PCR-RFLP Is a Useful Tool to Distinguish between C. Dubliniensis and C. Albicans in Cancer Patients in Iran

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Abstract

Introduction: Candida dubliniensis and C. albicans are very similar in morphology and phenotypic characteristics. Approximation of this yeast species has caused major problems in identifying these two correctly.

Materials and Methods: To distinguish among sixty yeast clinical isolates from patients with cancer, polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) was done.

Results: PCR-RFLP of the ITS region showed different patterns between Candida dubliniensis and C. albicans after digestion with enzymes BlnI. All of the Clinical isolates were diagnosed as C .albicans. The results were confirmed by sequencing and RAPD-PCR.

Conclusion: PCR RFLP would be a useful and applicable technique in clinical laboratories for discrimination of C. albicans and C. dubliniensis.

Keywords: PCR- RFLP, C .dubliniensis, C. albicans, BlnI

Introduction

One of the important issues in routine laboratories of medical mycology is the correct identification of C. albicans and C. dubliniensis together. C. dubliniensis is not detectable than yeast species of C .albicans morphology and phenotypically.

They are different in their epidemiology, virulence and antifungal susceptibility. Approximation of this yeast species has caused major problems in identifying the C. albicans correctly. Like C. albicans, C. dubliniensis can produce germ tubes and chlamydospores. These two species can be distinguished by examination of their phenotypic features, including growth at 45°C, intracellular β-D-glucosidase activity, and carbohydrate assimilation profiling.(1,2) However, these phenotypic tests are usually time-consuming and do not give completely reliable results.(2) In spite of the identified differences, a rapid and accurate discrimination between C. albicans and C. dubliniensis remains problematic in the most of clinical mycology laboratories.(3) Due to bugs in the phenotypic methods to identify C. albicans and C. dubliniensis recently, trends have increased about the use of molecular techniques. These techniques, are including 25S rDNA analysis(4) PCR-fingerprinting(3) fluorescent probe hybridization(5) amplified fragment length polymorphism.(6) However, these techniques often are laborious, time consuming and too expensive for routine use in medical laboratories. Mirhendi et.al,(2) introduced a simple polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) for differentiation between C. albicans and C. dubliniensis. The aim of this study is to distinguish C. albicans and C. dubliniensis in clinical yeast isolates from patients suffering cancer using PCR-RFLP.

Materials and methods
Sixty clinical isolates which originated from, lip, throat and tongue of patients suffering from cancer in four Mazandaran University Hospitals -Iran and two standard strains (C .albicans CBS 562; C. dubliniensis CBS 7987) which obtained from CBS-KNAW, fungal biodiversity centre, Utrecht, Netherlands were included in present study. Table one summarized the distribution of these isolates based on the origin of the specimens and type of malignancies. Primarily, these strains were identified by some phenotypic methods such as colony color on CHROMagar Candida medium (CHROMagar Company, Paris, France), germ-tube formation in horse serum, chlamydospore formation on cornmeal agar (DIFCO laboratories, Detroit, Mich., USA) with 1% Tween 80 (CMA-T_{80}) and some other molecular methods such as RAPD-PCR(7, 8) and sequencing for D1/D2 region of LSU rDNA gene.(9)
Stock cultures were initially grown on Sabouraoud's dextrose agar (LAB M, Bury, UK) at 32°C for 48 h, and then differentiated using RFLP PCR technique.

DNA Extraction: genomic DNA extracted using the method of glass bead disruption.(10) Briefly, a loop full of fresh yeasts was suspended in 300µl of lysis buffer (10mM Tris, 1mM EDTA pH 8, 1%SDS, 100mM NaCl, 2% Triton X-100), then 300µl of phenol-chloroform(1:1) solution and 300mg of 0.5 diameter glass bead were added to samples. For disrupting cell, samples were vortexed vigorously for 5min. Then, samples were centrifuged at 10,000 rpm for 5min, supernatant was separated and transferred to a new micro tube and equal volume of chloroform was added, centrifuged at 10,000 rpm for 5min and supernatant was separated and transferred to a new micro tube again. For alcohol precipitation, 2.5 volume of cold absolute ethanol were added and frozen in -20º C for 10 min. After freezing, samples were centrifuged at 12,000 for 12min. The precipitate was centrifuged and washed with 70% ethanol, air-dried, re-suspended in 100µl of TE (10mM Tris, 1mM EDTA) and was preserved at -20ºC until use. DNA was visualized by electrophoresis on 1% agarose gel stained with ethidium

PCR Conditions: PCR amplification of ITS1-5.8S -ITS2 rDNA spacers, was achieved using the ITS1 (forward, 5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (reverse, 5′-TCC TCC GCT TAT TGA TAT GC -3′) primer pairs (MWG-Biotech AG, Germany) to ampl ify ITS domains.
PCR amplification was performed in a final volume 50 µl .Each reaction consists of 2 µl template DNA, 0.5 µl of each primers at 25 µM, 1.25 µl of dNTP (BIORON GmbH, Germany) at 5mM, 0.5U Taq DNA polymerase (Roche Diagnostics GmbH, Mannheim,Germany) and 5µl 10×PCR. The amplification condition consists of 35cycles of denaturation at 94°C for 1min, primer annealing at 56°C for 1min, extension at 72°C for 1min. In the first cycle, the denaturation step was 94°C for 5min and in the final cycle the final extention step was 72°C for 7min. Amplified products were visualized by 1% agarose gel electrophoresis in TBE buffer. Gel was stained with ethidium bromide (0.5 µg/ml) and photographed by ultra violet photography.

Restriction Enzyme analyses: ITSI region sequences of several C. albicans and C. dubliniensis were received from NCBI's GenBank. The restriction sites of various restriction enzymes were determined by CLC workbench software (version 3) and the enzymes BlnI; (Roche diagnostics, Swiss) were selected which was similar to Mirhendi et al. study.(2) Digestion was performed by incubation 8.5 µl of PCR products with 0.5 µl of enzyme at 10U in a final reaction volume 0f 10 µl at 37ºC for 3 h. Restriction fragments were separated by 3% agarose gel in TBE buffer for 1h at 100 V. Gel was stained with ethidium bromide (0.5 µg/ml) and photographed by ultra violet photography.

Results
In this study, we apply a PCR-RFLP for distinguishing between C. dubliniensis and C. albicans using the universal primers ITSI and ITS4 for amplification the ITS region. The universal primer pair, ITS1 and ITS4 amplified DNA from all 60 clinical isolates and two standard strains and showed a unique band approximately 535bp. When the PCR products from ITS1/ITS4 amplifications were digested with BlnI enzyme C. dubliniensis and C. albicans showed different patterns of DNA fragments. The C. dubliniensis digestion produced two strong bands of about 200bp and 335bp and there were no cutting sites in C. albicans thus, only one fragment that has the same size as PCR has been created (535bp).(Figure- 1 and Table- 2)
PCR-RFLP analysis showed that all of the clinical strains were C. albicans. Results on the ITS region DNA sequence analysis did not show sequence variations comparing with NCBI GenBank and the

results were compatible with profiles which generated for the same species using RAPD(7) and had similarity when compared with two standard strains also.

Discussion

Opportunistic fungi are life-threatening infections in immunocompromised patients.(11) In recent years, Candida species have emerged as the major hospital pathogens.(12) Although Candida albicans remains the most frequently cause of Candidiasis, the incidence of the disease caused by other species of Candida that are less sensitive to azoles compounds has increased steadily.(11) Nowadays, Candida species have been identified based on routine laboratory techniques such as germ tube formation and bio chemical tests. These procedures require purification of the target organisms which are laborious and time-consuming and may not be species specific also.(13) Due to the high degree of phenotypic similarity between C. albicans and C. dubliniensis, accurate and rapid identification in routine laboratories remain problematic.(3) In this study, we apply a PCR-RFLP method using the universal primers ITS1 and ITS4 to amplify the ITS1 and ITS2 region and 5.8S in the rDNA gene and identify Candida species of suspected of C. albicans. Primarily, these strain were identified by phenotypic methods color of colony on CHROMagar Candida medium (CHROMagar, Company, Paris, France), germ-tube formation in horse serum, chlamydospore formation. Strains were identified by RAPD-PCR and sequencing definitively. Some of these strains were determined for D1/D2 region of LSU rDNA gene.(7-9) Several studies using PCR techniques together with restriction digestion enzymes for special identification of species have also expressed several techniques with universal primers for identification of various fungi have been reported.(2,14-17)

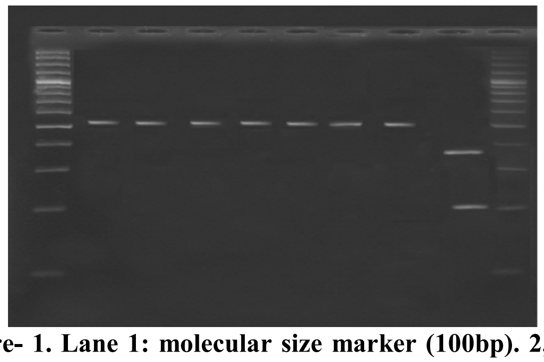

Figure- 1. Lane 1: molecular size marker (100bp). 2, 4, 6: PCR product of C.albicans. 3,5,7: digestion of PCR product of C. albicans with BlnI. 8: PCR product of C. dubliniensis. 9: digestion of PCR product of C. dubliniensis with BlnI. Lane 10: molecular size marker (100bp).

These primers have already demonstrated their efficiency.(2,14-17) In fact, they could amplify the complete part of ITS1, ITS2 and 5.8S rDNA regions and partial part of 18S and 28S rDNA and too many small fragments about 20-30bp. Consequently, using the universal primers, ITSI and ITS4, we created a fragment with variable length about 510- 879 bp of the ITS1-5.8S-ITS2 rDNA region from genomic DNA of several strains of Candida species.(8)

The standard PCR using universal primer pairs ITS1/ITS4 did not produce distinctive bands between C. albicans and C. dubliniensis and thus it is not practical to use them as a tool for species identification. The ITS DNA sequence in NCBI GenBank was clearly different and was used to select restriction enzymes that distinguish the two pathogens in disease diagnosis. PCR-RFLPusing restriction enzyme BlnI produced two fragments with C. dubliniensis and did not digest C. albicans. The PCR-RFLP is a very good tool to distinguish two pathogens (Table- 1).

BlnI makes DNA cleave where there is a CCTAGG sequence. As there is only one cutting site over ITS region of C. dubliniensis, two fragments have been created (200,340 bp). In other hand, since there is no cutting site over ITS region of C. albicans only one fragment that has the same size as PCR has been created.(Table 2)

The restriction patterns generated from ITS regions together with 5.8S rRNA genes have been strongly recommended to display underspecified differences among fungus species.(18)

Table- 1: Distribution of 60 clinical yeast isolates based on types of malignancy.

	Frequency	
Type of Malignancy	**No**	**%**
Brain tumor	**11**	**18.3**
Esophagus cancer	**11**	**18.3**
Acute Lymphoid Leukemia (ALL)	**5**	**8.3**
Breast tumor	**4**	**6.6**
Lymphoma	**5**	**8.3**
Acute Myeloid Leukemia (AML)	**2**	**3.3**
Larynx tumor	**4**	**6.6**
Neck tumor	**2**	**3.3**
Gastric tumor	**3**	**5**
A plastic Anemia	**1**	**1.6**
Lung cancer	**2**	**3.3**
Thyroid cancer	**1**	**1.6**
Liver cancer	**2**	**3.3**
Cheek tumor	**1**	**1.6**
Naso-Pharanx tumor	**2**	**3.3**
Gut tumor	**1**	**1.6**
Tongue tumor	**2**	**3.3**
Prostate tumor	**1**	**1.6**
Total	**60**	**100**

Table 2: Size of ITS1-ITS2 products for C. dubliniensis and C. albicans before and after digestion with BlnI

Candida species	Size of ITS-PCR product	Size of restriction product
C. albicans	**535**	**535**
C. dubliniensis	**535**	**200,335**

Williams et al,(19) evaluated ITS1 and ITS2 regions, together with the entire 5.8S rRNA genes. The sequence variations in the ITS regions were amplified by PCR, using primers ITS1 and ITS4. Although PCR products from both C. dubliniensis and C. albicans had been of similar size (about 540bp), sequence analysis revealed over 20 consistent base differences between the products of the two species. The restriction enzyme MspI yielded two distinct fragments from C. albicans PCR products at the same time as those from C. dubliniensis appeared undigested.(8) The same technique was used by Gee et al,(20) to confirm the existence of two distinct populations within the species C. dubliniensis, designed Cd25 group I and Cd25 group II, respectively, on the basis of DNA fingerprints generated with C. dubliniensis-specific probe Cd25. More recently, Graft et al,(18) established a PCR/RFLP-based system with amplification of regions ITS1 and ITS2 together with the 5.8S rRNA gene, followed by digestion with HpyF10VI, and separation of the DNA fragments on an agarose gel for differentiation of C. dubliniensis from C. albicans.

Based on the present and our previous studies,(9) none of clinical isolate mainly from mucus membrane of patients suffered from cancer identified as C. dubliniensis. Our results are quite consistent with Mirhendi et al.(2) They used the same approach to amplify the ITS region of for discrimination of C. dubliniensis and C. albicans although, their patient populations under study and location of lesions were different. In their study, clinical isolates which mainly originated from skin and nail of patient were suspected to superficial and cutaneous mycoses. Other researchers(21) who have used the same method noted a high prevalence of C. dubliniensis (10.9%) from complete denture wearers. Candida dubliniensis isolates have been primarily recovered from oral and mucosal surfaces, especially in HIV-positive patients. For the first time in Iran(22) Candida dubliniensis isolated (13.3%) from mucus membrane of HIV positive patient but with a different molecular methods.

However, there have been a number of recent reports of its isolation from non-HIV-positive patients.(2, 23) Mokadass et al,(24) reported an overall C. dubliniensis 4.9% among cancer patients by phenotypic and species specific and/or sequencing of ITS regions of rDNA.

However, in this study, we did not identify any C. dubliniensis among all suspected isolates despite of the capability of the method that we used, so further investigations of discrimination between these two morphologically similar species in patients suffering from cancer seem warranted.

Conclusion

In summary, PCR-RFLP of ITS regions with BlnI enzyme is easy and quick to differentiate between C. dubliniensis and C. albicans.

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