Rapid Identification and Differentiation of *Candida albicans* and *Candida dubliniensis* by Capillary-Based Amplification and Fluorescent Probe Hybridization

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We developed a rapid genotypic assay to differentiate the germ tube-positive yeasts *Candida albicans* and *Candida dubliniensis*. Fluorescently labeled nucleic acid probe binding and subsequent denaturation from the target site in the PCR amplicons produced characteristic peak melting temperatures (T_m) that identified each species. Peak T_m s of *C. albicans* ($n = 69$) and *C. dubliniensis* ($n = 28$) isolates produced in the presence of their respective probes were $61.04 \pm 0.64^\circ\text{C}$ and $60.52 \pm 1.01^\circ\text{C}$ (averages $\pm$ standard deviations). No signal was generated when the *C. albicans* or *C. dubliniensis* probes were tested against DNA from their counterparts. Both probes reacted with *Candida tropicalis* DNA, but the T_m was $51.85 \pm 0.05^\circ\text{C}$ with the *C. albicans* probe and $51.92 \pm 0.10^\circ\text{C}$ with the *C. dubliniensis* probe, differentiating *C. tropicalis* DNA from *C. albicans* and *C. dubliniensis*. A novel hybrid probe was designed to identify both species in a single reaction based on a 4°C difference in peak T_m s. Our assay is rapid (≤ 2 h) and allows reliable detection and differentiation of the two germ tube-positive *Candida* spp.

Candida albicans and *Candida dubliniensis* are two closely related, germ tube- and chlamydospore-positive *Candida* species (14, 30). A careful analysis of the germ tube-positive *C. albicans* from AIDS patients in Dublin, Ireland, revealed that a subset of them formed a homogenous cluster distinct from *C. albicans* by DNA fingerprinting analysis (30). This distinct subset of *C. albicans* was grouped into a new species and named *C. dubliniensis* sp. nov. This report was followed by numerous others from the United States and other parts of the world identifying *C. dubliniensis* as a clinically important species in human immunodeficiency virus (HIV)-seropositive patients (2, 5, 6, 8, 16, 26, 29; D. A. Stevens, Letter, J. Clin. Microbiol. **39**:416, 2001) and immunosuppressed patients (1, 9–12, 17, 24; D. Marriott, M. Laxton, and J. Harkness, Letter, Emerg. Infect. Dis. **7**:479, 2001). *C. dubliniensis* has been isolated from up to 30% of HIV-infected patients with oral candidiasis (28). Invasive infections have also been reported from AIDS and cancer patients (1, 27). It is important to understand the epidemiology of *C. dubliniensis*, as it is an emerging opportunistic pathogen capable of rapidly expressing stable fluconazole resistance in vitro and, in vivo, after prolonged therapy for oropharyngeal candidiasis in HIV-infected patients (19, 20, 25). To date, invasive *C. dubliniensis* isolates reported from the AIDS and cancer patients have not demonstrated any fluconazole resistance. *C. dubliniensis* is also found mixed with *C. albicans* in oropharyngeal samples from HIV-infected patients, leading to difficulty in isolating *C. dubliniensis* and understanding its contribution to clinical disease.

Information on the prevalence of the *C. dubliniensis* infec-

tions is accumulating due to the interest in screening immunocompromised patients for this pathogen and differentiating it from the other germ tube-positive yeast, *C. albicans* (10, 27). Several phenotypic assays may help to differentiate *C. dubliniensis* from *C. albicans*. The differentiating phenotypes include the capacity to grow at 45°C , the capacity to assimilate xylose or α -methyl-D-glucoside, and the type of colony color produced on CHROMagar *Candida* medium (7, 13, 21; I. F. Salkin, W. R. Pruitt, A. A. Padhye, D. Sullivan, D. Coleman, and D. H. Pincus, Letter, J. Clin. Microbiol. **36**:1467, 1998). These assays are time consuming and/or subjective in interpretation and therefore do not perform as optimal screening assays for the potentially fluconazole-resistant *C. dubliniensis*. Hence, several investigators have used genotypic rather than phenotypic assays to identify and differentiate these two closely related species. The genotypic assays have included ribosomal DNA analysis and PCR fingerprinting and molecular beacon assays (18, 22, 31), which produced reliable differentiation yet are labor intensive or time consuming.

Here we report a simple, rapid, and sensitive genotypic method to differentiate *C. albicans* from *C. dubliniensis*. We amplified the internal transcribed spacer region 2 (ITS2) DNA and identified *Candida* species by sequence-specific hybridization. The ITS2 is a spacer region flanked by the 5.8S and 28S rRNA gene. It was previously demonstrated that the sequence polymorphisms in the ITS regions 1 and 2 can be used to reliably identify medically important yeasts (3, 4). Our present methodology takes advantage of nucleotide differences between *C. albicans* and *C. dubliniensis* found in the ITS2 region. The assay involves rapid PCR thermal cycling using glass capillaries and real-time fluorimetric detection of the PCR products. DNA amplification was achieved by 45 thermal cycles in 45 min. The amplified products were identified by melting curve analysis (MCA) using various hybridization probes

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FIG. 1. Use of fluorescence resonance energy transfer to identify species-specific sequences in the ITS2 region DNA. Following amplification of the ITS2 region, the donor probe (CALB, CDUB, or CFLUC) and the acceptor probe (CANC) hybridize to target sequences in close proximity in a head-to-tail arrangement. Excitation of the donor fluorophore (fluorescein) on the 3' end of the donor probe emits light that is absorbed by the acceptor fluorophore (LC-Red 640) on the 5' end of the acceptor probe, which in turn emits light of a different wavelength that is filtered and measured.

bound to target sequences in the ITS region. The 3' end of the donor probe is coupled to the donor fluorophore (fluorescein) dye, and the 5' end of the acceptor probe is coupled with acceptor fluorophore (LC-Red 640) dye. Fluorescence resonance energy transfer occurs when the two probes hybridize to the amplicon in close proximity, allowing the fluorescein to emit light that is absorbed by the LC-Red 640 dye (Fig. 1).

TABLE 1. Average peak T_m s with corresponding donor probes

<i>Candida</i> sp. tested (no. of isolates)	Avg peak $T_m \pm$ SD with indicated probe ^a		
	<i>C. albicans</i>	<i>C. dubliniensis</i>	Hybrid
<i>C. albicans</i> (n = 69)	61.04 $\pm$ 0.64	NS	49.94 $\pm$ 0.54
<i>C. dubliniensis</i> (n = 28)	NS	60.52 $\pm$ 1.01	54.03 $\pm$ 1.01
<i>C. tropicalis</i> (n = 3)	51.85 $\pm$ 0.05	51.92 $\pm$ 0.10	53.46 $\pm$ 0.09
<i>C. parapsilosis</i> (n = 6)	NS	NS	47.81 $\pm$ 0.24
Other genera or species ^b	NS	NS	NS

^a Peak T_m values were statistically significant by Student's t test ($P \leq 0.005$) except between peak T_m s of *C. tropicalis* and *C. dubliniensis* when tested with the hybrid probe. NS, no signal.

^b Other species tested include *C. glabrata*, *C. guilliermondii*, *C. kefyr*, *C. krusei*, *C. lambica*, *C. lusitanae*, *Cryptococcus albidus*, and *Cryptococcus neoformans*.

MCA is performed after the completion of the amplification cycles to identify specific amplicons. The probe coupled with the LC-Red 640 hybridizes to conserved target sequence present in both *Candida* spp. and serves as the acceptor probe. The donor probes (*C. albicans*, *C. dubliniensis*, and Hybrid) coupled with the fluorescein dye hybridize to adjacent DNA containing species-identifying nucleotide polymorphisms. When the temperature is slowly increased, the donor probe melts off from the target sequence producing the characteristic melting peaks (T_m). The T_m is dependent on the length, G+C content, and degree of homology between the donor probe and the template. For our assay we designed one acceptor probe (CANC) and three donor probes: two probes were specific for either *C. albicans* (CALB) or *C. dubliniensis* (CDUB), and a

FIG. 2. Sensitivity and specificity of the *C. albicans* species-specific probe. Characteristic dose-dependent melting peaks were observed only in the presence of DNA from *C. albicans* (1 ng to 1 fg of DNA) and not in the presence of DNA from *C. dubliniensis* (1 ng of DNA) or in the negative control (water).

FIG. 3. Differentiation of germ tube-positive *C. albicans* and *C. dubliniensis* with a single hybrid probe (CFLUC). An $\approx 4^\circ\text{C}$ difference between the peak T_m produced in the presence of DNA from *C. albicans* and that produced in the presence of *C. dubliniensis* DNA differentiates these two germ tube-positive *Candida* spp. Water was used as a negative control.

third probe (CFLUC) included a single mismatch for each of the two species, allowing it to hybridize and produce characteristic T_m s with each target in a single reaction.

We validated our assay by using 69 *C. albicans* and 28 *C. dubliniensis* isolates characterized by ITS2 sequence polymorphisms (4). Eleven *C. dubliniensis* isolates were prospectively collected from our laboratory by examining the xylose assimilation-negative, germ tube-positive *Candida* species in the VITEK YBC system as suggested by Pincus et al. (23). These isolates were obtained from several specimens including mouth washes of stem cell transplant patients, vaginal culture samples, stool samples, and bloodstream infection. DNA was extracted from yeast colonies grown after 48 h on Sabouraud's agar by using the QIAspin Miniprep kit (Qiagen) following the manufacturer's protocol. Amplification was performed in 7 μl of total reaction volume consisting of 1X LightCycler master hybridization mix (Roche Diagnostics), plus an additional 2.0 mM of MgCl_2 and a 1 μM concentration of the ITS3-1 forward primer (5'-CATCGATGAAGAACGCAGC-3') and the ITS4 reverse primer (5'-TCCTCCGCTTATTGATATGC-3'). Product identification was achieved by adding a 0.1 μM concentration of one of the three donor probes, CALB (5'-TCCCTCA AACCCTGGGTTT-3'-FLU), CDUB (5'-TCCCTCAAACC CCTAGGGTTT-3'-FLU), or CFLUC (5'-TCCCTCAAACCC CTGGGTTT-3'-FLU), along with a 0.2 μM concentration of the acceptor probe, CANC (5'-LC-Red640-TGTTGAGCAA TACGACTTGGGTTTGP-3') to the reaction mixture. Thermal cycling conditions were as follows: 95°C initial denatur-

ation for 30 s was followed by 45 cycles of 95°C for 1 s, 55°C for 10 s, and 72°C for 25 s. MCA with species probes was performed by using the following thermal cycling profile: 95°C for 0 s, 45°C for 30 s, and ramping to 85°C. The MCA profile for the hybrid probe was 95°C for 10 s, 40°C for 30 s, and ramping to 70°C.

Specificity of the probes was verified by testing other yeast species previously characterized by ITS length and/or sequence polymorphism (4). These included *Candida tropicalis* (ATCC750), *Candida glabrata* (ATCC2001), *Candida krusei* (ATCC6258), *Candida lusitanae* (ATCC34449), *Candida guilliermondii* (ATCC6260), *Candida parapsilosis* (UWFP251), *Cryptococcus neoformans* (UWFP360), *C. lambica* (UWFP346), *Cryptococcus albidus* (ATCC10666), and *Candida kefyr* (UWFP208) (Table 1). Characteristic peak T_m s for the species probes with their cognate candidal species were obtained by MCA. The *C. albicans* probe gave a peak melting temperature of $61.04 \pm 0.64^\circ\text{C}$ (average $\pm$ standard deviation) only in the presence of *C. albicans* DNA (Fig. 2). Likewise, the *C. dubliniensis* probe gave a peak T_m of $60.52 \pm 1.01^\circ\text{C}$ for all the *C. dubliniensis* isolates tested (data not shown). Both the probes identified DNA from the respective species with an analytical sensitivity of ≤ 1 fg of DNA. These species probes did not produce any peak T_m s by MCA with any other species tested except for *C. tropicalis*. However, the T_m obtained with *C. tropicalis* DNA was approximately 52°C with either the *C. albicans* probe or the *C. dubliniensis* probe, and these values were readily distinguishable.

The hybrid probe, CFLUC, produced a peak T_m of $49.94 \pm 0.54^\circ\text{C}$ for *C. albicans* and $54.03 \pm 1.01^\circ\text{C}$ for *C. dubliniensis* (Fig. 3). Interestingly, the hybrid probe also produced a peak T_m of $53.46 \pm 0.09^\circ\text{C}$ with *C. tropicalis* and $47.81 \pm 0.24^\circ\text{C}$ for *C. parapsilosis*. Notably, the hybrid probe produces T_m s which may potentially overlap between *C. dubliniensis* and *C. tropicalis*. Hence, the use of the hybrid probe to differentiate between *C. albicans* and *C. dubliniensis* warrants pretesting and selection of germ tube-positive species only, thereby eliminating any *C. tropicalis* isolates. Alternatively, *C. dubliniensis* colonies can be preselected by their dark green color when CHROMagar medium is used for primary isolation. To identify the small proportion (up to 5%) of germ tube-negative *C. albicans* (15) and/or avoid germ tube testing, the *C. albicans* probe should be used.

We report the use of a rapid screening assay for *C. dubliniensis*. By targeting a specific sequence in the ITS2 region of both *C. albicans* and *C. dubliniensis*, we can specifically identify these two closely related species based on the characteristic T_m produced with specific donor probes. Thermal cycling in glass capillaries provides a rapid assay with real-time detection using hybridization of probes. The MCA is a particularly useful feature of the LightCycler system and adds specificity to identification of PCR amplicons. Additionally, the MCA is capable of identifying mutations by showing a shift in the peak T_m . The uniformity of the T_m s for 69 *C. albicans* and 28 *C. dubliniensis* isolates indicates that the target site is well conserved among isolates from the same species. Following DNA extraction, the amplification and detection can be completed in less than 1 h, making it the most rapid assay reported to date for reliably identifying these species. The above-mentioned characteristics, i.e., rapidity, specificity, reliability, and user friendliness, make this an adaptable assay in a laboratory setting. Unlike the rRNA genes, which evolve slowly and are relatively conserved, ITS region DNA contains sufficient sequence variation to make it a suitable molecular target for identification of fungi to the species level (3, 4). The availability of the rRNA genes in high copy numbers provides an added advantage, because it theoretically increases the sensitivity of the assay. Additionally, amplification and subsequent detection by probe hybridization in a closed system reduce the possibility of cross-contamination. Currently we are using this technology to identify the commonly encountered invasive *Candida* species from blood culture systems in our clinical laboratory. Doing so permits identification of the common *Candida* species on the same day that blood cultures become positive and eliminates the 2- to 3-day period for confirmatory identification by traditional methods. Such rapid reporting can have an impact on clinical care by assisting the clinician in making appropriate drug choices and patient management decisions.

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