

Species identification of medically important fungi by use of real-time LightCycler PCR

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Invasive fungal infection has become a major cause of morbidity and mortality in immunocompromised patients. Rapid identification of pathogenic fungi to species level is critical for disease treatment. A real-time LightCycler assay aiming at rapid detection and species identification of pathogenic fungi from clinical isolates was developed. Template DNAs of different species were amplified and detected in real time by employing SYBR Green fluorescent dye. The target sequences for species-level detection were located between the 18S and 28S rDNA. Seven fungal species encountered frequently in the clinical setting, *Candida albicans*, *Candida glabrata*, *Candida krusei*, *Candida parapsilosis*, *Candida tropicalis*, *Candida guilliermondii* and *Cryptococcus neoformans*, could be discriminated by species-specific primers and confirmed by melting-curve analyses. The range of linearity was from 1 ng to 1 pg (μl^{-1} water) and the sensitivity was 1 pg fungal DNA μl^{-1} . Identification by this real-time PCR method matched biochemical identification for all 58 clinical strains. Therefore, the method is simple, rapid and sensitive enough for detection and identification of several fungal species.

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INTRODUCTION

Invasive fungal infections have become major causes of morbidity and mortality among immunocompromised patients (Dasbach *et al.*, 2000; Ellis *et al.*, 2001), such as some neutropenic patients with haematological malignancies and recipients of allogenic bone marrow transplants (Denning, 1998) as well as individuals with AIDS (Mitchell & Perfect, 1995). *Candida* species are now the fourth most frequent cause of nosocomial blood-stream infections in critically ill patients in the United States. At a teaching hospital in Taiwan, yeast infection was identified as the leading cause of nosocomial infection (Chen *et al.*, 1997). The increasing prevalence of yeast infections highlights the need for simple and rapid methods to identify clinically important fungi in a microbiological laboratory.

Each *Candida* species has a different degree of susceptibility to common antifungal agents. For instance, *Candida krusei* is innately resistant and *Candida glabrata*, *Candida guilliermondii* and *Candida dubliniensis* are less susceptible to fluconazole than other *Candida* species (Orozco *et al.*, 1998; Piemonte *et al.*, 1996). Emergence of secondary resistance in *Candida lusitanae* to amphotericin B has also been observed and monitored closely (Pfaller *et al.*, 2003). Current recommendations suggest that invasive fungal in-

fections, such as candidiasis and aspergillosis, should be treated empirically, because the current diagnoses are difficult and time-consuming (Rex *et al.*, 2000; Stevens *et al.*, 2000). However, there is great concern that such practice would result in the emergence of resistant fungal pathogens.

As more and more alternative antifungal agents with various spectra of activities are developed and become available, treatment according to accurate diagnosis has become even more important. Therefore, rapid species identification will be more critical for effective disease therapy and control (Polak, 2003). Conventional diagnostic tests, such as blood culture and biochemical tests, which lack sufficient sensitivity and specificity for early diagnosis of invasive fungal infections, may often require 2 or more days and may be inaccurate (Espinel-Ingroff *et al.*, 1998; Goodwin *et al.*, 1992; Hazen, 1995). Serological tests have certain limitations, e.g. antibody response may be lacking or varied, since the patients most at risk of fungal infections are often immunosuppressed. As for antigen tests, there are no widely accepted *Candida* antigen tests and most cannot differentiate among *Candida* species. Other tests, such as those for cryptococcal, aspergillus and histoplasma antigen, are often hampered by low serum antigen concentrations (Morrison & Lindsley, 2002; Yeo & Wong, 2002).

Therefore, diagnostic assays based on *in vitro* amplification and detection of fungal DNA have been developed, among which PCR methods are particularly promising because of their high specificity and sensitivity. A number of studies have described restriction fragment length polymorphism, PCR amplification and hybridization with species-specific probes, amplicon size differences (Chen *et al.*, 2001; Fujita *et al.*, 2001; Henry *et al.*, 2000) or other methods to identify unique DNA sequences (Hopfer *et al.*, 1993; Kappe *et al.*, 1998; Martin *et al.*, 2000; Turenne *et al.*, 1999). Although these published PCR methods are quite useful for identification of fungal species, they still require a minimum of several hours for DNA amplification and visualization. More recently, real-time PCR techniques have been developed for the detection of fungal pathogens such as *Candida* species, *Cryptococcus neoformans* and *Aspergillus* species. All these assays demonstrate sensitivities better or at least comparable to previously described PCR methods. Real-

time PCR assays dramatically decrease the risk of false-positive results, because the PCR and detection systems are coupled and conducted in a closed system and no laborious post-PCR analyses are required. Various real-time PCR platforms have been developed. The signal to be analysed can be generated by double-stranded DNA-specific dyes, such as SYBR Green, or by sequence-specific fluorescence energy transfer probes. A couple of exonuclease-based Taq-Man PCR assays, capable of rapid identification and speciation of six *Candida* species (Guiver *et al.*, 2001) or *Aspergillus fumigatus* (Costa *et al.*, 2002; Kami *et al.*, 2001), have been described. DNA detection methods for *Candida albicans*, *A. fumigatus* and *Cryptococcus neoformans* using the Light-Cycler have also been reported (Bialek *et al.*, 2002; Loeffler *et al.*, 2000; Spiess *et al.*, 2003). White *et al.* (2003) described real-time and high-sensitivity detection of seven *Candida* species using the LightCycler system; however, they were not able to appreciate them.

Table 1. Clinical and reference strains used in this study

Strain	Source of isolation	Strain	Source of isolation
<i>Candida albicans</i>		<i>Candida guilliermondii</i>	
ATCC 14053	Reference strain	CDC-F276	Nail
CDC-F062	Urine	CDC-F317	Blood
CDC-F063	Blood	<i>Candida krusei</i>	
CDC-F064	Urine	ATCC 6258	Reference strain
CDC-F065	Blood	CDC-F069	Urine
CDC-F066	Blood	CDC-F072	Urine
CDC-F087	Urine	CDC-F191	Blood
CDC-F088	Sputum	CDC-F214	Blood
CDC-F089	Sputum	<i>Cryptococcus neoformans</i>	
CDC-F090	Sputum	Duke215	Reference strain
CDC-F091	Sputum	CDC-F360	Cerebrospinal fluid
CDC-F092	Wound	<i>Candida parapsilosis</i>	
CDC-F094	Sputum	ATCC 20515	Reference strain
CDC-F095	Sputum	ATCC 22019	Reference strain
CDC-F099	Sputum	CDC-F074	Sputum
CDC-F102	Sputum	CDC-F080	Wound
<i>Candida glabrata</i>		CDC-F084	Blood
ATCC 2001	Reference strain	CDC-F107	Blood
CDC-F070	Urine	CDC-F156	Wound
CDC-F073	Urine	CDC-F159	Urine
CDC-F075	Sputum	<i>Candida tropicalis</i>	
CDC-F078	Blood	ATCC 750	Reference strain
CDC-F093	Urine	CDC-F067	Sputum
CDC-F096	Sputum	CDC-F068	Sputum
CDC-F097	Urine	CDC-F076	Sputum
CDC-F098	Urine	CDC-F079	Wound tissue
<i>Candida guilliermondii</i>		CDC-F081	Urine
ATCC 20862	Reference strain	CDC-F082	Urine
CDC-F071	Sputum	CDC-F083	Urine
CDC-F119	Skin	CDC-F085	Sputum
CDC-F128	Sputum	CDC-F086	Urine
CDC-F171	Tinea	CDC-F085	Sputum
CDC-F261	Blood	CDC-F086	Urine

In this paper, we describe a simple real-time PCR assay with the LightCycler system employing species-specific primers and SYBR Green fluorescent dye for detection and species identification of fungal strains. This assay offers the advantage that the conventional PCR can be easily adapted to real-time format without the need for complicated probe design. The assay has been shown to be amply specific and sensitive and to facilitate the detection procedure significantly.

METHODS

Fungal strains. Eight reference strains and 50 clinical strains of *Candida albicans* ($n = 16$), *Candida glabrata* ($n = 9$), *Candida guilliermondii* ($n = 8$), *Candida krusei* ($n = 5$), *Candida parapsilosis* ($n = 8$), *Candida tropicalis* ($n = 10$) and *Cryptococcus neoformans* ($n = 2$) were used in this study (Table 1). Taiwan Surveillance of Antimicrobial Resistance of Yeasts (Lo *et al.*, 2001) collected clinical strains isolated from different body sites and from 22 hospitals located in different geographical locations in Taiwan from 15 April to 15 June 1999. Identification of fungal strains was done by germ-tube assay followed by VITEK yeast biochemical card and API-32C systems. Fungi were cultured on Sabouraud dextrose agar (SDA) plates for 72 h at 37 °C.

DNA extraction. DNA was extracted by using the PUREGENE DNA purification kit (Gentra). Briefly, two loops of fungal biomass from a 2- to 3-day-old culture on SDA were transferred to a 5-ml sterile tube with parafilm seals and re-suspended in 2 ml PBS containing 10–15 μ l lyticase and the mixture was incubated overnight at 37 °C. The samples were centrifuged at 13 000 g for 3 min and the supernatant was removed. Cell lysis solution (2 ml) was added to the cell pellet and gently pipetted up and down to lyse the cells. Next, 1 ml protein precipitation solution was added to the cell lysate and vortexed vigorously at high speed for 20 s and then centrifuged at 13 000 g for 10 min. The supernatant was mixed with 100% isopropanol to precipitate DNA. The DNA was washed with 70% ethanol, air-dried and dissolved in 50 μ l DNA hydration solution. DNA concentrations were measured with a spectrophotometer (A_{260}) and DNA samples were stored at -80 °C until used.

LightCycler-based PCR. The LightCycler PCR and detection system (Roche Diagnostics) was used for amplification and quantification. PCR was performed in glass capillaries and cycling was achieved by alternating heated air and air of ambient temperature, which ensures rapid equilibration between the air and the reaction components due to the high surface-to-volume ratio of the capillaries. The locations and sequences of the species-specific primers (CALB, CGL, CPA, CTR, CGU, CKRU and CN) are shown in Fig. 1 and Table 2 (Lindsley *et al.*,

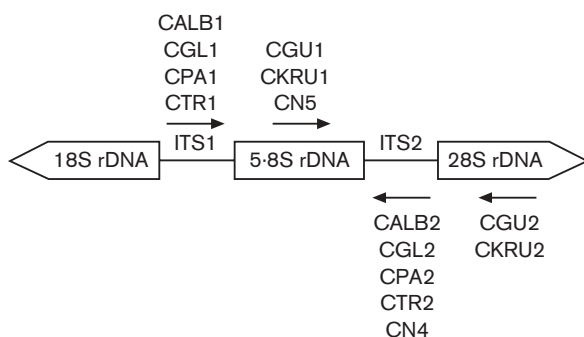


Fig. 1. Specific primer sites for the real-time PCR.

2001; Luo & Mitchell, 2002). For amplicon detection, the LightCycler FastStart DNA Master SYBR Green kit was used as described by the manufacturer. The PCR mixture (20 μ l) contained *Taq* polymerase, 1 \times LightCycler reaction buffer, 3 mM magnesium chloride and 0.5 μ M primers. Template DNA was added at a final concentration of 1 ng per 20 μ l reaction mixture. Samples were run in parallel by performing 35 cycles of repeated denaturation (5 s at 95 °C), annealing (5 s at 58 °C) and chain extension (25 s at 72 °C). This step was followed by a melting-curve analysis from 60 to 95 °C and, afterwards, cooling to 40 °C. The PCR was completed within 45 min. The PCR process was then monitored by fluorescence quantification of the DNA-binding dye SYBR Green 1 dye for the detection of double-stranded DNA.

Agarose gel electrophoresis. Gel electrophoresis was conducted in TBE buffer (0.1 M Tris/HCl, 0.09 M boric acid, 1 mM EDTA, pH 8.4) at 100 V cm^{-1} for 50 min in gels composed of 2.0% (w/v) agarose (BioWhittaker) and stained in 0.5 μ g ethidium bromide ml^{-1} for 15 min followed by washing for 15 min with distilled water.

RESULTS AND DISCUSSION

The increasing frequency of invasive fungal infections and the high mortality associated with disseminated fungal diseases have underscored the importance of rapid detection of pathogenic fungi. Prompt detection and accurate speciation may help to improve fungal disease management as a whole and lead to more rational use of antifungals. Traditional identification methods based on phenotypic features are often time-consuming and depend largely on the skill and experience of the technician. Therefore, we evaluated the feasibility of LightCycler PCR amplification of the rDNA region followed by melting-curve analysis for identification of clinically important yeasts.

The specificity of LightCycler amplification was tested by annealing the *Candida albicans*-specific primers CALB1 and CALB2 with DNA extracted from *Candida albicans* ATCC 14053. The detection limit was about 1 $pg \mu l^{-1}$ (Fig. 2). Similar results were observed with DNA of other species, i.e. the specific primers for each fungal species react only with DNA from the homologous fungal species (data not shown).

The range of linearity was tested by amplification of 10-fold serial dilutions of purified *Candida albicans* ATCC 14053 DNA with fluorescence plotted against number of cycles. Linearity was achieved over 4 logs of input fungal DNA amount, from 1 ng to 1 $pg \mu l^{-1}$ (Fig. 3).

Compared with bacteraemia or viraemia, fungaemia has much lower pathogen load. Therefore, high-sensitivity detection is pivotal for timely management of fungal diseases. The sensitivity of the assays was evaluated by amplifying DNAs of six *Candida* species and *Cryptococcus neoformans* by their respective primers with the LightCycler, and the detection limit of this method was about 1 $pg DNA \mu l^{-1}$. Serially diluted samples of reference strains ranging from 1 ng to 1 $pg DNA \mu l^{-1}$ showed a single band with the specific primers in all cases (Fig. 4).

In order to determine the utility of the LightCycler species-specific amplification method for accurate identification of fungal species, analyses of clinical strains were conducted.

Table 2. Primers for PCR amplification

Odd-numbered primers are forward primers and even-numbered primers are reverse primers.

Primer	Sequence (5'–3')	Amplicon (bp)	Reference/GenBank accession no.
<i>Candida albicans</i>			
CALB1	TTTATCAACTTGTACACCAGA	~273	L47111
CALB2	ATCCCGCCTTACCACTACCG		L28817
<i>Candida glabrata</i>			
CGL1	TTATCACACGACTCGACACT	~423	AB032177
CGL2	CCCACATACTGATATGGCCTACAA		AF167993
<i>Candida guilliermondii</i>			
CGU1	GCATCGATGAAGAACGCAGC	~315	AX592669
CGU2	GTTTGGTTGTTGTAAGGCCGGG		Elie <i>et al.</i> (1998)
<i>Candida krusei</i>			
CKRU1	GCATCGATGAAGAACGCAGC	~258	AX592669
CKRU2	AAAAGTCTAGTTCGCTCGGGCC		Elie <i>et al.</i> (1998)
<i>Candida parapsilosis</i>			
CPA1	GCCAGAGATTAAACTCAACCAA	~300	AF287909
CPA2	CCTATCCATTAGTTTATACTCCGC		L47109
<i>Candida tropicalis</i>			
CTR1	CAATCCTACCGCCAGAGGTTAT	~357	AF287910
CTR2	TGGCCACTAGCAAATAAGCGT		AF268095
<i>Cryptococcus neoformans</i>			
CN5	GAAGGGCATGCCTGTTTGAGAG	~136	M94516
CN4	ATCACCTTCCCACTAACACATT		M94517

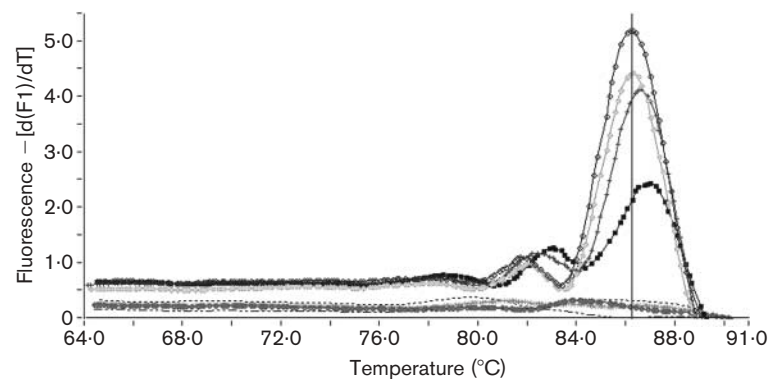


Fig. 2. Sensitivity of the *Candida albicans* ATCC 14053 DNA-specific primers CALB1 and CALB2. Characteristic dose-dependent melting peaks were observed in the presence of DNA from *Candida albicans* ATCC 14053 [1 ng (◇) to 1 pg (■) μl^{-1}].

The identification results from our real-time PCR method matched completely with biochemical identification results for all 58 tested samples. Characteristic peak T_m for species-specific primers with their respective fungal species were obtained by melting-curve analyses (Table 3).

Targets for species-level detection of fungal pathogens include the 18S rDNA, mitochondrial DNA (Yamada *et al.*, 2002), the internal transcribed spacer (ITS) regions and many other genes (Kanbe *et al.*, 2002). rDNA offers distinct advantages over other molecular targets because of greatly increased sensitivity due to the existence of approximately 100 copies per genome. Many *Candida* species can be differentiated by analysis of fragment length variation in

ITS1 (Chen *et al.*, 2001) or ITS2 (De Baere *et al.*, 2002). The LightCycler system offers another advantage of analysis of the melting temperature of amplicons. The melting temperature of the amplicon is dependent on the G+C content, sequence length and compositional variation in the nucleotide bases. Each fungal species has a characteristic T_m , which helps further to confirm its identity.

In conclusion, the real-time LightCycler PCR assay combines rapid amplification of DNA with real-time species determination. The routine block cyler PCR protocol can be easily transferred and adapted to the real-time protocol. This method is simple, rapid and sensitive and can therefore streamline the flow of diagnostic laboratory work.

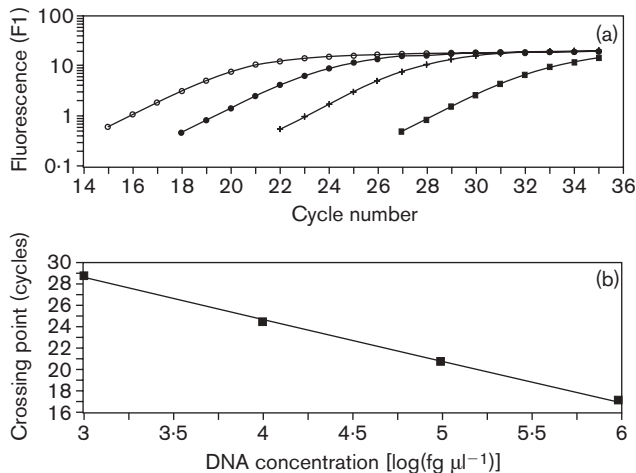


Fig. 3. (a) Quantification of serially diluted *Candida albicans* ATCC 14053 DNA [1 ng to $1 \text{ pg } \mu\text{l}^{-1}$] with CALB1 and CALB2 primers using the LightCycler software. (b) Log DNA concentration ($\text{fg } \mu\text{l}^{-1}$) against fluorescence. Slope (-3.924), y -intercept (40.31), mean squared error (0.100) and regression coefficient (-1.00) of the standard curve were calculated.

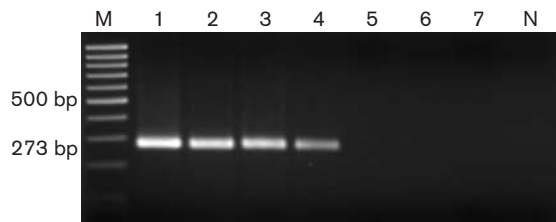


Fig. 4. Agarose electrophoretic gel of LightCycler-amplified DNA from *Candida albicans* ATCC 14053, showing a single, specific band of 275 bp. Lanes: M; marker; 1, $1 \text{ ng } \mu\text{l}^{-1}$; 2, $100 \text{ pg } \mu\text{l}^{-1}$; 3, $10 \text{ pg } \mu\text{l}^{-1}$; 4, $1 \text{ pg } \mu\text{l}^{-1}$; 5, $100 \text{ fg } \mu\text{l}^{-1}$; 6, $10 \text{ fg } \mu\text{l}^{-1}$; 7, $1 \text{ fg } \mu\text{l}^{-1}$; N, negative control (distilled water).

Table 3. T_m values of DNA products amplified with species-specific primers

Species tested	n	T_m (mean \pm SD) ($^{\circ}\text{C}$)
<i>Candida albicans</i>	16	85.71 ± 0.13
<i>Candida glabrata</i>	9	84.02 ± 0.14
<i>Candida guilliermondii</i>	8	84.01 ± 0.28
<i>Candida krusei</i>	5	90.32 ± 0.09
<i>Candida parapsilosis</i>	8	84.43 ± 0.16
<i>Candida tropicalis</i>	10	84.03 ± 0.13
<i>Cryptococcus neoformans</i>	2	84.47 ± 0.29

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