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Original Article

# Real-time LightCycler polymerase chain reaction and melting temperature analysis for identification of clinically important *Candida* spp.

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**Background and purpose:** Invasive candidiasis is a major fungal infection occurring in patients who have prolonged hospital admissions. The rapid detection and confirmation of *Candida* spp. in clinical specimens is essential for efficient management and improved prognosis of these patients. The purpose of this study was to develop a real-time LightCycler polymerase chain reaction (PCR) assay for the identification of *Candida* spp. commonly associated with invasive infections.
**Methods:** Using the LightCycler PCR System, the targets of genomic DNA isolated from the reference strains of 6 *Candida* spp. were amplified using genus- and species-specific primers, and detected in real-time employing SYBR Green fluorescent dye. The identity of *Candida* spp. was established by melting curve analysis. A similar analysis was performed with clinical isolates (n = 72) previously identified by conventional methods.
**Results:** The melting curve analysis of amplified DNA from the reference strains could differentiate between *Candida albicans*, *Candida parapsilosis*, *Candida tropicalis*, *Candida glabrata*, *Candida krusei*, and *Candida dubliniensis*. The specificity of the real-time PCR assay was validated by testing 72 clinical isolates of *Candida* spp. with 100% concordance, as compared with conventional identification methods. The notable findings of the study were differentiation of *C. krusei* from all other *Candida* spp. tested and of *C. dubliniensis* from *C. albicans* by melting temperature analysis; the latter 2 species share common phenotypic characteristics of germ-tube formation and chlamydospore production, so are often misidentified.
**Conclusion:** Real-time PCR using LightCycler and melting curve analysis are reliable methods for rapid identification of 6 *Candida* spp. frequently associated with candidemia and invasive candidiasis.

**Key words:** *Candida*; Laboratory techniques and procedures; Polymerase chain reaction; Transition temperature

## Introduction

The rapid detection and identification of *Candida* spp. in clinical laboratories is essential for the efficient management of patients with candidemia and hematogenous candidiasis. Invasive *Candida* infections have emerged as major causes of morbidity and mortality among patients admitted to intensive care units and other high-risk health care facilities [1-4]. In recent years, understanding of the epidemiology of these infections has increased because of more focused studies of specific patient groups [1,2]. While *Candida albicans* remains the predominant species in most countries, causing about 50% of bloodstream infections, non-*albicans Candida* spp. are emerging as significant pathogens posing new diagnostic and therapeutic challenges [2,5,6]. Notable among them are *Candida glabrata* and *Candida krusei,* which exhibit reduced susceptibility to fluconazole [2,5].

Diagnosis of invasive candidiasis remains problematic and challenging [7,8]. Since *Candida* spp. are part of the normal microbiota, their isolation in culture from clinical specimens may not always be indicative of infection, particularly if the specimen has originated from non-sterile sites. Several studies have discussed the usefulness of non-culture–based methods for early and

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specific diagnosis of invasive fungal infections, including candidiasis [8,9]. Recently, several polymerase chain reaction (PCR)–based protocols have been described for the diagnosis of invasive *Candida* infections in experimental and clinical settings, with encouraging results in terms of sensitivity over conventional culture methods [8-12]. Apart from greater sensitivity, PCR-based methods have the potential to simultaneously detect more than one incriminating species, and thus save time required for *Candida* isolation and speciation by conventional methods [11-14]. Additionally, commercially available yeast identification systems based on phenotypic characteristics may not always accurately identify *Candida* spp. and discrepant results are known to occur [15].

As for infectious diseases, the applications of real-time (RT)-PCR technology represent the most recent advances in the diagnosis of fungal infections [16-20]. The RT-PCR technique has the potential to substantially increase the sensitivity of PCR and provide the means to monitor response to therapy by measuring fungal burden at any given time [17,18,21]. RT-PCR assays also decrease the risk of carryover contamination, as the tests are conducted in a closed system, and no laborious post-PCR analyses are required [16-21]. In addition to detection and quantification of *Candida* spp. in clinical specimens, RT-PCR is also essential to accurately identify the etiologic species for initiation of specific antifungal therapy.

In this study, genus- and species-specific primers, previously used in end-point PCRs [11], were used to establish a melting temperature ($T_m$) analysis method using SYBR Green dye for identification of 6 *Candida* spp. using the real-time LightCycler PCR System (Roche Diagnostics Corporation, Indianapolis, IN, USA).

## Methods

### Fungal strains

Thirteen reference strains of 5 *Candida* spp. obtained from American Type Culture Collection (ATCC; Manassas, VA, USA) and Centraalbureau voor Schimmelcultures (CBS; Utrecht, The Netherlands) were used to standardize the PCR assays. The strains included *C. albicans* ATCC 2091, 56881, 90028, and 90029 (n = 4); *Candida parapsilosis* ATCC 10233, 7330, and 90018 (n = 3); *Candida tropicalis* ATCC 750 (n = 1); *C. glabrata* ATCC 15126, 90030, and 15545 (n = 3); *Candida dubliniensis* CD 36, CBS 7987 (n = 2); and *C. krusei* ATCC 6258 (n = 1). In addition, 72 *Candida* isolates cultured from different clinical specimens and identified to species level with the Vitek2 commercial yeast identification system (Bio-Mérieux, Marcy I'Etoile, France) and endpoint semi-nested PCR [11] were included in the study (Table 1).

### DNA isolation

DNA was isolated by using the QIAamp DNA Mini Kit (QIAGEN Sciences, Germantown, MD, USA). One loopful from a 2-day old yeast culture was suspended in 1 mL of phosphate-buffered saline (PBS), vortexed briefly, and centrifuged at 300 × g for 5 min. The pellet was resuspended in PBS to a final volume of 200 μL. After adding 20 μL of QIAGEN Proteinase K, the manufacturer's recommendations for DNA isolation by the blood and body fluid spin protocol were followed. The sensitivity of the assay was determined on the basis of the number of organisms required to isolate DNA for positive amplification by RT-PCR. The tests were positive for all the primers and the *Candida* spp. were tested with DNA isolated from 20 to 200 *Candida* cells.

### Real-time polymerase chain reaction

The LightCycler System was used for RT-PCR amplification. The locations and sequences of the universal and species-specific primers are shown in Fig. 1 and Table 2, and were derived from previous publications [11,22]. For amplicon detection, the LightCycler Fast-Start DNA Master SYBR Green kit was used as advised by the manufacturer. The PCR mixture (20 μL)

**Table 1.** Sources of *Candida* spp. isolates tested by LightCycler polymerase chain reaction.

| Source | *Candida* isolates | | | | | | |
|---|---|---|---|---|---|---|---|
| | *Candida albicans* | *Candida krusei* | *Candida parapsilosis* | *Candida tropicalis* | *Candida glabrata* | *Candida dubliniensis* | Total |
| Blood | 20 | 0 | 10 | 6 | 6 | 0 | 42 |
| Sputum | 0 | 0 | 0 | 2 | 3 | 14 | 19 |
| Endotracheal aspirate | 0 | 0 | 0 | 1 | 1 | 5 | 7 |
| Urine | 0 | 2 | 0 | 1 | 0 | 1 | 4 |
| Total | 20 | 2 | 10 | 10 | 10 | 20 | 72 |

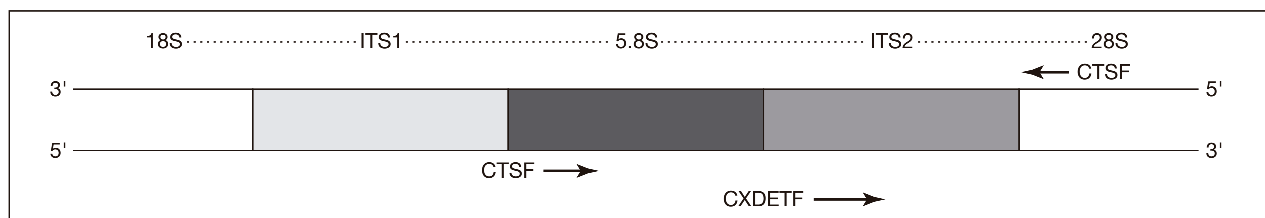


**Fig. 1.** Organization of the rDNA operon in *Candida* spp. and annealing sites for the genus-specific forward and genus-specific reverse primers (CTSF and CTSR, respectively), and species-specific forward primers (CXDETF, CADET, CPDET, CTDET, CGDET, and CDDET) with genus-specific reverse primer (CTSR).
Abbreviations: ITS = internally transcribed spacer; 18S, 5.8S, and 28S indicate 18S rRNA, 5.8S rRNA and 28S rRNA genes, respectively.

**Table 2.** Primers for amplification of *Candida* DNA using real-time LightCycler polymerase chain reaction.

| Primer pair | Nucleotide sequence | Specificity |
|---|---|---|
| CTSF | 5'-TCGCATCGATGAAGAACGCAGC-3' | *Candida* spp. |
| CTSR | 5'-TCTTTTCCTCCGCTTATTGATATGC-3' | |
| CADET | 5'-ATTGCTTGCGGCGGTAACGTCC-3' | *Candida albicans* |
| CTSR | 5'-TCTTTTCCTCCGCTTATTGATATGC-3' | |
| CPDET | 5'-ACAAACTCCAAAACTTCTTCCA-3' | *Candida parapsilosis* |
| CTSR | 5'-TCTTTTCCTCCGCTTATTGATATGC-3' | |
| CTDET | 5'-AACGCTTATTTTGCTAGTGGCC-3' | *Candida tropicalis* |
| CTSR | 5'-TCTTTTCCTCCGCTTATTGATATGC-3' | |
| CGDET | 5'-TAGGTTTTACCAACTCGGTGTT-3' | *Candida glabrata* |
| CTSR | 5'-TCTTTTCCTCCGCTTATTGATATGC-3' | |
| CDDET | 5'-GCTAAGGCGGTCTCTGGCGTCG-3' | *Candida dubliniensis* |
| CTSR | 5'-TCTTTTCCTCCGCTTATTGATATGC-3' | |

contained LightCycler PCR-grade water, magnesium chloride, SYBR Green, 5 pmol each of the respective forward and reverse primers, and 2 μL template DNA. In the seminested format, the PCR mixture was prepared in the same way as mentioned above, except that instead of the genomic DNA, 2 μL of the first PCR product (diluted 1:100 in trisaminomethane-ethylenediaminetetraacetic acid buffer) was added. The program consisted of an initial preincubation step (10 sec at 95°C), followed by 50 PCR cycles (15 sec at 95°C, 10 sec at 70°C, and 15 sec at 72°C) and a melting-curve step (65°C to 95°C) and, afterwards, cooling to 40°C. The PCR process was monitored by fluorescence quantification of the DNA-binding dye SYBR Green for detection of double-stranded amplified DNA in real time and the $T_m$ analysis was performed for identification of the species by a characteristic $T_m$ profile.

## Results

Genomic DNA isolated from 13 reference strains of 6 *Candida* spp. were used to standardize the RT-PCRs for species identification. The $T_m$ analysis showed characteristic melting peaks for each species of the reference *Candida* strains, with genus- as well as species-specific primers (Fig. 2 and Fig. 3).

In addition to the standard strains, 72 *Candida* isolates cultured from clinical specimens (Table 1) were also tested by the LightCycler PCR for species identification. The $T_m$ analysis of *Candida* isolates with genus-specific primers showed a characteristic peak for each species tested with different $T_m$ for *C. tropicalis* (83.03°C), *C. parapsilosis* (84.17°C), *C. glabrata* (84.20°C), *C. dubliniensis* (85.03°C), *C. albicans* (85.70°C), and *C. krusei* (89.59°C) [Table 3]. Since with genus-specific primers, the $T_m$ for *C. krusei* was clearly and distinctly higher than for the other *Candida* spp. (Fig. 2), this species was not included in further experiments with species-specific primers. With the remaining species, the differences in the interspecies $T_m$ with species-specific seminested PCR were as follows: 79.86°C for *C. parapsilosis*, 80.27°C for *C. tropicalis*, 81.62°C for *C. dubliniensis*, 82.24°C for *C. albicans*, and 83.18°C for *C. glabrata* (Table 3). The overall results of $T_m$ analysis showed that the *Candida* isolates representing 6 species were confirmed with 100% concordance, when compared with the Vitek-2 identification system and end-point PCR as shown in Table 1.

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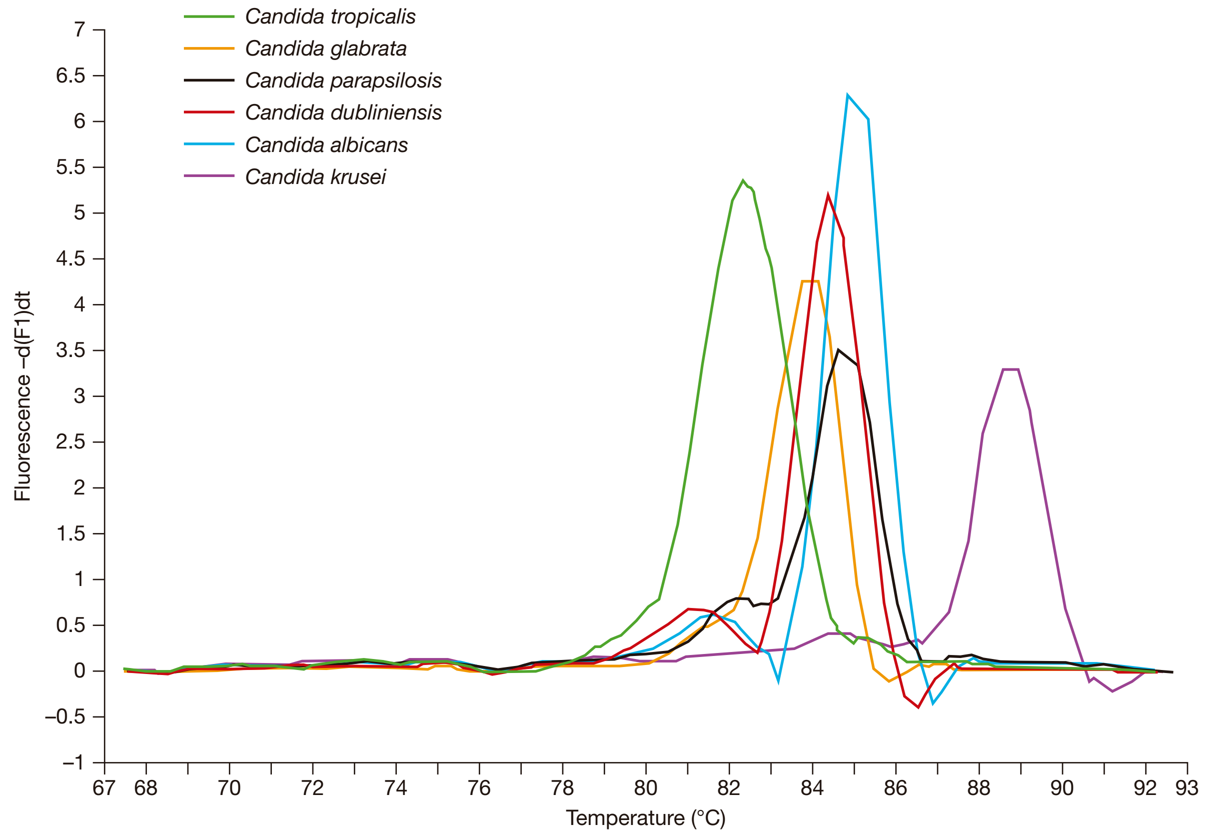


**Fig. 2.** Characteristic melting peaks with genus-specific forward and reverse primers for identification of 6 clinically important *Candida* spp.

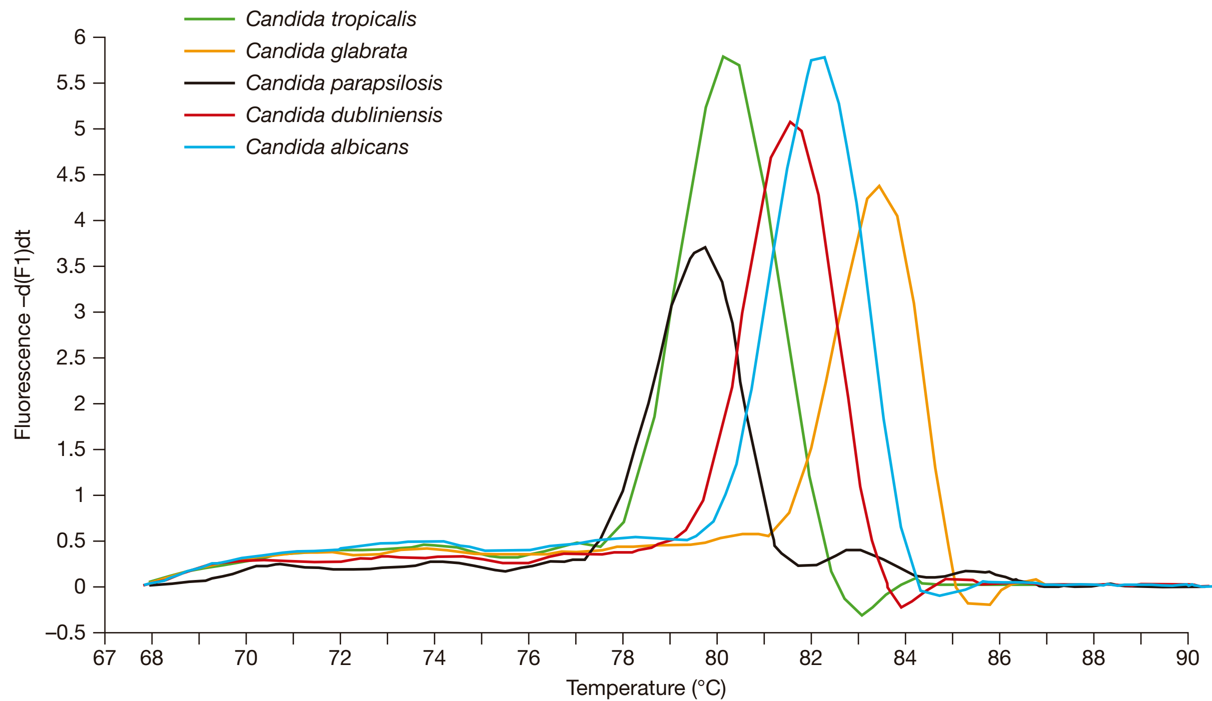


**Fig. 3.** Characteristic melting peaks with species-specific forward primers and the genus-specific reverse primer for 5 clinically important *Candida* spp.

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**Table 3.** Melting temperature values of DNA products amplified with genus- and species-specific primers.

| Species tested | No. of isolates | Genus-specific melting temperature (°C) Mean ± SD | Species-specific melting temperature (°C) Mean ± SD |
|---|---|---|---|
| *Candida albicans* | 20 | 85.70 ± 0.26 | 82.24 ± 0.10 |
| *Candida parapsilosis* | 10 | 84.17 ± 0.25 | 79.86 ± 0.08 |
| *Candida tropicalis* | 10 | 83.03 ± 0.68 | 80.27 ± 0.09 |
| *Candida glabrata* | 10 | 84.20 ± 0.42 | 83.18 ± 0.12 |
| *Candida dubliniensis* | 20 | 85.03 ± 0.41 | 81.62 ± 0.15 |
| *Candida krusei* | 2 | 89.59 ± 0.32 | Not required |

Abbreviation: SD = standard deviation.

## Discussion

The increasing incidence of invasive fungal infections and high mortality rate associated with these infections has underscored the importance of rapid detection of pathogenic fungi in clinical specimens [1]. Prompt detection and accurate speciation of the causative organism help with optimal management and rational use of antifungal agents [23]. With the development of RT-PCR technology for the diagnosis of infectious diseases, progress has also been made in its application to mycology for the detection and quantification of fungal pathogens [16-21]. To expand the application of this technology for species-specific identification, an RT-PCR assay using genus- and species-specific primers and SYBR Green dye in the LightCycler PCR System has been developed. By applying melting curve analysis, the identity of 6 clinically important *Candida* spp., namely *C. albicans*, *C. parapsilosis*, *C. tropicalis*, *C. glabrata*, *C. dubliniensis,* and *C. krusei* could be confirmed.

Isolation and identification of *Candida* spp. by conventional methods is time consuming. Moreover, these methods may misidentify *C. albicans* and *C. dubliniensis* as they share some common phenotypic characteristics [24]. In this study, genus- and species-specific primers were used to identify clinically important *Candida* spp. by $T_m$ analysis of PCR amplified DNA, a system that is similar in principle to that developed earlier by Hsu et al [25]. However, this system was able to differentiate between *C. albicans* and *C. dubliniensis*, which were not included in the RT-PCR developed by Hsu et al [25]. Moreover, the identification results from the RT-PCR method matched completely with the culture identification results for all 72 tested isolates, representing 100% concordance.

The analysis of $T_m$ profile suggests that while genus- and species-specific primers could be used to discriminate between different *Candida* spp., their reliability may be compromised for some *Candida* spp. due to the narrow range of melting temperatures. This was apparent for *C. glabrata* ($T_m$, 84.20°C) and *C. parapsilosis* ($T_m$, 84.17°C) with genus-specific primers, where the difference was not sufficiently discriminatory to distinguish between these 2 species by using genus-specific primers.

In contrast, *C. krusei* ($T_m$, 89.59°C) yielded the highest $T_m$ with genus-specific primer, thus excluding the need to do further experiments with species-specific primers with the DNA of this species. On the other hand, when species-specific primers for *C. glabrata* and *C. parapsilosis* were used, the $T_m$ values (83.18°C and 79.80°C, respectively) were sufficiently distinct to distinguish between these 2 species. Considering the aforementioned limitations, it is apparent that while melting curves can be used to confirm the identity of individual species, their ability to distinguish between some *Candida* spp. may not differ distinctly due to the narrow range of $T_m$ values.

A LightCycler-based RT-PCR assay has been developed for the rapid identification of *C. albicans*, *C. parapsilosis*, *C. tropicalis, C. glabrata*, *C. dubliniensis,* and *C. krusei*. A clear differentiation of *C. krusei* from other *Candida* spp. is therapeutically significant as it requires a different treatment strategy. Furthermore, the differentiation of *C. albicans* from *C. dubliniensis* is also noteworthy, since both of these species have similar phenotypic characteristics and may be misidentified in clinical microbiology laboratories.

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