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Multiplex Polymerase Chain Reaction Assay for Simultaneous Detection of *Candida albicans* and *Candida dubliniensis*

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A multiplex polymerase chain reaction (PCR) assay was developed for the identification of two *Candida* species-*albicans* and *dubliniensis*. Three sets of primers were selected from different genomic sequences to specifically amplify a 516 bp fragment within the *top2* gene, specific for several species of the genus *Candida* (CCL primers); a 239 bp fragment within the *cdNT1* gene, specific for *Candida albicans* (CAL primers); and a 175 bp fragment within the *ALSD1* gene, specific for *Candida dubliniensis* (CDL primers). Using the primers in conjunction (multiplex PCR), we were able to detect both *C. albicans* and *C. dubliniensis* and to differentiate between them. The detection limit of the PCR assay was approximately 10 cells per milliliter of saline. Thus, this multiplex PCR assay can be applied for differentiation of *C. albicans* and *C. dubliniensis* from clinical specimens.

Key words: *Candida albicans, Candida dubliniensis*, multiplex PCR

The opportunistic pathogen *Candida albicans* is the most common cause of candidiasis in neonates, diabetics, and immunosuppressed individuals (Fox, 1993) and the leading cause of infectious esophagitis in AIDS patients (Jones *et al.*, 1999). Candidiasis in persons with AIDS is not cured easily and the mortality rates are very high. Thus, defining the most effective diagnostic and therapeutic approach to curing candidiasis in such patients is especially important. Observation of chlamydospore and germ tube production has been used for the clinical diagnosis of *C. albicans* infections. Recently, commercial carbohydrate assimilation systems and chromogen agar for screening *C. albicans* have been developed and used as routine procedures for *Candida* species identification (Gales *et al.*, 1999; Kirkpatrick *et al.*, 1998). Although these methods represent progress in the detection of *C. albicans*, several days are still required to confirm *Candida* species.

C. dubliniensis is a recently recognized species, and the characteristics and identification of the species have been reviewed (Sullivan and Coleman, 1998). *C. dubliniensis* has increasingly been found in clinical specimens, especially in immunocompromised patients (Sullivan *et al.*, 1997), and strains resistant to the commonly used antifungal drug fluconazole have developed rapidly (Moran *et al.*, 1997). It has been reported that *C. dubliniensis* exhibits phenotypic characteristics similar to those of *C. albicans*, such as chlamydospore, germ tube production, and the appearance of colonies on Sabouraud dextrose agar or potato dextrose agar (Sullivan and Coleman, 1998). Thus, *C. dubliniensis* has often been misidentified as *C. albicans*. Although the phenotypic characteristics of the two strains are very close, molecular evidence shows that they are distinct species (Sullivan *et al.*, 1995). Since classical methods of clinical identification that rely on cultivation of presumptive pathogens are time-consuming and are often labor-intensive, a more rapid, sensitive, and specific method is needed to diagnose yeast infections. Accurate and rapid discrimination of *C. albicans* and *C. dubliniensis* would provide timely information for patient management, aid in defining the most effective diagnostic and therapeutic approach, allow effective and early antifungal therapy, and permit the measurement of resistance to the antifungal agents. Procedures suitable for identifying *Candida* directly from clinical specimens are now emerging. They are based on genomic amplification methods and seem to detect and identify a large number of related species of pathogenic yeast using distinct target DNA sequences (Kurzai *et al.*, 1999; Mannarelli and Kurtzman, 1998). Immunological methods have been reported as well (Marcilla *et al.*, 1999).

We believe that by using molecules that differ between *C. albicans* and *C. dubliniensis,* we should be able to distinguish the two species in a clinical assay. The existence of an integrin-like protein unique to *Candida albicans* has

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Table 1. Primers used in this study for *Candida* detection by the multiplex PCR assay

Primer	Target Gene	Length	Primer sequence (5'→3')	Size (bp)
CCL	*top2*	24	F-TACTGGTGGTAGAAATGGGTTTGG	516
		24	R-GTGGCAATGGAGTTGACAAAACTG	
CAL	*αINT1*	24	F-AAGCTCTGATACCTACACTAGCGA	239
		24	R-GTTAGGTCTAAAGTCGAAGTCATC	
CDL	*ALSD1*	24	F-GCATTTGGTACCGTAAGGATACCA	175
		24	R-CACTAGATGATTCCGGTGTTTTGG	

been reported recently and the gene *αINT1* was cloned and sequenced (Gale *et al.*, 1998; Gale *et al.*, 1996). Primers complementary to the species-specific DNA sequences within *αINT1* would be suitable for the detection of *C. albicans*. The *ALS* (agglutinin-like sequence) gene family of *Candida* species encodes cell-surface glycoproteins that function in adhesion to host surfaces (Fu *et al.*, 1998). *C. dubliniensis* has a unique sequence in the *ALS* gene (Hoyer *et al.*, 2001). The unique region of the *C. dubliniensis* agglutinin-like protein ALSD1P (*ALSD1*) gene could be used as a target sequence to detect the species. These species-specific sequences could potentially be excellent markers for simple identification assays. In this study, a multiplex PCR-based assay with the species-specific oligonucleotide is shown to be useful for the simultaneous identification and sensitive detection of the two major *Candida* species that cause candidiasis, *C. albicans* and *C. dubliniensis*, without DNA purification.

Materials and Methods

Yeast strains and culture medium

The strains of *C. albicans*, *C. dubliniensis*, and other yeast strains used are summarized in Table 2. Yeast strains were cultured for 2 days at 30°C on Sabouraud dextrose agar (SDA) from Difco (Detroit, MI. USA).

Preparation of genomic DNA

The DNA from the test strains was prepared as described by Holm *et al.* (1986). Several colonies from an SDA plate were added to 1 ml of sterilized distilled water and centrifuged at 10,000 × g for 10 min in an Eppendorf microcentrifuge, and the pellet was washed twice with sterilized distilled water. The pellets were resuspended in 100 μl of 1 M sorbitol-0.1 M EDTA (pH 8.0) containing 25 μg of Lyticase (Sigma, St. Louis, MO. USA) and 2% 2-mercaptoethanol, and the mixture was incubated at 37°C for 15 min. After centrifugation at 10,000 × g for 2 min, the spheroplasts were washed twice with 1 M sorbitol-0.1 M EDTA (pH 8.0). The spheroplasts were suspended in PCR buffer containing 0.1% Nonidet P-40 and 0.1 mg/ml of proteinase K (Sigma, St. Louis, MO. USA), and incubated at 55°C for 30 min. The samples were heated at 95°C for 10 min to remove enzyme activity and then centrifuged at 10,000 × g for 5 min. The crude DNA was used as a template for the PCR assay. Human DNA was purified from human brain neuroblastoma cell line (MC-IXC) (Herrmann and Frischauf, 1987).

PCR

For multiplex PCR, three primer pairs were used. All oligonucleotide primers were designed from published sequences. The CCL primer was selected from the type II DNA topoisomerase (*top2*) gene (GenBank Accession number AB049142) and was shown to be specific for several *Candida* species. The CAL primer was chosen from the integrin-like protein alpha INT 1p (*αINT1*) gene of *C. albicans* (GenBank Accession number U35070). The CDL primer was derived from the agglutinin-like protein AlsD1p (*ALSD1*) gene of *C. dubliniensis* (GenBank Accession number AF201685). All primer sequences used in this study are given in Table 1.

PCR was performed as follows: the reaction solution consisted of PCR reaction buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl [pH 8.3]), 1 μl of DNA (100 ng/μl), 200 μM dNTP, 1 μM of each primer, 0.5 U Taq DNA polymerase, and sterilized distilled water to a final volume to 25 μl. The PCR was performed in a Peltier Thermal Cycler (PTC-200, MJ Research Inc., Watertown, MA, USA). The first cycle was 5 min of denaturation at 92°C. This was followed by 30 cycles, each consisting of 1 min of denaturation at 92°C, 1 min of annealing at 65°C and 1 min of extension at 72°C. In the final cycle, an additional 5 min of incubation at 72°C was performed for complete extension. For each PCR run, a negative control was also included. The PCR product was electrophoresed on a 2% agarose gel using TAE buffer (40 mM Tris acetate, 1 mM EDTA [pH 8.4]), after which the gel was stained with ethidium bromide and photographed under UV light.

Results and Discussion

Specificity of the PCR for detecting C. albicans and C. dubliniensis

Three primer sets were used to detect the presence of *Candida* strains. The CAL PCR primer set was based on

Table 2. Yeast strains and human cell line used in this study and evaluation of the specificity of multiplex PCR

Strains	Strain no.	PCR results		
		CAL	CDL	CCL
Candida albicans	ATCC 10231[a]	+	-	+
Candida albicans	Clinical isolate[b]	+	-	+
Candida krusei	ATCC 32196	-	-	+
Candida famata	ATCC 12790	-	-	+
Candida kefyr	ATCC 9767	-	-	+
Candida dubliniensis	NRRL Y-17841[c]	-	+	+
Candida dubliniensis	CBS 7988[d]	-	+	+
Candida dubliniensis	CBS 7987	-	+	+
Candida glabrata	Clinical isolate	-	-	+
Candida parapsilosis	Clinical isolate	-	-	+
Candida intermedia	Clinical isolate	-	-	+
Candida tropicalis	Clinical isolate	-	-	+
Candida guilliermondii	Clinical isolate	-	-	-
Cryptococcus neoformans	ATCC 2344	-	-	-
Saccharomyces cerevisiae	Clinical isolate	-	-	-
Malassezia pachydermatis	Clinical isolate	-	-	-
Rhodotorula rubra	Clinical isolate	-	-	-
Hansenula anomala	Clinical isolate	-	-	-
Trichosporon cutaneum	Clinical isolate	-	-	-
Geotrichum candidum	Clinical isolate	-	-	-
Human cell, MX-IXC	ATCC CRL-2270	-	-	-

[a]American Type Culture Collection, Rockville, MD, USA
[b]Clinically isolated strains from Korea University Kuro Hospital
[c]Agricultural Research Service Cultural Collection, Peoria, IL, USA
[d]Centraal bureau voor Schimmelcultures, Baarn, The Netherlands

the sequence of the gene encoding the integrin-like protein α INT1p from *C. albicans*. We evaluated this primer set for specificity in detecting *C. albicans*. The primer amplified a 239 bp fragment from the *C. albicans* strain, whereas the primer did not amplify any product from the other *Candida* species nor from several other yeast strains (Table 2). However, in spite of its similarity to vertebrate leukocyte integrin genes (Gale *et al.*, 1996), no product was amplified from the human neuroblastoma MC-IXC cell line.

The CDL primer set was chosen from the *ALSD1* gene and was used to specifically detect *C. dubliniensis*. *C. dubliniensis* gave a 175 bp amplified fragment with the CDL primer sets (Table 2). No amplified product was found for the other *Candida* species, yeast strains, or human cells. These results suggest that *αINT1* and *ALSD1* are good candidates for the specific detection of *C. albicans* and *C. dubliniensis*, respectively.

Specificity of the multiplex PCR

To assess the specificity of the multiplex PCR, the primer sets CAL and CDL were used in the same reaction mixture, together with CCL primers specific for several *Candida* species including *C. albicans* and *C. dubliniensis*. All of the primers were the same length (24 bases) and

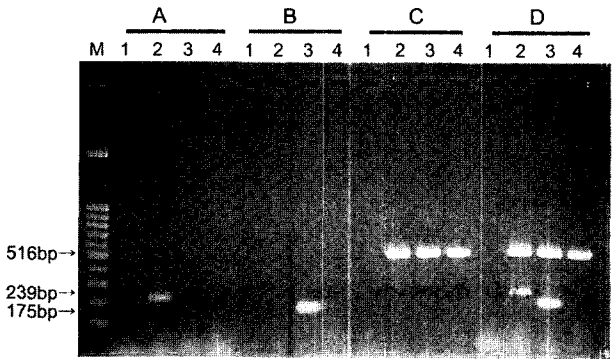

Fig. 1. Multiplex PCR assay with the three primer sets CAL (A), CDL (B), CCL (C), and all primer sets (D). Lane 1, sterile water used as a negative control; lane 2, *C. albicans*; lane 3, *C. dubliniensis*; lane 4, *C. intermedia*; M, DNA size standard (100-bp ladder).

GC content, and the melting temperatures (T_m) were similar for all primers used. This matching of GC content and T_m within the primer sets means that they all have similar physical properties. Thus, these primers are adapted for multiplex PCR. Four distinct results were obtained from the multiplex PCR: two amplified products of 239 bp and 516 bp for *C. albicans*; two products of 175 bp and 516 bp for *C. dubliniensis*; one amplified product of 516 bp for the other *Candida* species; and no amplified product for the negative control (Fig. 1). Thus, multiplex PCR yielded an amplified product for *C. albicans*, *C. dubliniensis*, and *C. intermedia* of 516 bp plus an additional distinct amplified product which allows the differential identification of *C. albicans* and *C. dubliniensis*.

Sensitivity of the PCR assay for detection of C. albicans DNA and C. dubliniensis DNA

To examine the detection sensitivity, serial 10-fold dilu-

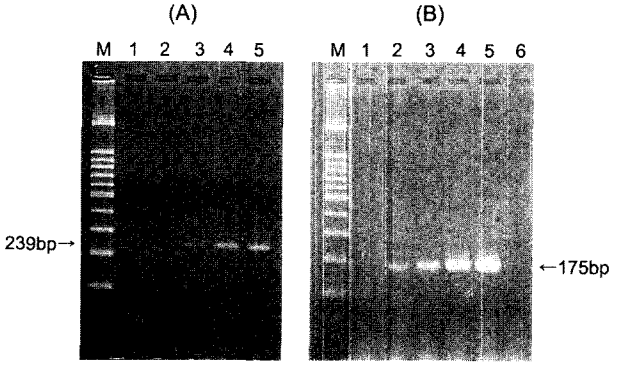

Fig. 2. Sensitivity of DNA detection by PCR of *C. albicans* (A) and *C. dubliniensis* (B) cells in saline, determined by ethidium bromide staining. Template DNAs were prepared from 1 ml of saline containing cells as follows: M, DNA size standard (100-bp ladder); lane 1, sterile water used as a negative control; (A) lane 2, no cells; lane 3, 10 cells/ml; lane 4, 10^2 cells/ml; lane 5, 10^3 cells/ml; (B) lane 2, 10 cells/ml; lane 3, 10^2 cells/ml; lane 4, 10^3 cells/ml; lane 5, 10^4 cells/ml; lane 6, no cells.

tions in saline were made of *C. albicans* and *C. dubliniensis* cells. PCR samples were prepared from each of the diluted samples as described in Materials and Methods. The PCR assay described here demonstrated a detection limit of approximately 10 cells/ml in saline for both strains (Fig. 2).

Candidiasis is an infection caused by one of many species of the genus *Candida,* the most common pathogenic species of which is *C. albicans*. However, the number of infections by the recently identified *C. dubliniensis* has increased, particularly in HIV-infected individuals and AIDS patients. It is therefore necessary to discriminate *C. dubliniensis* from other species in order to understand its clinical significance and epidemiology. When *C. dubliniensis* was examined for the utilization of xylose (XYL) and α-methyl-D-glucoside (MDG), it was found to lack the ability to use either, whereas *C. albicans* assimilates both XYL and MDG (Gales *et al.*, 1999). *C. dubliniensis* is also significantly more susceptible than *C. albicans* to fluconazole, an antifungal agent (McCullough *et al.*, 1999). Although *C. dubliniensis* exhibits similar phenotypic characteristics to *C. albicans*, such as the chlamydospore and germ tube production that is used mainly to identify *C. albicans*, these results suggest that carbohydrate assimilation and antifungal drug metabolism are different between the two species. The two species also show genetic differences, as shown by DNA fingerprinting and DNA sequence analysis of rRNA genes (McCullough *et al.*, 1999; Meyer *et al.*, 2001). An investigation of the molecular and phenotypic characterization of genotypic *C. albicans* subgroups indicated that those strains previously designated *C. albicans* genotype D should be assigned to the species *C. dubliniensis* (McCullough *et al.*, 1999). The discrimination of *C. dubliniensis* from *C. albicans* has been performed by incubation of the two species on Emmons' modified Sabouraud glucose agar for 24 h at 45°C (Pinjon *et al.*, 1998) and on chromogen agar for 48 h at 30°C (Schoofs *et al.*, 1997). These methods take time to culture and the strains can lose the ability to yield the colored colonies on the medium following subculture or storage. Detection of *C. albicans* by PCR has been shown to be a specific and sensitive diagnostic method (Miyakawa *et al.*, 1992; Wahyuningsih *et al.*, 2000). A PCR-based identification system has been developed to differentiate between *C. albicans* and *C. dubliniensis* with the pH-regulated *C. albicans PHR1* gene (Kurzai *et al.*, 1999), which detects a product from *C. albicans* but not *C. dubliniensis*.

A multiplex PCR system offers some advantages for the detection of *C. albicans* and *C. dubliniensis*, such as detecting coinfections simultaneously, simplifying diagnostic procedures, and saving labor time and costs. In this study, we used two sets of primers, one specific for *C. albicans* and the other for *C. dubliniensis,* as well as a third set that recognizes both. The products amplified from *C. albicans* and *C. dubliniensis* with these primers were sufficiently specific to allow for the differentiation of the two species. The high sensitivity of this method, which can detect as few as 10 cells/ml of saline, is another advantage of this method. It has been reported that the detection limit of saline samples using a PCR assay is 10-fold lower than that of urine samples using ethidium bromide staining (Miyakawa *et al.*, 1992). Many candidiasis patients have died following insufficient treatment of the disease because of the lack of simple, rapid, and sensitive identification procedures (Fraser *et al.*, 1992). The PCR assay developed in this study obviates the need for DNA purification, and is therefore expected to reduce the time to routine diagnosis.

In conclusion, we have developed a multiplex PCR assay to detect *C. albicans* and *C. dubliniensis* and to discriminate between them. With the primer sets used in this study, *C. albicans* and *C. dubliniensis* could be detected at a concentration of 10 cells/ml in saline by a method of simple and rapid DNA separation. Although more trials are needed concerning the detection of *C. albicans* and *C. dubliniensis* in clinical samples, it is expected that the multiplex PCR assay with the primers we used will be a useful and valuable tool for the detection of the two species in the clinical sample.

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