

# Advantages and Accuracy of Multiprobe Real-Time PCR Detection of Uncommon and Mixed *Candida* Species in Blood Culture Broth of Candidemic Patients

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## ABSTRACT

Candidemia is a fatal condition and usually caused by a single common *Candida* species. Mixed *Candida* blood stream infection is not common, but it has a higher morbidity and mortality. This study reported the advantages of multiprobe real-time PCR assay to identify uncommon and mixed *Candida* species in blood culture broth which were misidentified by phenotypic methods. The assay shows a perfect specificity for identification of 137 individual yeast positive blood culture samples. Eleven of these (8%) were misidentified by the routine methods. There were two samples of *Candida albicans*, *C. glabrata* and *C. glabrata*, *C. tropicalis* mixed infections that were detected by the real-time PCR assay. The other nine samples such as 2 *C. parapsilosis*, 2 *C. glabrata*, 2 *C. athensensis*, 1 *C. dublinensis*, 1 *C. aquatextoris* and 1 *C. haemulonis* show discordant results between real-time PCR and phenotype, whereas DNA sequencing confirmed the accuracy of the real-time PCR results. The advantages of the real-time PCR assay over traditional mycology make it valuable for use in a routine clinical setting.

**Keywords:** Real-time PCR, *Candida*, blood culture, mixed infection

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## INTRODUCTION

**B**lood stream infection caused by a pathogenic yeast, *Candida* species, or candidemia is currently increasing in hospitals and it is now the fourth most common agent of all hospital-acquired bloodstream infections in western countries.<sup>1</sup> Recently, there has been a change in the epidemiology of invasive *Candida* infections with a shift from *Candida albicans* toward the non-*albicans* *Candida* species, including *Candida tropicalis*, *Candida glabrata* and *Candida parapsilosis*. These non-*albicans* *Candida* spp. differ in their anti-fungal susceptibilities. *C. glabrata* is innately resistant to azole drug, *C. tropicalis* has been reported in fluconazole and

amphotericin B resistance, whereas some *C. parapsilosis* resists to echinocandins.<sup>2</sup> However, *Candida* other than these 4 species can occasionally cause diseases and the data regarding antifungal susceptibilities and treatment outcomes of candidemia caused by uncommon species are lacking.

Polymicrobial bloodstream infections have been reported for 6%-34% of microbial infection cases and it is associated with a higher morbidity and mortality rate than monomicrobial infection.<sup>3</sup> Polymicrobial candidemia has been reported in a few studies. The mixed infection of candidemia can be caused by *Candida* spp. combined with other bacterial pathogens such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Acinetobacter baumannii*<sup>4-6</sup>. *Candida*/mold mixed infection has also been reported, such as with dematiaceous molds, *Cladophialophora bantiana* and *Aspergillus* sp.<sup>7,8</sup> Furthermore, candidemia caused by 2 or more *Candida* species have occurred; for instance, mixed infection of *C. albicans*/*C. glabrata* and *C. albicans*/*C. dubliniensis*.<sup>9,10</sup>

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Identification of *Candida* species requires isolation of the infecting organism from specimens. For bloodstream infection, blood culturing technique is the most reliable marker of invasive candidiasis. Yeast positive blood cultures are confirmed by Gram staining before sub-culturing on Sabouraud dextrose agar to develop colonies for phenotypic species identification methods. Although, phenotypic assays are commonly used in routine laboratory test, they are complicated, time-consuming, less specific and they lack ability to identify mixed *Candida* infection and uncommon *Candida* species. Molecular identification techniques have been developed to overcome several limitations of phenotypic assays which are beneficial for early diagnosis of candidemia leading to appropriate anti-fungal therapy. These techniques improve the sensitivity, specificity, accuracy and reduce the turn-around time of species identification.

Several assays were performed based on conventional PCR followed by a post amplification analysis step which involved a potential risk of contamination or are labor-intensive due to post amplification analysis. Some studies developed real-time PCR with specific probes for *Candida* identification in blood culture samples. However, the assay could not detect mixed infection in the sample, because the amplification during PCR will bias detection in favor of the predominant yeast population in a mixed infection and the PCR product could not be interpreted by melting curve analysis.<sup>11</sup>

In the present study, we report herein an in-house developed single-tube multiprobe real-time PCR assay which is superior to the conventional methods in identifying mixed *Candida* infection in positive blood culture broth samples. It can also correctly identify *Candida* strains which were misidentified by biochemical assays.

## MATERIALS AND METHODS

### Clinical samples

Routine yeast positive blood culture BacT/ALERT MB broths (bioMérieux, Durham, USA) and BD BACTEC Myco/F-lytic were tested by in-house multiprobe real-time PCR.<sup>12</sup> These samples were obtained from the Mycology Laboratory, Department of Microbiology, Faculty of Medicine Siriraj Hospital during September 2010 to October 2011. The samples were collected and cultured on Sabouraud dextrose agar. The isolates were identified by CandiSelect Test (Bio-Rad Laboratories, USA) and RapID Yeast Plus system (Innovative Diagnostic Systems, USA). The leftover broth samples were one milliliter aliquot and stored at -20°C for DNA extraction.

### DNA extraction

DNA of yeast positive blood culture samples were extracted by High Pure<sup>®</sup> PCR Template Preparation Kit (Roche Applied Science, Germany) according to the manufacturer's instructions with modifications as follows. One milliliter of sample was centrifuged and the pellet was suspended in 200 µl PBS with 10 µl lyticase (0.5 mg/ml) and incubated at 37°C for 30 minutes. Subsequently, lysis buffer with proteinase K was added. DNA was washed

four times, and eluted twice with 100 µl pre-warmed elution buffer. The second eluted sample was kept at -20°C for the PCR assay.

### Identification of *Candida* in blood culture broths

The second eluted DNA of individual routine yeast positive blood culture broths were tested by the multiprobe real-time PCR assay. A set of primers and four hybridization probes for *C. albicans*, *C. tropicalis*, *C. parapsilosis* and *C. glabrata* were used in one reaction mix as described elsewhere<sup>12</sup>. Briefly, the reaction of real-time PCR mixture contained 6 µl of nuclease free water, 4 µl of LightCycler<sup>®</sup> FastStart DNA Master PLUS Hybridization Probes kit mix (Roche Molecular Biochemicals, Mannheim, Germany), 1 µl of 10 µM of each primer, 1 µl of 2 µM of each probe and 2 µl of DNA in a final volume of 20 µl. Each set of experiments included negative and positive controls. Nuclease-free water replaced the DNA template for negative controls.

Positive controls were prepared by adding 2 µl of template DNA from *Candida* reference strains, *C. albicans* ATCC 90028, *C. parapsilosis* ATCC 22019, *C. tropicalis* ATCC 750, *C. glabrata* Cg01 and *C. krusei* ATCC 6258. The reactions were performed on the LightCycler<sup>®</sup> real-time PCR system, version 2.0. Signals of amplification PCR and melting temperature analysis were detected in a specific channel of the real-time PCR instrument. All positive signals of the amplification curve and specific melting peak pattern were used for species identification by referring to the reference strains. Then, not only the four common *Candida* species, but also *C. krusei* were identified.

### 18S ribosomal RNA gene sequencing

DNA of the identified samples was sequenced to confirm the results. Conventional PCR was performed to amplify 18S ribosomal DNA by *Candida* specific primer.<sup>12</sup> The PCR product was purified by QIAquick PCR Purification Kit (QIAGEN, Germany) according to the manufacturer's instructions. These purified PCR products were used as a template for nucleotide sequencing by Ward Medic Company, Thailand.

## RESULTS

The multiprobe real-time PCR assay could identify 137 positive automated blood culture broths as *C. albicans* (n = 43, 31.4%), *C. tropicalis* (n = 36, 26.3%), *C. parapsilosis* (n = 25, 18.2%), *C. glabrata* (n = 25, 18.2%), *C. krusei* (n = 1, 0.7%), 5 other *Candida* species-positive samples (3.7%), and 2 samples containing mixed *C. glabrata* / *C. albicans* and *C. glabrata* / *C. tropicalis* infections (1.5%). The results of 126 samples matched the corresponding results from phenotypic identifications and / or sequencing assays, whereas 11 samples showed discordant results between the real-time PCR assay and phenotypic identification.

In term of discordant results, 2 samples were identified by the PCR assay as mixed *Candida* species infection. One sample showed a mix of *C. albicans* and *C. glabrata*.

The phenotypic methods (CandiSelect Test and RapID Yeast Plus System) misidentified this sample as single infection with *C. albicans*. Subsequent testing of this blood culture broth on chromogenic media yielded a mixed culture of *C. glabrata* and a few *C. albicans* colonies. The second sample was identified as mixed *C. glabrata* and *C. tropicalis*, but the phenotypic assay showed only *C. tropicalis* infection. Similarly, subsequent testing of the sample showed mixed organisms of *C. tropicalis* and a few small colonies of *C. glabrata*. The identification of mixed infections was confirmed by sequencing.

The real-time PCR assay identified two samples as *C. parapsilosis* and the other two samples as *C. glabrata*, whereas their phenotypes were compatible with *C. zeylanoides* and *C. krusei*, respectively. DNA sequences of these isolates confirmed that the PCR assay results were correct. The other five samples were identified as other *Candida* species, because the amplification curve and melting temperature of these samples did not match with the PCR criteria for specific species identification. Subsequently, they were identified by DNA sequencing as other *Candida* species; 1 *C. dubliniensis*, 1 *C. aquaetextoris*, 2 *C. athensensis* and 1 *C. haemulonis* (Table 1).

## DISCUSSION

Systemic candidiasis has become a major cause of infection-related morbidity and mortality in immunocompromised and critically-ill patients. In general, candidemia is caused by only one pathogenic species. However, mixed species infection has been reported in some studies.<sup>9,10</sup> Mixed *Candida* infection has a higher morbidity and is more difficult to treat compared with those of single infection and may lead to inappropriate antifungal therapy. To solve these problems, an accurate and rapid diagnosis of *Candida* species is crucial. The molecular identification methods based on conventional PCR were able to identify the causative pathogens, but they were not practical for routine laboratory testing. Therefore, several real-time PCR techniques have been developed to reduce several problems of conventional identification methods such as time consuming and contamination in the post-PCR analysis step. Using an specific primer and specific probes in real-time PCR lead to high sensitivity, specificity and accuracy of the identification. In addition, this technique can detect a very small amount of nucleic acid.

In this study, we developed an in-house multiprobe real-time PCR technology for *Candida* identification to improve sensitivity, specificity, and turn-around time.<sup>12</sup> The PCR assay showed higher specificity than the phenotypic assay. Ten samples showed discordant results between the real-time PCR and phenotypic methods. Two samples were identified as *C. zeylanoides* by RapID Yeast Plus System methods with positive in glucose and proline tests, whereas *C. parapsilosis* were shown positive in glucose, proline and  $\alpha$ -glucose with this phenotypic method. However they were *C. parapsilosis* by real-time PCR assay and sequencing. It might be a false negative of the  $\alpha$ -glucose test in the RapID Yeast Plus System methods.<sup>13-15</sup> The other two samples had phenotypic features of *C. krusei*, because they showed positive only to glucose assimilation. However, they were *C. glabrata* confirmed by both real-time PCR assay and sequencing. *C. glabrata* has an ability to ferment and assimilate both glucose and trehalose.<sup>16</sup> These results suggested that the organism may contain phenotypic variation or had a false negative result in trehalose testing.

The multiprobe real-time PCR assay showed more effectiveness in mixed *Candida* infections presented in two samples, which phenotypic identification failed to identify. These were confirmed by direct subculture from broth samples to retrieve single separate colonies on the Candiselect<sup>®</sup> plate. The first sample had few *C. albicans* mixed with *C. glabrata*. The identifiable phenotype belonged to *C. albicans*, which was more predominant due to its ability to ferment and assimilate more carbon sources. The second sample was mixed infection of few small colonies of *C. glabrata* and *C. tropicalis* and phenotypic assays identified as *C. tropicalis*. These two samples were mixed *Candida* infection with *C. glabrata* which exhibits an innate resistance to most antifungals.<sup>1</sup> *C. glabrata* can survive through most antifungal agents and outgrows other species in mixed infections. Some studies reported that mixed infections with *C. glabrata* and *C. albicans* can cause more severe symptoms and are more difficult to treat.<sup>17-18</sup> Therefore, these results underscored the advantages of real-time PCR for the detection of *C. glabrata* in mixed *Candida* infection.

Five other strains were identified as other *Candida* species by multiprobe real-time PCR, whereas the phenotypic assay identified them as *C. albicans*, *C. glabrata*, *C. parapsilosis* and *Candida* species. The sequence

**TABLE 1.** Summary of 11 samples with discordant results between phenotypic assay, real-time PCR and sequencing.

| Number of isolates | Phenotypic identification | Genotypic identification                  |   |
|--------------------|---------------------------|---|---|
|                    |                           | Multiprobe Real-time PCR                  | Sequencing                                |
| 1                  | <i>C. tropicalis</i>      | <i>C. glabrata</i> & <i>C. tropicalis</i> | <i>C. glabrata</i> & <i>C. tropicalis</i> |
| 1                  | <i>C. albicans</i>        | <i>C. glabrata</i> & <i>C. albicans</i>   | <i>C. glabrata</i> & <i>C. albicans</i>   |
| 2                  | <i>C. zeylanoides</i>     | <i>C. parapsilosis</i>                    | <i>C. parapsilosis</i>                    |
| 2                  | <i>C. krusei</i>          | <i>C. glabrata</i>                        | <i>C. glabrata</i>                        |
| 1                  | <i>C. albicans</i>        | <i>Candida</i> spp.                       | <i>C. dubliniensis</i>                    |
| 1                  | <i>C. glabrata</i>        | <i>Candida</i> spp.                       | <i>C. aquae-textoris</i>                  |
| 1                  | <i>Candida</i> spp.       | <i>Candida</i> spp.                       | <i>C. athensensis</i>                     |
| 1                  | <i>C. parapsilosis</i>    | <i>Candida</i> spp.                       | <i>C. athensensis</i>                     |
| 1                  | <i>Candida</i> spp.       | <i>Candida</i> spp.                       | <i>C. haemulonis</i>                      |

analysis of 18s ribosomal DNA confirmed the PCR identification. The results of sequencing identified as 1 *C. dublinensis*, 1 *C. aquae-textoris*, 1 *C. athensensis*, and 1 *C. haemulonis* respectively. These occurrences revealed that multiprobe real-time PCR has the limitation that it can identify only the four most common species, while the assay showed a high specificity and accuracy (100%) of identification compared with phenotypic identification methods (93%).

From these studies, we conclude that the multiprobe real-time PCR is highly specific and accurate for identification of common *Candida* species. Also, the real-time PCR assay requires a costly real-time PCR instrument and specific reagents which lead to higher cost than conventional methods. The estimated consumable cost for this assay is approximately 500 baht compared with 300 baht for conventional identification methods. However, the assay shows the reproducible results better than phenotypic characterization in which species variations can affect the identification and mixed infection detection. The time of species identification by real time PCR was 3 hours including 1.5 hours for DNA extraction, 30 minutes for PCR preparation and 1 hour for PCR amplification and identification.

Therefore, the multiprobe real-time PCR assay was suitable in routine laboratory testing in terms of precision and cost effectiveness for management of candidemia.

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