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Rapid PCR Test for Discriminating between *Candida albicans* and *Candida dubliniensis* Isolates Using Primers Derived from the pH-Regulated *PHR1* and *PHR2* Genes of *C. albicans*

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The development of a satisfactory means to reliably distinguish between the two closely related species *Candida albicans* and *Candida dubliniensis* in the clinical mycology laboratory has proved difficult because these two species are phenotypically so similar. In this study, we have detected homologues of the pH-regulated *C. albicans PHR1* and *PHR2* genes in *C. dubliniensis*. Restriction fragment length polymorphism analysis suggests that there are significant sequence differences between the genes of the two species. In order to exploit this apparent difference, oligonucleotide primers based on the coding sequence of the *C. albicans PHR1* structural gene were designed and used in PCR experiments. Use of these primers with *C. albicans* template DNA from 17 strains yielded a predicted 1.6-kb product, while *C. dubliniensis* template DNA from 19 strains yielded no product. We therefore propose that PCR using these primers is a rapid and reliable means of distinguishing the two germ tube- and chlamydospore-producing species *C. albicans* and *C. dubliniensis*.

Opportunistic fungal infections have gained considerable importance during recent years, and oral candidosis is among the most common opportunistic infections encountered in human immunodeficiency virus-infected patients (13). *Candida dubliniensis* is a chlamydospore-positive, germ tube-positive species with many characteristics in common with *Candida albicans*. To date, *C. dubliniensis* isolates have been primarily recovered from the oral cavities of immunosuppressed individuals (20), although isolates have also been recovered from other anatomic sites (19). It has been shown to be a low-level constituent of the human oral flora and has the potential to cause oral candidosis (1, 18). Although *C. albicans* and *C. dubliniensis* are phenotypically very similar, they differ in their carbohydrate assimilation profiles, growth patterns at elevated temperatures, and intracellular β-glucosidase activities (1, 12, 14). In addition, *C. dubliniensis* possesses a very distinct genomic organization (1, 4, 19). In spite of these differences, rapid discrimination between *C. albicans* and *C. dubliniensis* in the clinical mycology laboratory is still problematic and usually only achieved effectively by using a combination of standard laboratory procedures (19). The incidence of *C. dubliniensis* and its role in disease have yet to be firmly established. In order to effectively address this situation, it is important to be able to differentiate this organism from *C. albicans*. For this reason, rapid techniques suitable for the accurate and reliable identification of *C. dubliniensis* in clinical laboratories are required. Because of the difficulty in using methods based on phenotypic parameters to distinguish efficiently between *C. dubliniensis* and *C. albicans*, we proposed to develop a rapid and reliable discriminatory method based on the use of molecular tools.

While analyzing the expression and genomic organization of genes regulated in response to the ambient pH in *C. dubliniensis*, we found that the genomic organization of the *PHR1* and *PHR2* genes differed between *C. dubliniensis* and *C. albicans*. In *C. albicans*, the expression of these functionally homologous genes is regulated by environmental pH and deletion analysis suggests that they play a role in morphogenesis and cell survival in vivo (2, 8, 15). The unique organization of these genes in *C. dubliniensis* suggested the opportunity to use these sequences as targets for species-specific oligonucleotides in PCR experiments to discriminate between *C. dubliniensis* and *C. albicans*.

Identification of *PHR* homologs in *C. dubliniensis*. In order to investigate the presence of *PHR1* and *PHR2* homologous genes in *C. dubliniensis*, genomic DNA from reference strains and clinical isolates (Table 1) was isolated as previously described (16). DNA was digested with the restriction enzymes *Eco*RI and *Hin*dIII and subsequently used for Southern blot analysis. The membranes were hybridized by using an 800-bp *Cla*I fragment of the *C. albicans PHR1* gene or a 1.6-kb *Hin*dIII fragment of the *C. albicans PHR2* gene as a probe under stringent conditions (8, 15). Both probes hybridized with the digested *C. dubliniensis* DNA under these conditions (Fig. 1 and Table 1). However, the sizes of the hybridizing bands differed between the two species in experiments using *Eco*RI or *Hin*dIII (Fig. 1 and Table 1). These findings demonstrate that *PHR1* and *PHR2* homologous genes are present in the *C. dubliniensis* genome. The differences in the restriction fragment lengths point to an architecture of the *PHR1-PHR2* genomic locus in *C. dubliniensis* different from that in *C. albicans*. In agreement with the observations of others who suggested that *Candida stellatoidea* is a variant or synonym of *C. albicans*, the *C. stellatoidea* reference strains tested were indistinguishable from *C. albicans* in this study (11) (Fig. 1 and Table 1). DNA from other chlamydospore-negative pathogenic or nonpathogenic species either did not hybridize at all or produced barely detectable signals of different sizes (Table 1). This may well be due to the existence of *PHR* homologous

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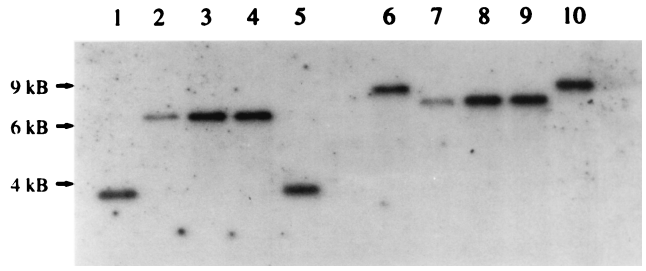

FIG. 1. Southern blot hybridization with the *PHR1* gene probe. DNA from *C. albicans* SC5314 (lanes 1 and 6); *C. dubliniensis* CBS 7987 (lanes 2 and 7), CBS 7988 (lanes 3 and 8), and CD 33 (lanes 4 and 9); and *C. stellatoidea* ATCC 11006 (lanes 5 and 10) digested with *Eco*RI (lanes 1 to 5) or *Hin*dIII (lanes 6 to 10) was analyzed. The electrophoretic positions and sizes deduced from DNA standards are indicated on the left.

genes in these species. Indeed, using PCR with degenerate primers deduced from the *C. albicans PHR1* and *PHR2* sequences, we were able to amplify fragments with homologies to the *PHR1* and *PHR2* genes in the pathogenic yeast *Candida glabrata* (22). Furthermore, *PHR* homologous genes have also been described for *Candida maltosa* and *Saccharomyces cerevisiae* (9, 21). However, so far *C. albicans* is the only species demonstrating strong pH-dependent inverse expression of these genes.

pH-dependent differential expression of the *C. dubliniensis PHR* homologs. To determine whether the expression of the *PHR* homologous genes of *C. dubliniensis* resembles the pattern demonstrated for the *C. albicans PHR1* and *PHR2* genes, total RNA was isolated from the *C. dubliniensis* reference strains at pH 4 and pH 8 as previously described (6). Northern blot hybridization was performed with either the *PHR1* or the *PHR2* probe under stringent conditions. With each of the probes, a transcript with a size comparable to that of the *PHR1* or *PHR2* mRNA was detected in the *C. dubliniensis* reference strains (data not shown). Furthermore, the *PHR* homologous genes of *C. dubliniensis* exhibited a pH-dependent pattern of expression similar to that of *PHR1* and *PHR2*. Thus, it seems likely that the pH-balanced system of the two functional homologues *PHR1* and *PHR2* of *C. albicans* is conserved in *C. dubliniensis*.

Amplification of *PHR1* for differentiation between *C. albicans* and *C. dubliniensis*. In order to investigate whether sequence differences between the *PHR1* and *PHR2* genes of *C. albicans* and their *C. dubliniensis* homologues might be useful in terms of differentiation between these two species, we performed PCR with primers derived from the *PHR1* sequence of *C. albicans*. In addition to the strains listed in Table 1, 12 *C.*

TABLE 1. Reference yeast strains, the sizes of restriction fragments in Southern blot analysis using *PHR1* and *PHR2* probes, and the sizes of PCR products in experiments using *C. albicans PHR1*-specific primers

Species	Strain[a]	Reference	Restriction fragment size (kb) by:				
			Southern blotting				PCR
			PHR1 probe		*PHR2* probe		
			*Eco*RI	*Hin*dIII	*Eco*RI	*Hin*dIII	
Candida albicans	SC5314	Gillum et al. (5)	3.7	8	3.9	1	1.6
	CA019	This study	3.7	8	ND[b]	ND	1.6
	CA024	This study	3.7	8	ND	ND	1.6
	132A	Gallagher et al. (3)	3.7	8	ND	ND	1.6
	3153	Odds (10)	3.7	8	ND	ND	1.6
Candida stellatoidea	ATCC 11006		3.7	8	3.9	1	1.6
	ATCC 20408		3.7	8	ND	ND	1.6
Candida dubliniensis	CBS 7987		6.5	7	20	0.8	NS[c]
	CBS 7988		6.5	7	20	0.8	NS
	CD 33	Sullivan et al. (20)	6.5	7	ND	ND	NS
	CD 38	Sullivan et al. (20)	6.5	7	ND	ND	NS
	CM 1	Sullivan et al. (20)	6.5	7	ND	ND	NS
	LP	This study	6.5	7	ND	ND	NS
Candida glabrata	ATCC 90876		NS	NS	12	7.5	0.7
Candida tropicalis	CBS 94		5.5	14	7.5	13	NS
Candida parapsilosis	CBS 604		11	13	NS	NS	NS
Issatchenkia orientalis	CBS 673		NS	NS	NS	NS	2.1
Pichia guilliermondii	CBS 566		NS	NS	NS	NS	NS
Kluyveromyces marxianus	CBS 834		NS	NS	NS	NS	2.2
Candida maltosa	CBS 5611		NS	NS	ND	ND	2.2

[a] ATCC, American Type Culture Collection, Manassas, Va.; CBS, Centralbureau voor Schimmelcultures, Delft, The Netherlands.
[b] ND, not determined.
[c] NS, no signal detected.

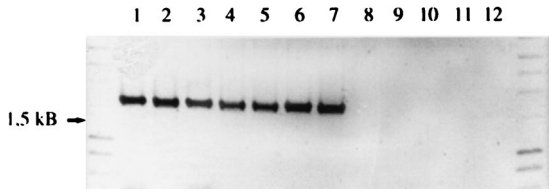

FIG. 2. Amplification products obtained following PCR with the primer set OK3-OK4 and *Candida* template DNA. Lanes 1 to 5, *C. albicans* SC5314, CA019, CA024, 132A, and 3153, respectively; lanes 6 and 7, *C. stellatoidea* ATCC 11006 and ATCC 20408, respectively; lanes 8 to 12, *C. dubliniensis* CBS 7987, CBS 7988, CD 33, CD 38, and LP, respectively. The 1.5-kb band deduced from DNA standards is indicated on the left.

albicans and 13 *C. dubliniensis* fresh clinical isolates from the oral cavities of different human immunodeficiency virus-infected individuals from Ireland were subjected to PCR analysis. Identification of the strains was based on previously described phenotypic criteria (19). The sequence of the forward primer OK3 was 5′ ATG TAT TCA TTA ATC AAA TCA 3′. The sequence of the reverse primer OK4 was 5′ ATT TAA AAA ACA ACG GAC AT 3′. These primers are capable of amplifying the entire 1,644-bp open reading frame of *PHR1*. The PCR mixture (total volume, 100 μl) contained 100 ng of genomic template DNA, 0.2 mM each deoxynucleoside triphosphate, 1× AmpliTaq buffer, 1.5 mM $MgCl_2$, 100 pmol of each primer, and 5 U of AmpliTaq polymerase (Perkin-Elmer, Weiterstadt, Germany). Amplification was performed after denaturation at 95°C for 5 min for 30 cycles with a 20-s denaturation at 95°C, primer annealing at 50°C for 60 s, and extension for 90 s at 72°C, followed by a final extension at 72°C for 10 min in a Trio Thermoblock system (Biometra, Göttingen, Germany). Each experiment included negative controls with all reagents except template DNA. As a positive control, plasmid pSMS-24 containing the complete *PHR1* gene was used as the template DNA (15). Since assignment of isolates to *C. dubliniensis* is based on the missing amplification of the *PHR1* homologue, proper adjustment of the controls is necessary. Products were separated on an 0.8% (wt/vol) agarose gel, stained with ethidium bromide, and compared to the positive control. Template DNA from all of the 17 *C. albicans* isolates and both *C. stellatoidea* strains led to the specific amplification of a fragment with the predicted size of approximately 1.6 kb that was identical to the positive control. When the same conditions were used with 19 *C. dubliniensis* isolates, no amplimers were detected (Fig. 2 and data not shown). In addition, the PCR test was applied to 109 oral isolates previously classified as *C. albicans* based on germ tube and chlamydospore formation. Differentiation between *C. albicans* and *C. dubliniensis* was then achieved in a blinded fashion based on either phenotypic characteristics or the PCR test. The results obtained by the two methods were shown to correlate (unpublished data). Identical results were obtained when colony material from the *C. dubliniensis* and *C. albicans* reference strains was used as a template for the PCR (data not shown). In addition, DNA from reference strains of the chlamydospore-negative species *Candida tropicalis*, *Candida parapsilosis*, *C. glabrata*, *Kluyveromyces marxianus* (*Candida kefyr*), *Issatchenkia orientalis* (*Candida krusei*), and *Pichia guilliermondii* (*Candida guilliermondii*) was isolated and used as a template in this PCR. Similar to the results obtained in the Southern blot analysis, no products were generated or there was amplification of fragments that clearly differed in size from the positive control (Table 1). This, however, does not interfere with the diagnostic value of the procedure since only chlamydospore-positive species are considered.

Conclusion. *C. dubliniensis* was only recently identified as a separate species, and since it is difficult to distinguish it from *C. albicans* in clinical samples there is still very little information available concerning its epidemiology and clinical significance. It has been shown that *C. dubliniensis* is able to readily develop fluconazole resistance under selective pressure (7). To determine whether this or other factors are implicated in the emergence of *C. dubliniensis* as a pathogen, and to measure what the true incidence of this species is in humans, novel methods for discriminating between *C. dubliniensis* and *C. albicans* are required. Until now, identification of *C. dubliniensis* was achieved by time-consuming investigation of a combination of potentially variable phenotypic characteristics (12, 17, 19). In this study, we describe for the first time the use of a structural gene, *PHR1*, as a target for a rapid and reliable PCR-based identification system with the potential to facilitate the analysis of the epidemiology of *C. dubliniensis*.

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