Identification of *Candida albicans* by polymerase chain reaction amplification of a *CaYST1* gene intron fragment

Claudia Baquero, Mercedes Montero, Rafael Sentandreu & Eulogio Valentin

Departamento de Microbiologia y Ecología, Facultad de Farmacia, Universidad de Valencia, Burjassot, Valencia, Spain

Summary

A single pair of primers, deduced from the intron nucleotide sequence of the *Candida albicans CaYST1* gene, was used in PCR analysis performed with both genomic DNA and whole cells of clinical isolates of *Candida* species and other microorganisms. All the clinical *C. albicans* isolates generated the expected 310 bp amplicon; other *Candida* species as well as laboratory strains belonging to other fungal genera failed to amplify any DNA fragment, except for *Candida pseudotropicalis* (amplicon of 1200 bp), *Kluyveromices marxianus* (amplicon of 1250 bp) and *Cryptococcus neoformans* (several amplicons longer than 1200 bp). Unusual *C. albicans* isolates from Africa also yielded the expected 310 bp amplicon. These results indicate that genes containing intron sequences may be useful to design species-specific primers for identification of fungal strains by PCR. The sensitivity of the method was evaluated for *C. albicans* genomic DNA by using both various DNA concentrations (224 ng to 2.7 pg) and different cell amounts (10^7 to 5 cells). The results obtained may be useful in earlier detection of candidiasis.

Key words *Candida albicans, CaYST1*, PCR, Yeast identification

Identificación de *Candida albicans* por amplificación del intron del gen *CaYST1* mediante reaccion en cadena de la polimerasa

Resumen

A partir de la secuencia nucleotídica del intrón del gen *CaYST1* de *Candida albicans*, se ha diseñado una pareja de oligonucleótidos cebadores que se ha empleado en un análisis de PCR sobre ADN genómico y células enteras de especies del género *Candida* y otros microorganismos. En todos los aislados clínicos de *C. albicans* se generó el amplicón de 310 pb esperado; en otras especies de *Candida*, así como en otras cepas de laboratorio, pertenecientes a otros hongos, no se generó ningún amplicón excepto en los casos de *Candida pseudotropicalis* (amplicón de 1200 pb), *Kluyveromices marxianus* (amplicón de 1250 pb) y *Cryptococcus neoformans* (varios amplicones mayores de 1250 pb). En aislados, poco comunes, de *C. albicans* procedentes de Africa también se generó el amplicón de 310 pb esperado. Estos resultados indican que los genes que presentan intrones pueden ser de utilidad en el diseño de oligonucleótidos cebadores específicos de especie para la identificación mediante PCR de cepas fúngicas. La sensibilidad del método se evaluó empleando diferentes cantidades (de 224 ng a 2,7 pg) de ADN de *C. albicans* así como de distintas cantidades de células (de 10^7 a 5). Los resultados obtenidos pueden ser útiles en una detección temprana de candidiasis.

Palabras clave *Candida albicans, CaYST1*, PCR, Identificación de levaduras

Dirección para correspondencia:

Dr. Eulogio Valentin
Departamento de Microbiologia y Ecología
Facultad de Farmacia
Universidad de Valencia
Av. Vicente Andres Estelles s/n
46100-Burjassot, Valencia, Spain.
Tel.: +34 963 983 614
Fax: +34963 864 299
E-mail: Eulogio.Valentin@uv.es

Aceptado para publicación el 10 de Abril de 2002

1130-1406/01/10.00 Euros

The frequency of systemic candidiasis infections in patients with other serious illnesses has risen dramatically in the past two decades [1] with a mortality rate from 50 to 80%, despite an adequate treatment [2]. In the absence of pathognomic signs or symptoms, the diagnosis of candidiasis is usually based on the isolation and identification of *Candida* by conventional morphology and assimilation tests which can require three to five days, or even longer for more difficult or unusual species [3]. The prognosis is better with an early antifungal treatment [4], but such empirical antifungal therapy may be toxic and costly and, on the other hand, may increase selection of more-resistant *Candida* species [5]. Thus, rapid and accurate identification methods of pathogenic fungi to the species level would be clinically very helpful. The increasing knowledge on the molecular genetics of *Candida albicans* has allowed the use of molecular approaches to identify *Candida* species. One of these involves the polymerase chain reaction (PCR) method wherein different targets of *Candida* DNA have been tested: either single copy genes such as actin [6], chitin synthase [7], HSP 90 [8], and EFB1 [9], or multicopy genes such as the gene coding for rRNA [10-13].

In the present study we describe the use of primers deduced from the *C. albicans CaYST1* intron to specifically amplify by PCR assay a DNA fragment in *C. albicans* strains. The *CaYST1* gene codes for a protein which is a component of the translational machinery and is well-conserved among species [14].

MATERIALS AND METHODS

Microorganisms, media and growth conditions. The microorganisms used in this study were obtained from the CECT [15] (Table 1) except *Candida dubliniensis* NCPF 3649 (CD36) [16]. Clinical isolates identified as *Candida* spp., mainly *C. albicans*, by standard microbiological methods (isolation of yeast colonies on Sabouraud-dextrose agar added with chloramphenicol, induction of hyphal formation by serum and assimilation tests) were also assayed, as were four unusual vaginal isolates of *C. albicans* (glucosamine and N-acetylglucosamine negative) from Africa [17]: three from Madagascar (M82, M335, M8621) and two from Angola (A1587, A1622a). Laboratory strains and clinical isolates were grown on solid or liquid YPD medium [18] at 30 °C. *Cryptococcus neoformans* was grown similarly, except that YPD broth was supplemented with 2.9% (wt/vol) NaCl to reduce capsule formation.

Preparation of DNA. Rapid DNA preparation of different microorganisms was made as described by Ausubel *et al.* [19] for *Saccharomyces cerevisiae*. Briefly, the cell suspensions were vortexed thoroughly following addition of 0.3 g of glass beads (425-600 mm of diameter, Sigma) and an equal volume of phenol-chloroform-isoamyl alcohol (24:24:1). After centrifugation, the aqueous phase was collected and precipitated by adding ethanol. The pellet was resuspended in TE (10 mM Tris-HCl, 1mM Na_2EDTA, pH 8.0) and used for PCR analysis.

Labeling of DNA probe and Southern blot hybridization. The 325 bp DNA fragment, CaYST1-INT, derived from *C. albicans CaYST1* was labeled with digoxigenin (DIG) by using a DIG-DNA Labeling Kit (Roche Diagnostics). The PCR products were electrophoresed on an agarose gel, transferred onto a Hybond-N nylon membrane positively charged (Amersham Pharmacia) by capillary action and baked at 120 °C for 30 min. The membrane was subjected to hybridization with the labeled probe at 68 °C and then processed as described by Ramon *et al.* [20].

Table 1. Order and designation of strains of various *Candida* species and other non *Candida* microorganisms tested by PCR using INT1 and INT2 primers.

Lane No.[a]	Microorganism	Strain designation
1	*C. boidinii*	CECT 1014
2	*Torulospora delbruekii*	CECT 1015
3	*C. atlántica*	CECT 1016
4	*C. glabrata*	CECT 1448
5	*Pichia guillermondii*	CECT 1437
6	*Issatchenkia orientalis*	CECT 1433
7	*C. intermedia* var *intermedia*	CECT 1431
8	*Kluyveromices marxianus*	CECT 1432
9	*C. zeylanoides* var *zeylanoides*	CECT 1441
10	*C. sake*	CECT 1044
11	*C. parapsilosis*	CECT 1449
12	*C. mesenterica*	CECT 1025
13	*C. catenulata*	CECT 1428
14	*C. maritima*	CECT 1435
15	*C. zeylanoides*	CECT 1434
16	*Pichia jadinii*	CECT 1430
17	*C. tropicalis*	CECT 1440
18	*C. inconspicua*	CECT 1980
19	*C. utilis*	CECT 1060
20	*Cryptococcus neoformans*	CECT 1697
21	*C. dubliniensis* CD36	NCPF 3649
22	*C. guillermondii*	CECT 1021
23	*C. krusei*	CECT 10668
24	*C. kefyr*	CECT 1436
25	*C. glabrata*	CECT 1698
26	*C. pseudotropicalis*	CECT 1018
27	*C. tropicalis*	CECT 1005
28	*Schizosaccharomyces pombe*	CECT 1375
29	*C. albicans* ATCC 26555	CECT 1472

a: Lane numbers in figure 2.

Rapid procedure for preparation of PCR samples. Yeast cells were taken with a loop and resuspended in 29.7 ml of 10 mM NaOH, 0.5 % (vol/vol) Tween 20, 0.5% (vol/vol) Nonidet P-40, and then incubated 15 min at 95 °C. Following the treatment the samples were added to 20.3 ml of the PCR reaction mixture.

PCR assay. The synthetic oligonuleotides used were primers INT1 (5'-AAGTATTTGGGAGAAGGGA-AAGGG-3') and INT2 (5'-AAAATGGGCATTAAGGA-AAAGAGC-3') deduced from the *CaYST1* gene sequence of *C. albicans* (EMBL accession number AJ251858).

One unit of EcoTaq polymerase (Ecogen, Spain) was added to 49.7 ml of a solution consisting of 16.6 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 67 mM Tris-HCl (pH 8.8), 0.01% vol/vol Tween-20, 0.2 mM each of dATP, dCTP, dGTP, dTTP, and unless otherwise stated, 500 ng of target DNA and 0.3 mM of each primer (INT1 and INT2). DNA was amplified in a PCR thermal cycler (Perkin Elmer Gene Amp PCR System 2400) by using 1 cycle at 95 °C for 3 min, and then 40 cycles as follows: 60 s of denaturation at 94 °C, 30 s of annealing at 55 °C, and 45 s of primer extension at 72 °C. At the final cycle an additional 5 min of incubation at 72 °C was carried out ensure complete polymerization of any remaining PCR products.

RESULTS AND DISCUSSION

Primers INT1 and INT2 deduced from *CaYST1* intron sequence produced the expected 310 amplicon from different *C. albicans* laboratory strains (data not shown); this amplicon was sequenced and presented a 100% identity with *CaYST1* intron (Figure 1). Since the intron sequences are poorly conserved among microorganism strains, we decided to use this 310 bp amplicon, which we termed CaYST1-INT, to identify *C. albicans* from different sources.

In order to determine the specificity of INT1 and INT2 primers, laboratory strains other than *C. albicans* and other microorganisms were used. Under the experimental conditions described in Materials and Methods,

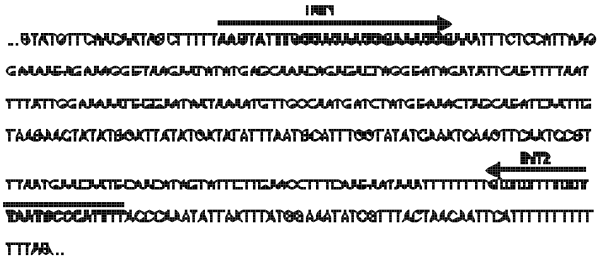

Figure 1. Nucleotide sequence of CaYST1-INT. The primers INT 1 and INT2 are in bold.

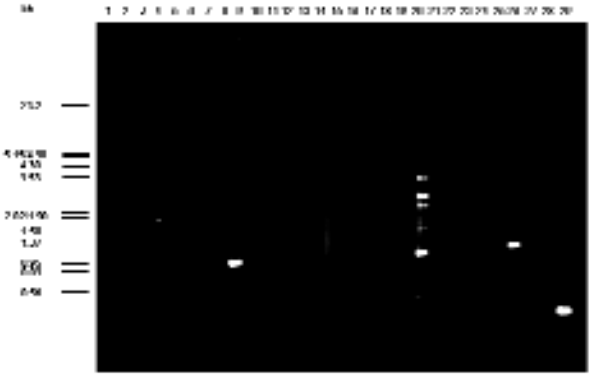

Figure 2. PCR-generated DNA products obtained with primers INT1 and INT2 deduced from the *C. albicans CaYST1* intron sequence, electrophoresed on an agarose gel and ethidium bromide stained. DNA digested with *Eco*RI / *Hind*III is shown in kb (DNA standard size). Lane numbers in table 1.

these primers were unable to amplify DNA extracted from *Candida atlantica, Candida boidinii, Candida catenulata, C. dubliniensis, Candida glabrata, Candida guilermondii, Candida inconspicua, Candida intermedia, Candida kefyr, Candida krusei, Candida maritima, Candida mesenterica, Candida parapsilosis, Candida sake, Candida tropicalis, Candida utilis* and *Candida zeylanoides*, as well as *Issatchenkia orientalis* (sexual form of *C. krusei*), *Pichia guillermondii* (sexual form of *C. guillermondii*), *Pichia jadinii* (sexual form of *C. utilis*), *Schizosaccharomyces pombe* and *Torulospora delbrueckii* (sexual form of *Candida colliculosa*) (Figure 2). We obtained positive PCR products from *Candida pseudotropicalis* (an amplicon of 1050 bp), *Kluyveromyces marxianus* (an amplicon of 1250 bp) and *C. neoformans* (different amplicons longer than 1100 bp) (Figure 2). *C. pseudotropicalis* and *C. kefyr* are synonymus and anamorphic stages of *K. marxianus*. The fact that these organisms given different results could be indicative of a substantial level of polimorphism in the target region. In *C. albicans* the expected 310 bp amplicon was obtained. Although some species other than *C. albicans* gave PCR amplified products, their different lengths when compared with the amplicon from *C. albicans*, suggests that combination of INT1 and INT2 primers in PCR assays could be useful to identify *C. albicans* strains.

To further check this observation, and to evaluate its potential in identification, PCR analysis using DNA from clinical *C. albicans* isolates was performed. As shown in Figure 3, the 310 bp fragments were amplified in all 82 *C. albicans* isolates tested, indicating a high sensitivity for the method. To confirm that these amplicons were derived from the CaYST1-INT region, products from 24 PCR-positive isolates were subjected to Southern blot hybridization probed with CaYST1-INT, hybridizing with all the amplified 310 bp fragments (data not shown). Thus, we conclude that the 310 bp amplicons are derived from the CaYST1-INT and suggest that they are contained in all the *C. albicans* isolates tested. Four clinical isolates of *Candida* species other than *C. albicans* (*C. parapsilosis* and *C. tropicalis*) failed to amplify any DNA fragment. The discriminatory power of the described test is strongly supported by the fact that all five tested unusual vaginal isolates of *C. albicans* from Africa yielded the expected PCR-generated amplicons; these strains are unable to utilize the amino sugars (glucosamine and N-acetylglucosamine) as sole carbon source, and their correct identification as *C. albicans* was made possible by using a PCR-based method of DNA-fingerprinting [17].

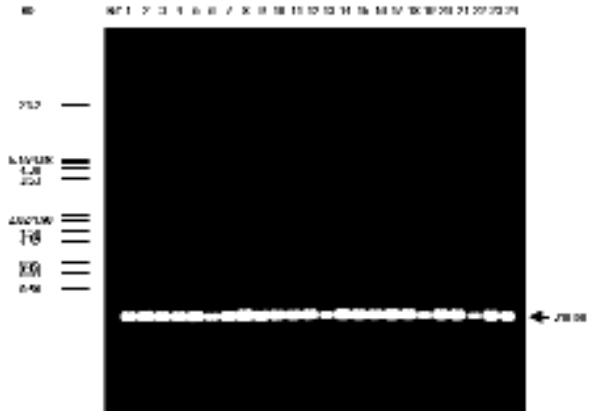

Figure 3. Ethidium bromide-stained PCR amplicons from genomic DNAs of different *C. albicans* clinical isolates. l DNA digested with *Eco*RI / *Hind*III is shown in kb (DNA standard size), NC means negative control.

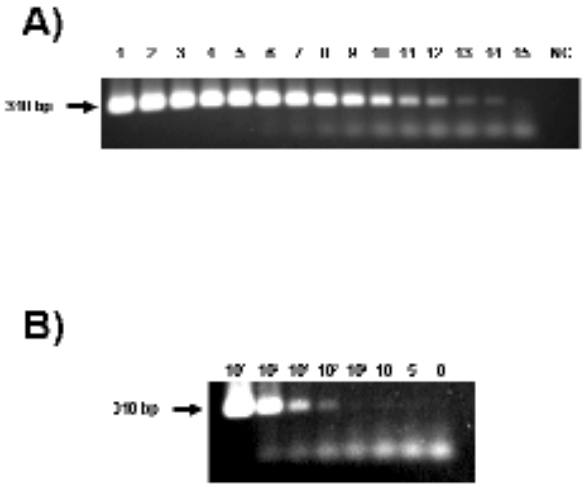

Figure 4. (A) Sensitivity of the PCR assay determined by ethidium bromide staining. Lanes 1 through 15, PCR amplicons obtained from: 224 ng, 22.4 ng, 11.2 ng, 5.6 ng, 2.8 ng, 1.4 ng, 0.7 ng, 350 pg, 175 pg, 87.5 pg, 43.7 pg, 21.8 pg, 10.9 pg, 5.4 pg and 2.7 pg of *C. albicans* ATCC 26555 genomic DNA. NC means negative control. (B) Sensitivity of DNA detection by PCR of *C. albicans* ATCC 26555 determined by ethidium bromide staining. Template DNAs were prepared from 10^7 to 5 cells by a rapid procedure, as described in Materials and Methods.

The sensitivity of the PCR assay was evaluated with isolated DNA at various concentrations (from 224 ng to 2.7 pg) of *C. albicans* genomic DNA. Our primer set (INT1 / INT2) was able to amplify 5.4 pg of *C. albicans* DNA (Figure 4A), and Southern blot analysis with the CaYST1-INT probe confirmed the specificity of the amplicon (data not shown).

To examine the utility of *C. albicans*-specific PCR test for clinical samples we attempted to establish a rapid procedure for preparation of PCR samples from *C. albicans* cells. The treatment of these cells with a solution containing NaOH, Tween 20 and Nonidet P-40 (see Materials and Methods), followed by the PCR assay, resulted in an amplification of the target sequence of the 310 bp DNA fragment (Figure 4B). To determine the detection limit, 10-fold dilutions of *C. albicans* were made. As shown in Figure 4B, the detection limit of *C. albicans* cells was in the order of 10^4 cells/ml. Since fewer than 10^4 yeast cells per ml of urine are suspected to colonize in an asymptomatic patient with renal candidiasis [21], our PCR assay could be useful for detecting *C. albicans* cells in these cases.

The results in this report indicate that genes containing intron sequences could well be useful to design specific primers for identification of *Candida* strains at the species level. A PCR method to identify *C. albicans* and *C. dubliniensis* isolates, which uses species-specific primers from *EFB1* and *ACT1* intron sequences respectively [9,22] has recently been described. Identification of *Candida* species could be clinically very beneficial from a prognostic and therapeutic point of view [23-26]. Further, these PCR approaches could also be important in epidemiological and taxonomic studies.

We are grateful to Dr. Enrique Herrero for C. albicans isolates. The unusual vaginal isolates of C. albicans from Africa were a generous gift from Dr. G. Schönian. C.B. is supported by a fellowship from the ALFA program (B2 type No. ALR/B7-3011/90.04-2342-8, Mycology Network) of the European Union. M.M. is supported by a fellowship from the Fundacion Ramon Areces, Madrid, Spain. This work was supported by grants from the Secretaría de Estado de Universidades, Investigación y Desarrollo, Spain (PM96/0019 and PM97/0103), Fundación Vicente Ricardo Cantin (Alicante, Spain) and Gastea S.L. (Alicante, Spain).

References

1. Pfaller MA**.** Nosocomial candidiasis: emerging species, reservoirs and modes of transmission. Clin Infect Dis 1996; 22: 89-94.
2. Fraser VJ, Jones M, Dunkel J, Storfer S, Medoff G, Dunagan WC. Candidemia in a tertiary care hospital: epidemiolgy, risk factors , and predictors of mortality. Clin Infect Dis 1992; 15: 414-421.
3. Warren NG, Hazen KC. *Candida, Cryptococcus* and other yeasts of medical importance. In: Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH (Eds.) Manual of Clinical Microbiology, 6th ed., Washington D.C., American Society for Microbiology, 1995: 723-737.
4. Karp JE, Merz WG, Churache P. Response to empiric amphotericin B during antileukemic therapy-induced granulocytopenia. Rev Infect Dis 1991; 13: 592-599.
5. Wingard JR. Infections due to resistant *Candida* species in patients with cancer who are receiving chemotherapy. Clin Infect Dis 1994; 19: 49-53.
6. Kan VL. Polymerase chain reaction for the diagnosis of candidemia. J Infect Dis 1993; 168: 779-783.
7. Jordan JA. PCR identification of four medically important *Candida* species by using a single primer pair. J Clin Microbiol 1994; 32: 2962-2967.
8. Crampin AC, Matthews RC. Application of the polymerase chain reaction to the diagnosis of candidiosis by amplification of an HSP 90 gene fragment. J Med Microbiol 1993; 39: 1353-1360.
9. Maneu V, Martinez J, Gozalbo D. Identification of *Candida albicans* clinical isolates by PCR amplification of an *EFB1* gene fragment containing an intron-interrupted open reading frame. Med Mycol 2000; 38: 123-127.
10. Fujita SI, Lasker BA, Lott TJ, Reiss E, Morrison CJ. Microtitration plate enzyme immunoassay to detect PCR-amplfied DNA from *Candida* species in blood. J Clin Microbiol 1995; 33: 962-967.
11. Haynes KA, Westerneng TJ, Fell JW, Moens W. Rapid detection and identification of pathogenic fungi by polymerase chain reaction amplification of large subunit ribosomal DNA. J Med Vet Mycol 1995; 33: 319-329.
12. Shin JH, Notle FS, Morrison CJ. Rapid identification of *Candida* species in blood cultures by a clinically useful PCR method. J Clin Microbiol 1997; 35: 1454-1459.
13. van Deventer AJM, Goessens WHF, van Belkum A, van Vliet HJA, van Teten EWM, Verbrugh HA. Improved detection of *Candida albicans* by PCR in blood of neutropenic mice with systemic candidiasis. J Clin Microbiol 1995; 33: 625-628.
14. Montero M, Marcilla A, Sentandreu R, Valentin E. A *Candida albicans* 37 kDa polypeptide with homology to the laminin receptor is a component of the translational machinery. Microbiology 1998; 144: 839-847.
15. Belloch C, Lopez L, Esteve B, Martinez PV, Garcia-Lopez MD, Uruburu F (Eds.) Spanish type culture collection, 4th ed. University of Valencia, Valencia, Spain, 1998.
16. Bikandi J, San Millan R, Moragues MD *et al.* Rapid identification of *Candida dubliniensis* by indirect immunofluorescence based on differential localization of antigens on *C. dubliniensis* blastospores and *Candida albicans* germ tubes. J Clin Microbiol 1998; 36: 2428-2433.
17. Tietz HJ, Küssner A, Thanos M, Pinto de Andrade M, Presber W, Schönian G. Phenotypic and genotypic characterization of unusual vaginal isolates of *Candida albicans* from Africa. J Clin Microbiol 1995; 33: 2462-2465.
18. Sherman F. Getting started with yeast. In: Guthrie C, Fink G R (Eds.) Guide to yeast genetics and molecular biology. San Diego, Academic Press, 1991: 3-21.
19. Ausubel FM, Brent R, Kingston RE *et al.* Current protocols in molecular biology, vol. 2, New York, John Wiley and Sons, 1996: 13.11.1-13.13.8.
20. Ramon AM, Gil MR, Burgal M, Sentandreu R, Valentin E. A novel cell wall protein specific to the mycelial form of *Yarrowia lipolytica*. Yeast 1996; 12: 1535-1548.
21. Dupont B. Clinical manifestations and management of candidosis in the compromised patient. In: Warnock DW, Richardson MD (Eds.) Fungal infection in the compromised patient, 2nd ed. New York, John Wiley & Sons 1990: 55-84.
22. Donnelly SM, Sullivan DJ, Shanley DB, Coleman DC. Phylogenetic analysis and rapid identification of *Candida dubliniensis* based on analysis of *ACT1* intron and exon sequences. Microbiology 1999; 145:1871-1882.
23. Bodey GP. Candidiasis, pathogenesis, diagnosis and treatment. Raven Press, New York, 1993.
24. Meunier F. Candidiasis. Eur J Clin Microbiol Infect 1989; 8: 438-447.
25. Odds FC . *Candida* and candidosis, London, 2nd ed. Bailliere Tindal, 1988.
26. Odds FC. Antifungal susceptibility testing of *Candida* spp. by relative growth measurement at single concentrations of antifungal agents. Antimicrob Agents Chemother 1992; 36:1727-1737.