JOURNAL OF CLINICAL MICROBIOLOGY, July 2006, p. 2590–2592 Vol. 44, No. 7
0095-1137/06/$08.00+0 doi:10.1128/JCM.00125-06

Amplification of the Hyphal Wall Protein 1 Gene To Distinguish *Candida albicans* from *Candida dubliniensis*

Orazio Romeo, Cosimo Racco, and Giuseppe Criseo*

Department of Microbiological, Genetic, and Molecular Sciences, University of Messina, Salita Sperone, 31 98166 Messina, Italy

Received 20 January 2006/Returned for modification 2 March 2006/Accepted 9 May 2006

The authors developed a new, simple, and reliable PCR/restriction fragment length polymorphism technique, using amplification of the hyphal wall protein 1 gene of *Candida albicans* and its gene homologue in *Candida dubliniensis*, to differentiate the two species of *Candida*. Performed with a new primer set, CRR-f/CRR-r, PCR produced two different fragments: one of 1,180 bp for *C. albicans*, and one of 930 bp for *C. dubliniensis*.

In 1995, Sullivan et al. (14) described for the first time a new species of *Candida* called *Candida dubliniensis.* Like *Candida albicans*, *C. dubliniensis* isolates produce germ tubes and chlamydospores and cannot be differentiated using conventional methods (9, 12, 13). Because phenotypic methods may not unequivocally differentiate *C. albicans* from *C. dubliniensis*, more-reliable tests based on molecular techniques such as specific PCR are used for discriminating between these two *Candida* species (2, 4, 5, 8).

Hyphal wall protein 1 (HWP1) is a gene that is required for virulence in systemic candidiasis (10). This gene encodes a surface protein that has been demonstrated to serve as a substrate for mammalian transglutaminase, which cross-links *C. albicans* to epithelial cells (10). An HWP1 homologue is also present in *C. dubliniensis*, as shown in the GenBank database. We decided to compare the nucleotide sequence of the HWP1 gene of *C. albicans* (GenBank accession number U64206) and its homologue in *C. dubliniensis* (GenBank accession number AJ632273) to differentiate between these two organisms.

Twenty strains of *C. albicans* were obtained from various body sites of patients hospitalized at the OORR of Reggio Calabria, Italy. A total of 16 *C. dubliniensis* strains from the oral cavities of human immunodeficiency virus-infected individuals came from different geographic locations. Three strains of *C. dubliniensis* (V3, V4, and V5) were obtained from the laboratory of José Ponton of the Universidad del Paìs Vasco, Bilbao, Spain; four strains (CD33, CD36, CD519, and CAN6) were kind gifts from Derek J. Sullivan of the Dublin Dental School and Hospital, University of Dublin, Ireland; six strains (05-87 h, 05-110 h, 05-111 h, 05-112 h, 05-131 h, and 05-139 h) were obtained from stock cultures of V. Vidotto of the Department of Medical and Surgical Sciences, University of Turin, Italy; and three strains (MAL CD1, MAL CD2, and MAL CD3) were isolated from patients hospitalized at the OORR, Reggio Calabria, Italy. *C. albicans* ATCC 10231 and *C. dubliniensis* CBS 7987 were used as reference strains. The identity of all the strains was determined by the ID 32C system (bioMérieux, Marcy l'Etoile, France) and other phenotypic (1, 3, 7, 11, 15) and molecular methods (5, 8).

The Vector NTI program (version 9.0.0; Invitrogen, San Giuliano Milanese, Italy) was used for gene alignment and primer design. Using the new primers, we calculated the lengths of the resulting PCR products as 1,180 bp for *C. albicans* and 930 bp for *C. dubliniensis*. The 1,180-bp fragment of *C. albicans* contains a restriction site specific for BamHI, a restriction enzyme which cuts the *C. albicans* PCR product into two fragments of the expected lengths of 793 and 387 bp. BamHI does not digest the 930-bp PCR product of *C. dubliniensis*.

DNA was extracted from yeast isolates as described by Hoskins (6), with the following small modifications made to enhance the speed of growth of the yeast strains: the yeast cells were cultured in 10 ml of yeast extract-peptone-dextrose broth and incubated overnight at 37°C under shaking conditions (C25 incubator shaker; New Brunswick Scientific, Edison, N.J.).

The primers used for PCR were CRR-f 5′-GTTTTTGCAA CTTCTCTTTGTA-3′ and CRR-r 5′-ACAGTTGTATCATG TTCAGT-3′. The PCR mixture (total volume, 100 μl) contained 3 μl of genomic DNA template (2 μg ml^{-1}), 0.2 mM (each) deoxynucleoside triphosphate, 100 mM (each) primer, 5 U of EuroTaq polymerase (Euroclone, Pero-Milan, Italy), 1× reaction buffer, and 1.5 mM $MgCl_2$. Amplification was performed after denaturation at 95°C for 5 min followed by 34 cycles of denaturation at 94°C for 45 s, primer annealing at 50°C for 60 s, and an extension at 72°C for 90 s, followed by a final extension at 72°C for 10 min in a GeneAmp PCR 2400 system (Perkin Elmer, Monza-Milan, Italy).

PCR products were separated on a 1.3% (wt/vol) agarose gel, stained with ethidium bromide, and compared to the DNA size marker (2-Log DNA Ladder [0.1-10.0 kb]; BioLabs, Pero-Milan, Italy). The PCR products were digested for 3 h at 37°C with BamHI (MBI Fermentas, St. Leon-Rot, Germany) (20 U per 10 μl of PCR product). The digested products were run on a 1.3% (wt/vol) agarose gel and analyzed using a transilluminator (Foto/PrepI; Fotodyne-Celbio, Milan, Italy). PCR and restriction fragment length polymorphism (RFLP) were repeated three times.

All examined isolates produced chlamydospores on cornmeal agar with 1% Tween 80 and germ tubes in bovine serum (3 h at 37°C). Twenty clinical isolates and *C. albicans* ATCC 10231 showed a carbohydrate assimilation profile that is typical

* Corresponding author. Mailing address: Dipartimento di Scienze Microbiologiche, Genetiche e Molecolari, Università degli studi di Messina, Salita Sperone, 31 98166 Messina, Italy. Phone: 39-0906765195. Fax: 39-090392733. E-mail: gcriseo@unime.it.

TABLE 1. Comparison of phenotypic and molecular methods to differentiate *C. albicans* from *C. dubliniensis*

Species and strain	Origin	Length of PCR product (bp)	Length of RLFP product fragments (bp)	Appearance of colony on Pal's agar	Color of colony on TTC agar[c]
C. albicans ATCC 10231[a]	Reference strain[a]	1,180	793, 387	Smooth	Pale pink to whitish
C. albicans L 30390	Ascitic liquid	1,180	793, 387	Smooth	Pale pink to whitish
C. albicans L 18907	Ascitic liquid	1,180	793, 387	Smooth	Pale pink to whitish
C. albicans T 215123	Oral swab	1,180	793, 387	Smooth	Pale pink to whitish
C. albicans T 25780	Oral swab	1,180	793, 387	Smooth	Pale pink to whitish
C. albicans T 30378	Oral swab	1,180	793, 387	Smooth	Not grown
C. albicans T 215140	Oral swab	1,180	793, 387	Smooth	Pale pink to whitish
C. albicans T 25781	Oral swab	1,180	793, 387	Smooth	Pale pink to whitish
C. albicans T-V1	Oral swab	1,180	793, 387	Smooth	Pale pink to whitish
C. albicans TF 29361	Wound swab	1,180	793, 387	Hyphal fringe	Pale pink to whitish
C. albicans TR 29562	Rectal swab	1,180	793, 387	Smooth	Pale pink to whitish
C. albicans E 18894	Sputum	1,180	793, 387	Smooth	Pale pink to whitish
C. albicans E 30384	Sputum	1,180	793, 387	Smooth	Pale pink to whitish
C. albicans E 29351	Sputum	1,180	793, 387	Smooth	Not grown
C. albicans E 29459	Sputum	1,180	793, 387	Smooth	Pale pink to whitish
C. albicans E 18522	Sputum	1,180	793, 387	Hyphal fringe	Pale pink to whitish
C. albicans AG 29355	Gastric aspirate	1,180	793, 387	Smooth	Pale pink to whitish
C. albicans A 29463	Gastric aspirate	1,180	793, 387	Smooth	Pale pink to whitish
C. albicans MAL 012	Vaginal swab	1,180	793, 387	Smooth	Pale pink to whitish
C. albicans MAL 139	Vaginal swab	1,180	793, 387	Smooth	Pale pink to whitish
C. albicans S 28274	Blood	1,180	793, 387	Smooth	Pale pink to whitish
C. dubliniensis CBS 7987[b]	Reference strain[b]	930	930	Hyphal fringe	Red to maroon
C. dubliniensis V3	Oral swab	930	930	Hyphal fringe	Red to maroon
C. dubliniensis V4	Oral swab	930	930	Hyphal fringe	Red to maroon
C. dubliniensis V5	Oral swab	930	930	Hyphal fringe	Red to maroon
C. dubliniensis CD33	Oral swab	930	930	Hyphal fringe	Red to maroon
C. dubliniensis CD36	Oral swab	930	930	Hyphal fringe	Red to maroon
C. dubliniensis CD519	Oral swab	930	930	Hyphal fringe	Red to maroon
C. dubliniensis CAN6	Oral swab	930	930	Hyphal fringe	Red to maroon
C. dubliniensis 05-87h	Oral swab	930	930	Hyphal fringe	Red to maroon
C. dubliniensis 05-110h	Oral swab	930	930	Hyphal fringe	Red to maroon
C. dubliniensis 05-111h	Oral swab	930	930	Hyphal fringe	Red to maroon
C. dubliniensis 05-112h	Oral swab	930	930	Hyphal fringe	Red to maroon
C. dubliniensis 05-131h	Oral swab	930	930	Hyphal fringe	Red to maroon
C. dubliniensis 05-139h	Oral swab	930	930	Hyphal fringe	Pale pink
C. dubliniensis MAL CD1	Oral swab	930	930	Hyphal fringe	Pale pink
C. dubliniensis MAL CD2	Oral swab	930	930	Hyphal fringe	Red to maroon
C. dubliniensis MAL CD3	Oral swab	930	930	Hyphal fringe	Red to maroon

[a] The reference strain *C. albicans* ATCC 10231 is from the American Type Culture Collection.
[b] The reference strain *C. dubliniensis* CBS 7987 is from the Centraalbureau voor Schimmelcultures.
[c] TTC, triphenyltetrazolium chloride (15).

of *C. albicans*, whereas 7 of 17 (41.2%) *C. dubliniensis* strains identified by ID 32C (version 2.0 database) did not correspond to any *C. dubliniensis* profiles in the database. Four strains showed an identification profile that matched that of *C. sake* (7142-7100-15 and 7342-7100-15), and three strains showed an unacceptable profile (7145-3004-15, 7147-1006-15, and 7343-7000-15). Excellent identification (99.9%) results were obtained for 10 *C. dubliniensis* isolates: 6 strains showed a 7042-1000-15 profile, 3 strains a 7142-1000-15 profile, and 1 strain a 7042-1000-11 profile. Further phenotypic and molecular identification results are shown in Table 1.

A fragment of 1,180 bp identical to that of the reference strain *C. albicans* ATCC 10231 was detected in all the *C. albicans* isolates, whereas all the *C. dubliniensis* strains produced a fragment of 930 bp. In addition, the 1,180-bp fragment of *C. albicans* produced two fragments (793 and 387 bp) after digestion with the BamHI restriction enzyme for 3 h at 37°C. No restriction fragments were recovered from the 930-bp PCR product of *C. dubliniensis* (Fig. 1). Unequivocal PCR and RFLP profiles were obtained in all the assayed strains.

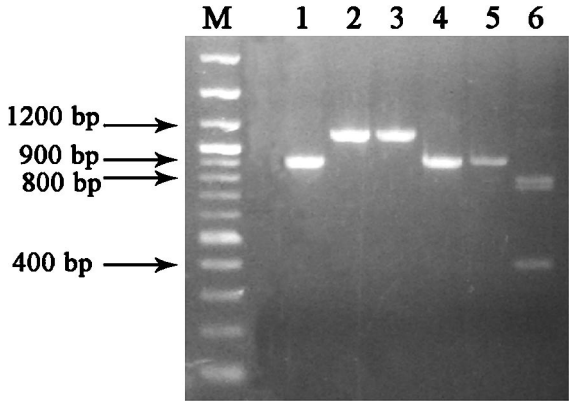

FIG. 1. Differentiation of *C. dubliniensis* and *C. albicans* by the PCR/RFLP method. Lanes 1 and 4, PCR products of *C. dubliniensis* CBS 7987; lanes 2 and 3, PCR products of *C. albicans* ATCC 10231; lane 5, PCR products of *C. dubliniensis* CBS 7987 digested with the BamHI restriction enzyme; lane 6, PCR products of *C. albicans* ATCC 10231 digested with the BamHI restriction enzyme; lane M, molecular size marker.

Several research groups have described rapid and reliable PCR for the identification of *C. dubliniensis*, with specific primers that bind a specific gene and amplify one fragment in one of the two species (2, 8). In addition, Donnelly et al. (4) have developed a discriminative PCR that uses *C. dubliniensis*-specific primers producing a fragment of 288 bp, but universal fungal primers are also used as an internal positive control. Our method uses only a pair of primers that produces different DNA fragments in the two *Candida* species. It excludes the possibility of false-negative results, avoiding misidentification due to possible experimentation errors, particularly when many strains have to be examined.

Graf et al. (5) have recently described a simple, rapid, and inexpensive PCR/RFLP method to discriminate between *C. albicans* and *C. dubliniensis*. After PCR this method uses digestion with the HpyF10VI restriction enzyme to discriminate between the two species of *Candida.* In our study, the RFLP method can be omitted because discrimination between *C. albicans* and *C. dubliniensis* is already made on analysis of the PCR products. RFLP analysis performed with the BamHI restriction enzyme could represent a useful tool for further confirmation in cases of doubtful PCR results.

To our knowledge, this is the first report that uses the HWP1 gene of *C. albicans* and its homologue from *C. dubliniensis* as targets for reliable and unequivocal discrimination between the two *Candida* species. The present study shows a new, relevant role for the HWP1 gene as an important target in rapid, unequivocal differentiation of *C. dubliniensis* and *C. albicans*.

We thank G. Bolignano and his staff of the OORR, Reggio Calabria, Italy; J. Ponton, Departemento de Microbiologia y Inmunologia Facultad de Medicina y Odontologia, Universidad del Pais Vasco, Bilbao, Spain; V. Vidotto, Department of Medical and Surgical Sciences, University of Turin, Italy; and D. J. Sullivan, Dublin Dental School and Hospital, University of Dublin, Ireland, for providing *C. albicans* and *C. dubliniensis* strains. The authors are also grateful to Derek J. Sullivan for useful comments on the manuscript.

REFERENCES

1. **Al Mosaid, A., D. J. Sullivan, and D. C. Coleman.** 2003. Differentiation of *Candida dubliniensis* from *Candida albicans* on Pal's Agar. J. Clin. Microbiol. **41:**4787–4789.
2. **Biswas, S. K., K. Yokoyama, L. Wang, K. Nishimura, and M. Miyaji.** 2001. Identification of *Candida dubliniensis* based on the specific amplification of mitochondrial cytochrome *b* gene. Nippon Ishinkin Gakkai Zasshi. **42:**95–98.
3. **Boerlin, P., F. Boerlin-Petzold, C. Durussel, M. Addo, J. L. Pagani, J. P. Chave, and J. Bille.** 1995. Cluster of oral atypical *Candida albicans* isolates in a group of human immunodeficiency virus-positive drug users. J. Clin. Microbiol. **33:**1129–1135.
4. **Donnelly, S. M., D. J. Sullivan, D. B. Shanley, and D. C. Coleman.** 1999. Phylogenetic analysis and rapid identification of *Candida dubliniensis* based on analysis of *ACT1* intron and exon sequences. Microbiology **145:**1871–1882.
5. **Graf, B., A. Trost, J. Eucker, U. B. Göbel, and T. Adam.** 2004. Rapid and simple differentation of *C. dubliniensis* from *C. albicans*. Diagn. Microbiol. Infect. Dis. **48:**149–151.
6. **Hoskins, L.** 16 October 1998. Quick yeast DNA prep: isolation of total DNA (genomic and plasmid). Hahn Laboratory [Online.] http://www.fhcrc.org/labs/hahn/methods/mol_bio_meth/yeast_quick_dna.html.
7. **Kurtzman, C. P., and J. W. Fell.** 1998. The yeasts, a taxonomy study, 4th ed., p. 476–479. Elsevier Science B.V., Amsterdam, The Netherlands.
8. **Kurzai, O., W. J. Heinz, D. J. Sullivan, D. C. Coleman, M. Frosch, and F. A. Mühlschlegel.** 1999. 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*. J. Clin. Microbiol. **37:**1587–1590.
9. **Pincus, D. H., D. C. Coleman, W. R. Pruitt, A. A. Padhye, I. F. Salkin, M. Geimer, A. Bassel, D. J. Sullivan, M. Clarke, and V. Hearn.** 1999. Rapid identification of *Candida dubliniensis* with commercial yeast identification systems. J. Clin. Microbiol. **37:**3533–3539.
10. **Staab, J. F., S. D. Bradway, P. L. Fidel, and P. Sundstrom.** 1999. Adhesive and mammalian transglutaminase substrate properties of *Candida albicans* Hwp1. Science **283:**1535–1538.
11. **Staib, P., and J. Morschhäuser.** 1999. Chlamydospore formation on Staib agar as species-specific characteristic of *Candida dubliniensis*. Mycoses **42:**521–524.
12. **Sullivan, D., G. P. Moran, and D. C. Coleman.** 2005. *Candida dubliniensis*: ten years on. FEMS Microbiol. Lett. **253:**9–17.
13. **Sullivan, D., and D. Coleman.** 1997. *Candida dubliniensis*: an emerging opportunistic pathogen. Curr. Top. Med. Mycol. **8:**15–25.
14. **Sullivan, D. J., T. J. Westerneng, K. A. Haynes, D. E. Bennett, and D. C. Coleman.** 1995. *Candida dubliniensis* sp. nov: phenotypic and molecular characterization of a novel species associated with oral candidosis in HIV-infected individuals. Microbiology **141:**1507–1521.
15. **Velegraki, A., and M. Logotheti.** 1998. Presumptive identification of an emerging yeast pathogen, Candida dubliniensis (sp. nov.) reduces 2,3,5-triphenyltetrazolium chloride. FEMS Immunol. Med. Microbiol. **20:**239–241.