ <i>C. parapsilosis</i> d+ND	<i>C. tropicalis</i>
32. Neurology	<i>C. tropicalis</i>	Positive/ <i>C. parapsilosis</i> d+ND	<i>C. tropicalis</i>
33. Plastic surgery	<i>C. parapsilosis</i>	Positive/ <i>C. parapsilosis</i>	<i>C. parapsilosis</i>
34. Dermatology	<i>C. albicans</i>	Positive/ <i>C. albicans</i>	<i>C. albicans</i>
35. Pediatrics unit	<i>C. parapsilosis</i>	Positive/ <i>C. parapsilosis</i>	<i>C. parapsilosis</i>
36. Internal medicine	<i>C. parapsilosis</i>	Positive/ <i>C. parapsilosis</i>	<i>C. parapsilosis</i>
37. Pediatrics unit	<i>C. parapsilosis</i>	Positive/ <i>C. parapsilosis</i>	<i>C. parapsilosis</i>
38. Intensive care	<i>C. albicans</i> + <i>C. parapsilosis</i>	Positive/ <i>C. albicans</i> + <i>C. parapsilosis</i>	<i>C. parapsilosis</i>
39. Neurology	<i>C. parapsilosis</i>	Positive/ <i>C. parapsilosis</i>	<i>C. parapsilosis</i>
40. Cardiovascular surgery	<i>C. parapsilosis</i>	Positive/ <i>C. parapsilosis</i>	<i>C. parapsilosis</i>
41. Intensive care	<i>C. parapsilosis</i>	Positive/ <i>C. parapsilosis</i>	<i>C. parapsilosis</i>
42. Urology	<i>C. albicans</i>	Positive/ <i>C. albicans</i>	<i>C. albicans</i>
43. Neurosurgery	<i>C. parapsilosis</i>	Positive/ <i>C. parapsilosis</i>	<i>C. parapsilosis</i>
44. Intensive care	<i>C. albicans</i>	Positive/ <i>C. albicans</i>	<i>C. albicans</i>
45. Intensive care	<i>C. parapsilosis</i>	Positive/ <i>C. albicans</i> d+ <i>C. parapsilosis</i>	<i>C. parapsilosis</i>
46. Intensive care	<i>C. parapsilosis</i>	Positive/ <i>C. parapsilosis</i>	<i>C. parapsilosis</i>
47. Cardiovascular surgery	<i>C. parapsilosis</i> + <i>R. mucilaginosa</i>	Positive/ <i>C. parapsilosis</i>	<i>C. parapsilosis</i>
48. Internal medicine	<i>C. glabrata</i>	Positive/ <i>C. glabrata</i>	<i>C. glabrata</i>
49. Intensive care	<i>C. albicans</i>	Positive/ <i>C. albicans</i>	<i>C. albicans</i>
50. Intensive care	<i>C. parapsilosis</i>	Positive/ <i>C. parapsilosis</i>	<i>C. parapsilosis</i>

ND: Not determined (because we did not use any probe for *C. tropicalis* in this study); a: Wrong interpretation in the first culture; b: Correct interpretation in the second culture. c: This sample was not determined because of morphological structure of the yeast cell deteriorated; d: Small numbers of second *Candida* species in mixed samples were observed.

Table 2. The consistency of FISH, PCR-RFLP and conventional methods for single species and multi-species *Candida* spp.

<i>Candida</i> spp.	Positive (%)	Kappa value	<i>p</i>	Consistency	Results
<i>Candida albicans</i>					
Conventional methods - FISH	15 (100)	0.926	<0.001	Yes	Excellent
Conventional methods - PCR	14 (93.3)	0.922	<0.001	Yes	Excellent
FISH-PCR	15 (100)	0.926	<0.001	Yes	Excellent
<i>Candida parapsilosis</i>					
Conventional methods - FISH	24 (100)	0.760	<0.001	Yes	Good
Conventional methods - PCR	24 (100)	0.872	<0.001	Yes	Excellent
FISH-PCR	29 (85.3)	0.884	<0.001	Yes	Excellent
<i>Candida tropicalis</i>					
Conventional methods - PCR	5 (71.4)	0.823	<0.001	Yes	Excellent
<i>Candida glabrata</i>					
Conventional methods - FISH	1 (50)	0.662	<0.001	Yes	Good
Conventional methods - PCR	1 (50)	0.662	<0.001	Yes	Good
FISH-PCR	1 (100)	1.000	<0.001	Yes	Excellent
Multi-species					
Conventional methods - FISH	1 (50)	0.138	>0.001	No	-
Conventional methods - PCR	0 (0)	-0.25	>0.001	No	-
FISH-PCR	3 (30)	0.435	<0.001	Yes	Medium

other fungal species. Three of 10 mix cultures detected with FISH were identified with PCR-RFLP method and also one of these was determined accurately with conventional methods.

PCR-RFLP Results

Twenty eight (56%) out of 50 positive blood culture samples were successfully analyzed after the first-round amplification. After re-amplification, only one sample was negative. In 22 *C. parapsilosis*, 12 *C. albicans*, one *C. glabrata*, and one *C. albicans* plus *C. parapsilosis* determined with conventional methods were accurately identified with PCR-RFLP. In one blood sample, *C. parapsilosis* plus *R. mucilaginosa* were determined with conventional methods, whereas only *C. parapsilosis* was identified in this sample with PCR-RFLP methods. In 3 of 7 blood samples, *C. tropicalis* was identified with both conventional methods and PCR-RFLP. In 3 of 7 *C. tropicalis* isolates, *C. tropicalis* plus *C. parapsilosis* were identified with PCR-RFLP. In one blood sample, *C. tropicalis*, which had been identified as *K. japonica* with conventional methods, was determined with PCR-RFLP. *Debaryomyces carsoni* (n=1) and *C. glabrata* (n=1) misidentified with conventional methods were *C. parapsilosis* with PCR-RFLP (Table 1). Fifty blood culture negative

samples were also negative with PCR-RFLP. With this method, multi-species candidemia was found in 3 of 50 (6%) blood culture positive samples.

The comparison of FISH with PCR-RFLP and conventional culture methods

In the present study, of the 50 samples, the two blood samples misidentified as *D. carsonii* and *C. glabrata* in the first culture were then identified correctly with FISH, PCR-RFLP and in a second culture as *C. parapsilosis*. In one blood sample, *C. tropicalis*, which had been determined positive with culture methods, was found to be negative with PCR-RFLP. In one blood sample, *C. parapsilosis* plus *R. mucilaginosa* were determined with conventional methods, whereas only *C. parapsilosis* was identified in this sample with both FISH and PCR-RFLP methods. Only one sample was evaluated as negative with FISH because morphological structure of the yeast cell deteriorated. Initially, in 22 (44%) out of 50 positive blood culture samples were not obtained DNA with MagNA Pure LC DNA isolation kit in PCR-RFLP. Fifty (100%) out of 50 blood culture negative samples were also found negative with conventional methods, FISH and RFLP-PCR. The consistency of three methods is showed in Table 2. All three methods were compatible

Table 3. Comparison of FISH with PCR-RFLP and conventional methods

Methods	Time	Cost per sample	Multi-species candidemia (%)
FISH	2.5 h	9.1 \$	20
PCR-RFLP	6-8 h	33.8 \$	6
Conventional methods	1-3 days	16.9 \$	4

for single species *Candida* spp. (*Candida glabrata*, *Candida parapsilosis*, *Candida albicans*) ($p < 0.001$). The consistency of FISH for *Candida tropicalis* was not evaluated because there was not specific probe while PCR-RFLP and conventional methods for *Candida tropicalis* were compatible ($p < 0.001$). FISH and PCR-RFLP for multi-species *Candida* spp. were found compatible ($p < 0.001$).

In addition, the period of identification by FISH was also very short and the cost per sample was lower in FISH. The cost per sample for FISH, PCR-RFLP and conventional methods were 9.1 \$, 33.8 \$ and 16.9 \$, respectively. Multi-species candidemia was also observed to be higher with FISH than the other two methods (Table 3).

DISCUSSION

Although several molecular-based methods have been developed for the identification of *Candida* spp., recently, new molecular approaches for the diagnosis of fungal infections have been asserted that are straightforward, rapid to apply, and are inexpensive in routine laboratories. These tests are required for the identification of *Candida* spp., which is essential in order to reduce morbidity and mortality, and for the treatment of patients suffering from systemic candidiasis.^[10,18,25]

In the present study, the identification of *Candida* spp. from blood samples of patients with systemic candidiasis was performed with three different methods. The identification of *Candida* spp. with conventional methods takes at least 1-3 days, and sometimes it may lead to misinterpretation of the results. In this study, two blood samples were misidentified with conventional methods. Furthermore, experienced personnel are essential in the evaluation of the microscopic and culture.

Using one restriction enzyme with PCR-RFLP method allows the identification of six medically important *Candida* spp. It was applied by Mirhendi et al.^[18] using only one enzyme for the identification of six *Candida* spp. in 137 clinical isolates. However, MspI enzyme did not discriminate the species level of *C. albicans* and *C. dubliniensis* because the digestion of *C. albicans* and *C. dubliniensis* with MspI enzyme had similar patterns. Mirhendi et al.^[17] used BlnI enzyme in their studies to discriminate these two *Candida* species. In the present study, BlnI enzyme was not used to discriminate *C. dubliniensis* in the samples identified as *C. albicans* with PCR-RFLP because these samples were confirmed with both FISH and conventional methods. We found that PCR-RFLP was compatible with both FISH and conventional methods, except multi-species candidemia. Although PCR-based methods are also highly sensitive, it may be associated with several problems. An inter-laboratory study involving 30 laboratories in 18 countries to estimate the performance of amplification tests for routine diagnosis has reported that only five laboratories correctly identified the presence or absence of DNA.^[26] In this study, 56% of blood culture positive sam-

ples could be successfully analyzed in the first-round amplification with RFLP-PCR. The failure in first-round amplification may have three reasons: storage and transport of samples, inhibitors in blood and the presence of a small amount of the DNA in the sample. Van Deventer et al.^[27] demonstrated that storage conditions of blood samples influence sensitivity and reproducibility of the PCR assays, although they could not clarify the negative effect of freezing on PCR detection, since under microscopy the *Candida* cells looked intact and were still viable when checked by culture. Wolff et al.^[28] showed that the PCR reactions were completely inhibited by hemoglobin of 0.1 mg/ml and by a heparin concentration of 1.3×10^{-3} mg/ml. Moreover, sodium polyanetholesulfonate, which is ingredient of blood culture broth for neutralization of antibiotics, also appeared to represent a potent inhibitor in PCR techniques.^[29] Nucleic acid amplification assays might therefore be affected by inhibitors and can cause false-negative results. Yet, it has been determined that there was no inhibition of PCR with MagNA Pure LC DNA isolation kit III (Bacteria, Fungi) which is used in this study.^[30] We thought that the small amount of the DNA in the samples might be affected from the storage and transport conditions or a small amount of DNA in the samples were not isolated with MagNA Pure LC DNA isolation kit III (Bacteria, Fungi). That there is an increase in the positive, after second-round amplification supported this view.

Many studies have shown that FISH has a very high sensitivity and specificity for the identification of micro-organism.^[11,20,21] It has also been determined that PNA FISH test for *C. albicans* is more accurate than standard culture methods and results in significant cost saving.^[31] Our results showed that FISH was compatible with PCR-RFLP and conventional methods, except multi-species candidemia. FISH and PCR-RFLP were found compatible in the detection of multi-species candidemia. In this study, only one sample was evaluated as negative with FISH. Negative sample determined by FISH may be affected from prolonged storage. Prolonged storage may have decreased the sensitivity of FISH because long time storage of fixed cells is not recommended.^[19] Moreover, FISH was better than the other two methods regarding the identification time and the cost.

Molecular methods (FISH, seminested PCR) are more sensitive in the detection of multi-species candidemia than conventional culture methods.^[32,33] Haase et al.^[33] and Perry-O'Keefe et al.^[34] have shown that mixtures of isolates in cultures can be detected by FISH method and it has been indicated that PNA-FISH is more sensitive than conventional methods in the detection of mixtures of isolates in blood cultures.^[33,34] Similarly, the rate of multi-species candidemia with FISH is higher than PCR-RFLP and conventional methods in the present study. With FISH, the rate of multi-species candidemia was 3.4 times higher than that of PCR-RFLP, and about

10.2 times higher than that of conventional methods. The rate of multi-species candidemia with PCR-RFLP has not been found similar to that of FISH, which might be caused by the difficulty in obtaining DNA from cells. However, both FISH and PCR-RFLP were more compatible in the detection of multi-species candidemia than conventional methods in this study. Unfortunately, we could not make sequence analysis to confirm of these results. We thought that the reason for high rate of multi-species candidemia may be due to catheter-related nosocomial infections because all patients had catheter (not shown data). However, if clinical features of patients with multi-species candidemia are investigated, it may help to clean up the multi-species cases.

Although a chromogenic agar (CHROMagar *Candida*) was used to detect multi-species candidemia it was found that the rate of multi-species candidemia with conventional methods was low than FISH in the present study. There may be two reasons for the discrepancy with FISH and Chromagar *Candida* medium in mixed cultures. First, second species in mixed cultures may be not grown in the medium. Some studies have shown that microorganisms are not able to grow on medium while they are presence in microscopy.^[35,36] Second reason is that mixtures of similar-appearing yeast isolates could have been missed. Hospenthal et al.^[37] have determined that some *Candida* spp. can be hampered in mixtures of yeast isolates that produce pink colonies. Molecular methods appears more reliable to detect mixture isolates than phenotyping methods, especially FISH.

Our study has some limitations. First, the number of cases with candidemia was small. Another limitation was that there was no *C. tropicalis*-specific probe for FISH in this study. Yet, this limitation did not affect the results because all blood samples were definitely confirmed with all yeast probe, PCR-RFLP and conventional methods.

In conclusion, both FISH and PCR-RFLP methods are efficient methods to rapidly identify *Candida* spp. from blood culture positive samples in routine laboratories. PCR-RFLP method is simple and practical. FISH might also be preferred to the other two methods for routine applications in mycology laboratories; because FISH has advantages in terms of being practical, easy, cheap and quick. Moreover, this method appears to be more suitable, especially in the detection of multi-species candidemia.

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