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A SIMPLE AND RELIABLE PCR-RESTRICTION FRAGMENT LENGTH POLYMORPHISM ASSAY TO IDENTIFY *CANDIDA ALBICANS* AND ITS CLOSELY RELATED *CANDIDA DUBLINIENSIS*

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ABSTRACT

Candida dubliniensis is an emerging pathogen capable of causing superficial as well as systemic infections. Due to its close similarity to *C. albcians*, conventional methods based on phenotypic traits are not always reliable in identification of *C. dubliniensis*. In this study, we developed a PCR-restriction fragment length polymorphism (RFLP) assay to identify and discriminate between the two closely related species. The D1/D2 region of 28S rDNA was amplified by PCR and enzymatically digested by *Apa*I and *BsiE*I respectively. PCR products of both species were digested into two fragments by *Apa*I, but those of other yeast species were undigested. *BsiE*I cut the PCR products of *C. albicans* into two fragments but not those of *C. dubliniensis*. Thus two species were differentiated. We evaluated 10 reference strains representing 10 yeast species, among which *C. albicans* and *C. dubliniensis* were successfully identified. A total of 56 phenotypically characterized clinical isolates (42 *C. albicans* isolates and 14 *C. dubliniensis* isolates) were also investigated for intra-species variability. All tested isolates produced identical RFLP patterns to their respective reference strains except one initially misidentified isolate. Our method offers a simple, rapid and reliable molecular method for the identification of *C. albicans* and *C. dubliniensis*.

Key words: *Candida albicans*, *Candida dubliniensis*, PCR-restriction fragment length polymorphism, identification, differentiation

INTRODUCTION

Candida species usually reside as commensals at mucosal membranes in healthy individuals and can be detected in approximately 50% of the population in this non-virulent form. However, under conditions when the host's normal flora is disrupted or the immunity is impaired, *Candida* species often become pathogenic. *Candida* infections have become a problem of growing significance. The incidence of infections has increased dramatically over the past a few decades. *C. albcians* is the most common pathogen in this genus and fourth leading cause of nonsocomial bloodstream infections (4, 21). However, several non-albicans *Candida* species, e.g. *C. glabrata*, *C. tropicalis* and *C. parapsilosis*, have emerged as causative agents of candidiasis (20). Another *Candida* species of growing clinical importance is *C. dubliniensis*, a novel

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opportunistic pathogen first described as a distinct taxon by Sullivan in 1995 (26). *C. dubliniensis* was mainly associated with oropharyngeal candidiasis in HIV-infected patients. Recent evidence, however, indicates that it is also a cause of superficial and systemic infections in HIV-negative individuals with an estimated prevalence rate below 5% (7, 10, 27). It is important to study the epidemiology of *C. dubliniensis* due to its capability of rapid acquisition of stable fluconazole resistance, both *in vitro* and *in vivo*, after prolonged therapy in HIV-seropositive patients (15, 16, 24).

Identification of *C. dubliniensis* can be problematic due to its close phenotypic similarity to *C. albicans*. Both species produces germ tubes, chlamydospores and true hyphae (26). Several phenotypic assays have been developed to differentiate *C. dubliniensis* from *C. albicans*, including the capacity to grow at 45°C, formation of chlamydospores on selected media, the ability to assimilate xylose, lactate or α-methyl-D-glucoside and different colony color produced on CHROMagar Candida medium (2,5,23,28). These assays can serve as rapid methods to screen for potential *C. dubliniensis* isolates. But none of these assays have proved to be efficient and entirely reliable. At present, the most accurate means of differentiating between these two closely related species requires the use of molecular biology-based techniques, such as electrophoretic karyotyping, DNA fingerprinting analysis with repetitive sequence-containing DNA probes, randomly amplified polymorphic DNA analysis, restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism, conventional and real-time PCR analysis, or pulsed-field gel electrophoresis(18,25). These methods proved very effective. Among them, PCR-RFLP analysis is a simple and reliable one. In this study we developed and evaluated a PCR-RFLP assay to identify and discriminate between *C. albicans* and *C. dubliniensis*.

MATERIALS AND METHODS

Yeast Strains

C. albicans (SC 5314), *C. dubliniensis* (CBS 7987), *C. glabrata* (ATCC 2001), *C. guilliermondii* (CBS 6021), *C. krusei* (ATCC 6258), *C. kefyr* (CBS 6432), *C. lusitaniae* (CBS 6936), *C. parapsilosis* (ATCC 22019), C. *tropicalis* (CBS 8072) and *Trichosporon asahii* (CBS 2479) were used as reference yeast strains. Fourteen clinical isolates of presumptive *C. dubliniensis* originating from sputum (n=10), vaginal swabs (n=3) and urine (n=1) were studied in comparison with 42 clinical strains of *C. albicans*. Presumptive *C. dubliniensis* isolates were characterized by phenotypic methods including growth on cornmeal Tween-80 agar, growth at 45°C, characteristic growth on CHROMagar Candida (CHROMagar, Paris, France) and confirmed by Vitek 2 system (biomerieux, Marcy l'Etoile, France).

Culture Conditions and Genomic DNA Extraction

Yeast cells were cultured on YPD broth (1% yeast extract, 2% peptone, and 2% dextrose) and were incubated for 24-36 hours at 30°C under shaking conditions (200rpm). The yeast cells were collected by centrifugation(2ml of the broth culture at 12000×g for 2 min), suspended in 600μl of 1 M sorbitol-50 mM phosphate buffer (pH 7.5) containing 50U Lyticase (Sigma-Aldrich, US). After 30 min of incubation at 30°C, the cells were centrifuged at 1500×g for 10 min. The supernatant was then discarded and pellet was collected. TianGen Yeast Genomic DNA Extraction Kit (TianGen Biotech, Beijing, China) was used to extract genomic DNA from tested isolates by following the enclosed protocol. DNA obtained was finally suspended in 100 μl TE buffer and stored at -20°C before use.

Sequence Analyses and Selection of Restriction Enzymes

A total of 30 sequences of the 28S ribosomal DNA (rDNA) D1/D2 region of 10 tested yeast species were retrieved from GenBank database (data not shown). Each sequence was then analyzed for restriction sites using the MapDraw program of DNA Star Lasergene Version 7.0. Restriction enzymes were selected as to generate *C. albcians*-specific and *C. dubliniensis*-specific RFLP patterns.

PCR Amplification of the D1/D2 region

Primers NL-1 (5'-GCATATCAATAAGCGGAGGAAA AG-3') and NL-4 (5'-GGTCCGTGTTTCAAGACGG-3') were used to amplify D1/D2 region of the 28S rDNA genes. PCR amplifications were carried out in 50-µl volumes containing 1.5µl of each 10µmol/l primer, 25µl of GoTaq Green Master Mix (Promega, Madison, WI, USA), 3µl DNA template and corresponding amount of ultra-pure distilled water. PCR was performed in a PTC-200 DNA Engine thermal cycler (Bio-Rad) with following parameters: 94°C for 3 min; 94° for 1 min, 52°C for 30 s and 72°C for 1 min, repeated for a total of 32 cycles; 72°C for 10 min and 4°C hold.

Restriction Digests of PCR Products

RFLP analyses were performed in 20µl volumes with 100-200 ng of amplified DNA products, 25U *Apa*I or 5U *BsiE*I(New England Biolabs, Beverly, MA, USA), 2µl 10×digestion buffer, 0.2 µl bovine serum albumin and corresponding amount of water. Digestion mixtures were incubated for 0.5-1 h at 37°C for *Apa*I or at 60°C for *BsiE*I. The resulting fragments were separated on 2% agarose gels and visualized under UV light after ethidium bromide staining, with a 100bp DNA ladder for fragment size comparison.

RESULT

Sequence Analyses

An extensive analysis of database entries of yeast species tested in this study was performed with respect to calculated fragment lengths of the D1/D2 regions generated by commercially available restriction enzymes. Minor differences in calculated lengths of PCR products were observed. Finally, *Apa*I and *BsiE*I were selected to be evaluated in experiment. Predicted fragment lengths of *Apa*I and *BsiE*I-digested PCR products are given in Table 1. *Apa*I was expected to cut the PCR amplicons of *C. albicans* and *C. dubliniensis* into two fragments, but leave those of other yeast species intact. *BsiE*I was selected to digest the amplicon of *C. albicans* into two fragments and those of *C. dubliniensis* would remain undigested. Thus two related *Candida* species can be identified and differentiated by distinctive and specific RFLP patterns.

Table 1. Comparison of PCR-RFLP assays to differentiate between *C.albicans* and *C. dubliniensis*

Target of PCR amplification and primers	Length of PCR products (bp) CA / CD	Fragments' length after enzymatic digestion (bp) Enzyme: CA / CD	No. of isolates tested CA / CD	Reference
ITS region: ITS5 and NL4	approximately 1200	*Dde*I: 450, 350, 210, 150 / 450, 350, 210, 110 *Bfa*I and *Hae*III: differentiable but not specified	78 / 10	8
ITS region:CA-INT-L(R)	approximately 600	*Dde*I: one fragment / two fragments	8 / 2	13
V3 region: CA25SV3L(R)	approximately 500	*Hae*III: differentiable but not specified	8 / 2	13
ITS2 region: ITS3 and ITS4	approximately 340	*NspB*II: approximately 160, 180 / 340 *BsmA*I: approximately 340 / 100, 240	17 / 8	19
ITS2 region: CTSF and CTSR	345 / 350	*MspA*1I: 35, 143, 167 / 35, 315	1 / 9	6
ITS region: UNI1 and UNI2	586 / 589	*Hpy*F10VI: 141, 184, 261 / 264, 325	61/ 23	1
ITS region: ITS1 and ITS4	540 / 540	*Bln*I: 540 / 200,340	146 /12	14
D1-D2 region: NL-1 and NL-4	615 / 614	*Apa*I: 134 , 481/ 134, 480 *BsiE*I: 181, 434 / 614	43/ 15	This study

CA : *C. albicans*, CD : *C. dubliniensis*

PCR Amplification of D1/D2 Regions

As shown in Fig.1, intended DNA fragments of all reference strains were successfully amplified with primers NL-1 and NL-4. PCR products were found to reach 550-600 bp in length as predicted from sequence analysis. However, most tested species, except *C. lusitaniae*, were inseparable due to similar PCR amplicon sizes.

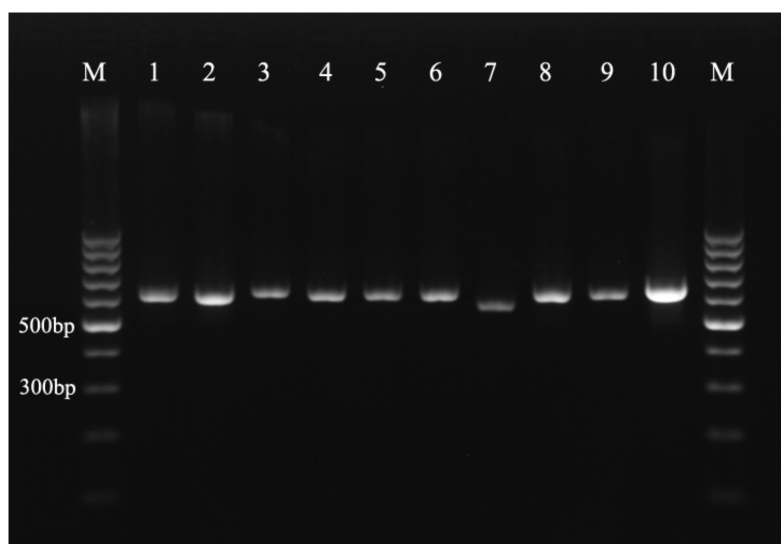

Figure 1. PCR products from 10 yeast species: Lane 1: *C. albicans* (SC 5314) ; Lane 2: *C. dubliniensis* (CBS 7987); Lane 3: *C. glabrata* (ATCC 2001); Lane 4: *C. guilliermondii* (CBS 6021); Lane 5: *C. kefyr* (CBS 6432); Lane 6: *C. krusei* (ATCC 6258); Lane 7: *C. lusitaniae* (CBS 6936); Lane 8: *C. parapsilosis* (ATCC 22019); Lane 9: C. *tropicalis* (CBS 8072); Lane 10: *T. asahii* (CBS 2479) ; Lane M: 100-bp ladder.

RFLP Analyses of Reference Strains

When digested with *Apa*I, D1/D2 regions of *C. albicans* and *C. dubliniensis* strains shared the same restriction pattern, with two bands of almost identical sizes (134bp, 481bp for *C. albicans* and 134bp, 480bp for *C. dubliniensis*) (Fig.2). Nevertheless, amplicons of other yeast strains were undigested by *Apa*I, which easily distinguished *C. albicans* and *C. dubliniensis* from other tested species. Digestion of the D1/D2 region with *BsiE*I generated distinctive restriction profiles for *C. albicans*: two fragments of 181bp and 434bp (Fig.3). In addition, *BsiE*I also cut into two fragments the PCR-products of *C. glabrata* (149bp and 475bp), *C. krusei* (59bp and 548bp) and *C. lusitaniae* (126bp and 433bp). As was seen in Fig.3, the differences in fragment lengths were sufficient to discriminate *C. albicans* from *C. glabrata*, *C. krusei* and *C. lusitaniae*. Other tested species were undigested by *BsiE*I, including *C. dubliniensis*. Therefore, two separate enzymatic digestions produced species-specific RFLP profiles for *C. albicans* and *C. dubliniensis*.

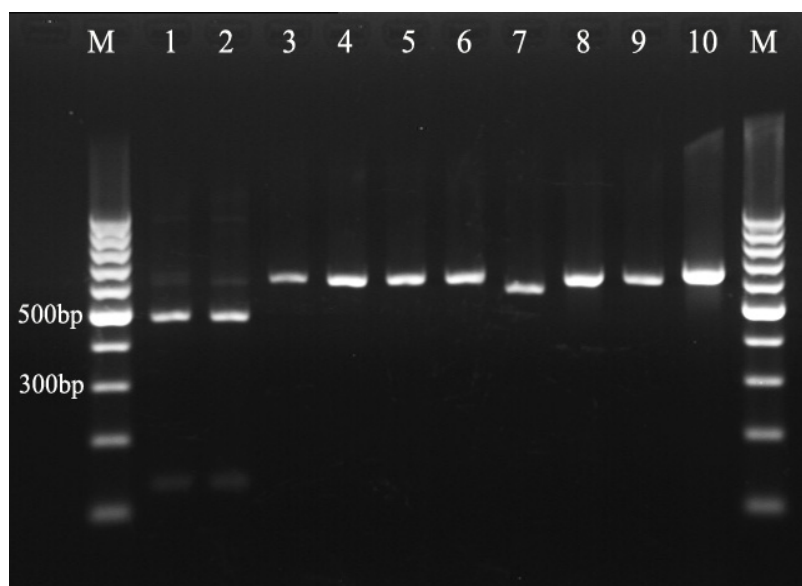

Figure 2. Restriction digestion of PCR products of reference yeast strains with *Apa*I : Lane 1: *C. albicans* (SC 5314) ; Lane 2: *C. dubliniensis* (CBS 7987); Lane 3: *C. glabrata* (ATCC 2001); Lane 4: *C. guilliermondii* (CBS 6021); Lane 5: *C. kefyr* (CBS 6432); Lane 6: *C. krusei* (ATCC 6258); Lane 7: *C. lusitaniae* (CBS 6936); Lane 8: *C. parapsilosis* (ATCC 22019); Lane 9: C. *tropicalis* (CBS 8072); Lane 10: *T. asahii* (CBS 2479) ; Lane M: 100-bp ladder.

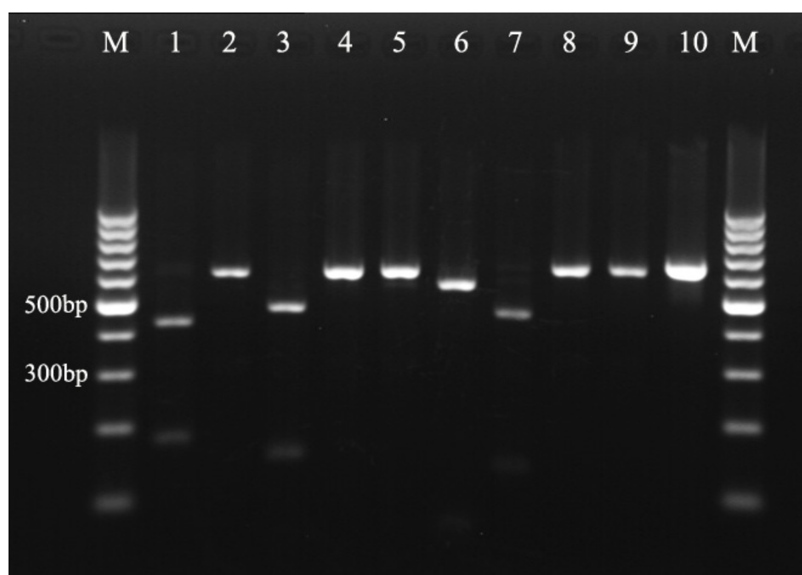

Figure 3. Restriction digestion of PCR products of reference yeast strains with *BsiE*I : Lane 1: *C. albicans* (SC 5314) ; Lane 2: *C. dubliniensis* (CBS 7987); Lane 3: *C. glabrata* (ATCC 2001); Lane 4: *C. guilliermondii* (CBS 6021); Lane 5: *C. kefyr* (CBS 6432); Lane 6: *C. krusei* (ATCC 6258); Lane 7: *C. lusitaniae* (CBS 6936); Lane 8: *C. parapsilosis* (ATCC 22019); Lane 9: C. *tropicalis* (CBS 8072); Lane 10: *T. asahii* (CBS 2479) ; Lane M: 100-bp ladder.

Evaluation for Intra-species Variation of Restriction Sites

To assess intra-species variability of the two restriction sites, a total of 56 clinical isolates of *C. albicans* and *C. dubliniensis* were also investigated. Most tested isolates, after digestion with *Apa*I and *BsiE*I respectively, showed identical and consistent RFLP patterns to their respective reference strains (data not shown). However, one of the 14 presumptive *C. dubliniensis* isolates showed RFLP pattern indicative of *C. albicans*. Further sequencing of the D1/D2 region of this isolate confirmed the identification of *C. albicans*.

DISCUSSION

Rapid and accurate identification of *C. dubliniensis* is crucial for the study of epidemiology and clinical management of infections caused by this opportunistic pathogen. However, this is hampered by lack of easy and reliable methods for definite identification. Most phenotypic methods are presumptive and often subject to error (28). Although several commercial identification systems such as Vitek 2 system, have demonstrated useful in separation of *C. albicans* and *C. dubliniensis*, results are not always reliable as was seen in our study (3, 12, 23). Confirmatory identification of *C. dubliniensis* always requires the molecular methods.

PCR-RFLP assays have been successfully applied to the identification of *Candida* species (17, 22, 29). Compared with other molecular methods, PCR-RFLP analysis is generally easy and rapid to perform. Although more complex RFLP methods have previously been used for the identification of *C. dubliniensis*, their use may be limited since they were time-consuming or the results were difficult to interpret (18).Thus in this study, simpler but more advantageous PCR-RFLP is preferred due to its increased applicability in clinical laboratories.

Ribosomal regions, such as the internal transcribed spacer (ITS) region and 28S rDNA, exhibit a low intraspecific polymorphism and a high interspecific variability, making them ideal targets for species identification purposes (9). In this study, we selected the D1/D2 variable region at the 5’ end of the 28S rDNA gene as target for PCR amplification. Sequencing of this region has been demonstrated to be sufficient for accurate identification of most yeast species (11). Between *C. albicans* and *C. dubliniensis*, there are 13 nucleotide differences in the region D1/D2, sufficiently variable for reliable differentiation. Two enzymes, *Apa*I and *BsiE*I, were selected based on the restriction profiles generated

by these nucleotide differences. Despite that only a limited number of *C. albicans* and *C. dubliniensis* isolates were investigated, those restriction sites for *Apa*I and *BsiE*I seemed to be well conserved in these two species.

Several PCR-RFLP assays have been described so far to discriminate between *C. albicans* and *C. dubliniensis* (Table 1). Irobi et al amplified the ITS regions (ITS1, 5.8S, ITS2) of several medically important *Candida* species, including *C. dubliniensis* (8). Further RFLP analysis with *Bfa*I, *Dde*I or *Hae*III revealed distinct differences between the two species. McCullough et al amplified the ITS regions and restricted them with *Dde*I. *C. albicans* produced one fragment while *C. dubliniensis* produced two fragments (13). In the same study, McCullough and his group also targeted the V3 region of 25S/28S rDNA and cut the PCR amplicons with *Hae*III; the discrimination could be made based on fragments of different sizes. Park and his co-authors amplified a conserved part of the 5.8S rDNA, the adjacent ITS2 region and a part of 28S rDNA. The differentiation was achieved by analysis of the PCR products with *Bsm*AI (*C. dubliniensis*-specific) and *NspB*III (*C. albicans*-specific) (19). Three other PCR-RFLP assays also used a similar strategy by targeting a part or whole of the ITS region (1, 6, 14). All these methods proved effective for accurate identification. In comparison, our study used a slightly different strategy. We first identified a restriction site for *Apa*I which was specific to both species, instantly separating them from other yeast species. Subsequently *BsiE*I was found to produce *C. albicans* specific pattern and *C. dubliniensis* was identified by absence of the restriction site.

An advantage of the method described here is the stable and easy-to-read RFLP patterns. Unlike previous reports, this method involves only one or two DNA fragments. Besides, it is a simple and rapid method to perform. With the aid of time-saving restriction enzymes, the whole process can be accomplished in less than 6h, requiring no sophisticated equipments except a conventional thermal cycler. Considering that DNA sequencer may not be readily available to most clinical laboratories, this molecular method is applicable for unequivocal identification and differentiation of *C. albicans* and *C. dubliniensis*.

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