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Molecular Identification of *Candida dubliniensis* among *Candida albicans* Isolated from Oral Cavity of Cancer Patients using PCR-RFLP, in a Tertiary Care Hospital in Kashmir, India

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Authors' contributions

This work was carried out in collaboration between all authors. Author GB designed the study, wrote and standardized the protocol, supervised the work and managed literature searches. Author BAF helped in the study design and literature searches. Author AJ performed the experiments, managed the analyses of the study and literature searches, and wrote the first draft of the manuscript. Author MSL helped in collection and phenotypic identification of isolates. Author AF helped in the experiments and literature searches and author SR managed the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To retrospectively evaluate 186 stock strains of *C. albicans* strains isolated from oral cavity of HIV negative patients with various malignancies for the presence of *C. dubliniensis* isolates among them by PCR-RFLP.
Place and Duration of Study: Department of Microbiology, Sher-i-Kashmir Institute of Medical Sciences, Srinagar, between October 2013 and October 2014.
Methodology: This study included 186 stock strains of *C. albicans* tentatively identified by phenotypic methods like germ tube formation in human serum, colony color on chromogenic

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candida differential agar, characteristic morphology on corn meal agar and assimilation of sugars isolated from HIV negative patients with various malignancies. DNA extraction was performed by chemical method. PCR amplification of ITS1-5.8S-ITS2 rDNA region was achieved using the ITS1 and ITS4 primer pairs which amplify the ITS region of both species, providing a single PCR product of expected size (540 bp). There is no visible difference between these two species with regard to their ITS PCR products. Digestion of amplified products was performed by using restriction enzyme *Bln*I *(Avr*II*)* which cleaves DNA where there is a CCTAGG sequence. The products of digestion generate one band of 540 bp for *C. albicans*, and two bands of 200 bp and 340 bp for *C. dubliniensis because Bln*I has one recognition site within the ITS region of *C. dubliniensis*, whereas none within that of *C. albicans.*

Results: Of the 186 isolates tested, no *C. dubliniensis* was found by PCR-RFLP.

Conclusion: Our results of not finding *C. dubliniensis* in this subset of patients support the need for further investigations into the prevalence of this species among other clinical samples and other susceptible patient populations.

Keywords: Candida dubliniensis; BlnI (ArvII); oral candidiasis; cancer patients; PCR-RFLP.

1. INTRODUCTION

Candida dubliniensis is a novel *Candida* species first described as a distinct taxon in 1995 by Sullivan et al. in Dublin, Ireland and was subsequently named after its place of origin [1]. He identified this organism while performing an epidemiological investigation of oral candidiasis in HIV-infected and AIDS patients in the early 1990s [1,2]. The earliest known isolates of *C. dubliniensis* precede the AIDS pandemics with one isolate deposited in the Central Bureau voor Schimmel cultures in Holland as *C. albicans* in 1952 [3] and another in the British National Collection for Pathogenic Fungi as *C. stellatoidea* in 1957 [1].

Although the first isolate of *C. dubliniensis* had been recovered way back in 1950s, it was not until the late 1980s or early 1990s that the next isolates of *C. dubliniensis* were identified [3]. This clearly highlights the fact that due to phenotypic similarity with *C. albicans, C. dubliniensis* is generally misidentified. Afterwards, *C. dubliniensis* isolates were identified in a wide range of clinical settings [4]. *C. dubliniensis* is primarily associated with recurrent episodes of oral candidiasis in AIDS and HIV-infected patients. It has also been implicated in cases of superficial and disseminated candidiasis in patients without HIV infection [1]. The incidence of this yeast species is increasing whereas its epidemiology still remains to be elucidated.

Most microbiology laboratories make use of germ tube test, color on chromogenic agar and chlamydoconidia formation for routine identification of *C. albicans*. These tests giving similar results in *C. dubliniensis* may be responsible for its isolates being overlooked and misidentified. To gain a more complete understanding of the precise epidemiological role played by *C. dubliniensis* in human disease, it is essential that rapid and reliable tests for its identification be available in routine clinical microbiology laboratory. Various phenotypic characteristics that have been used for identification of *C. dubliniensis* include absence of β glucosidase activity, non-fluorescent colonies on methyl-blue Sabouraud agar, dark green colonies on CHROM agar, absence of growth at 42–45ºC and the reduction of tetrazolium salts [5]. The identification methods based on phenotypic criteria may be subject to variable expression and lead to incorrect identification of isolates [6].

Genotyping approaches are capable of detecting differences directly in the genetic information [7]. At present, the most accurate means of differentiating between *C. albicans* and *C. dubliniensis* 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. Among them, PCR-RFLP analysis is a simple and reliable one [8]. Single-enzyme PCR-RFLP using universal primers to the coding regions of fungal rRNA genes, amplifies the ITS region of both *C. albicans* and *C. dubliniensis* species and provides a single PCR product of size 540 bp. Then the enzyme *BlnI* is used to achieve the best species-specific fragment length pattern [9].

C. dubliniensis is mainly associated with oropharyngeal candidosis in HIV- infected patients although more recently; it has been isolated from oral cavity of cancer patients [10]. Keeping in view the low prevalence of HIV in Kashmir Valley this study was undertaken in cancer patients with oral candidosis. Yeast isolates recovered from oral cavity of cancer patients presumptively identified as *C. albicans* were reevaluated by genotypic method for presence of *C. dubliniensis*.

2. MATERIALS AND METHODS

A total of 500 HIV negative patients with various malignancies, admitted in SKIMS were screened for oral candidiasis/colonization in a previous study. Patients who had taken antifungal drugs in the past 4 weeks and patients screened once were excluded. A total of 186 strains of *C. albicans* tentatively identified by phenotypic methods like germ tube formation in human serum at 37℃ for 3 hrs, colony color on HiCrome Candida Differential agar (chromogenic agar from Himedia) after 24-48 hours of incubation and characteristic morphology on corn meal agar were isolated. All the test isolates were sensitive to both fluconazole and voriconazole (disc diffusion test performed according to CLSI M44-A guidelines). These test strains were held in stock collection of Mycology laboratory, Department of Microbiology, SKIMS, Srinagar. In the present study these test strains were reevaluated by PCR-RFLP for presence of *C. dubliniensis* over a period of one year starting from October 2013.

Reference strains: *C. albicans* 90028 was obtained from National culture collection of pathogenic fungi (NCCPF), Department of Medical Microbiology PGIMER Chandigarh and *C. dubliniensis* (type strain CD36) and *C. dubliniensis* (CBS 7987) were kindly provided by Dr. Ziauddin Khan (Professor and Chairman, Department of Microbiology, Kuwait University).

2.1 DNA Extraction

Before DNA extraction, both the study and control strains preserved in 10% glycerol at -70℃ were subcultured on Sabouraud's Dextrose agar and incubated at 37℃ for 24 hours. DNA extraction was performed from all the clinical isolates and standard strains by phenol chloroform method [11].

2.2 PCR Amplification

For PCR amplification reaction mixture for one sample included 40.50 µl distilled water, 5 µl Buffer (10X), 1 µl dNTPs (10 mM), 0.5 µl ITS-1 (10 µM), 0.5 µl ITS-4 (10 µM) and 0.5 µl Taq polymerase (5U/µl). To above 48 µl reaction mixture 2 µl of DNA template was added. The thermocycler was set with the following amplification parameters: Initial denaturation at 94℃ for 5 min; denaturation at 94℃ for 1 min; annealing at 56℃ for 1 min; extension at 72˚C for 1 minute for 35 cycles; final extension at 72˚C for 7 min. Amplified products were electrophoresed in 1.5% agarose in TBE buffer for 45 minutes at 100V and viewed in Gel Documentation system (AlphaImager, USA). The size of PCR products was determined directly by comparison with 100 bp molecular size marker.

2.3 RFLP Analysis

Digestion of amplified products was performed by using restriction enzyme *Bln*I (*Avr*II) [9] which cleaves DNA where there is a CCTAGG sequence [12]. Reaction mixture for one sample included 18µl distilled water, 2 µl restriction buffer (10X), 1 µl *Bln*I enzyme and 10 µl PCR mixture. The reaction mixture was mixed gently, spinned down for few seconds and incubated at 37℃ for 3 h. The reaction was stopped by incubating at 80℃ in a dry bath for 20 min. The restriction products were loaded alongside the PCR products onto 2.5% agarose gel, run in TBE buffer for 1 hour at 60 V and viewed in the Gel Documentation system.

3. RESULTS

The universal primer pairs, ITS1 and ITS4 amplified DNA from all test isolates and standard strains and showed a unique band of 540 bp [9]. When the PCR products from ITS1 /ITS4 amplifications were digested with *Bln*I enzyme, *C. dubliniensis* and *C. albicans* standard strains showed different patterns of DNA fragments. The PCR product of *C. dubliniensis* on digestion produced two strong bands of about 200 bp and 340 bp and that of *C. albicans* produced only one fragment that had same size as 540 bp [9] PCR product (Fig. 1). PCR-RFLP analysis of all the clinical strains showed that they were *C. albicans* (Fig. 2).

4. DISCUSSION

Molecular methods with high discriminatory power are required for reliable identification of

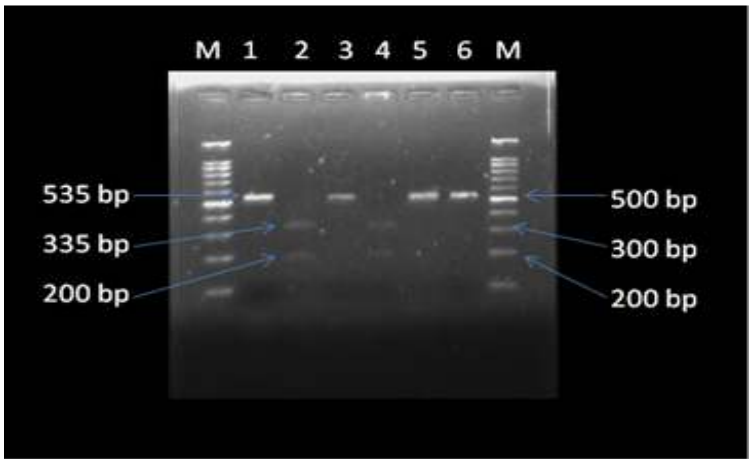

Fig. 1. Reference strains. Lane M: 100 bp DNA ladder. Lanes 1, 3: PCR products of *C. dubliniensis,* Lanes 2, 4: RFLP products of *C. dubliniensis,* Lane 5: PCR product *C. albicans,* Lane 6: RFLP product of *C. albicans*

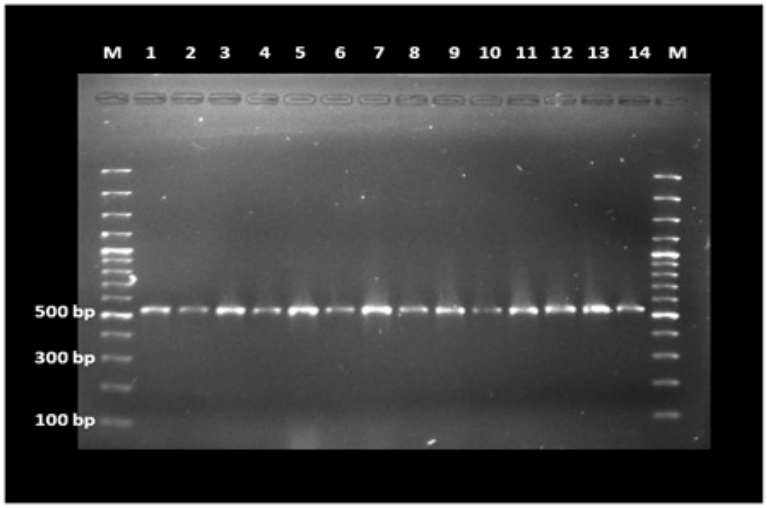

Fig. 2. Lane M: 100 bp DNA ladder, Lanes 1,3,5,7,9,11,13: PCR products of test strains, Lanes 2,4,6,8,10,12,14: RFLP products of test strains

Candida at the species level [12]. Various molecular techniques have been developed such as DNA fingerprinting with repetitive-sequence containing DNA probes, pulsed-field gel electrophoresis, real-time PCR assay and others. Most of these methods are expensive and need skilled workers trained in such techniques [13]. It is documented that PCR-RFLP is a simple, sensitive, specific, fast and cost-effective method for detection and differentiation of the medically important Candida species [8] and is considered the ‘‘gold standard’’ for identification of *C. dubliniensis* and discriminating it from *C. albicans* [14].

Several PCR-RFLP assays using different targets and restriction enzymes have been described so far to discriminate between *C. dubliniensis* and *C. albicans* [8]. In the present study ITS region of rDNA (ITS1-5.8S-ITS2 rDNA) was amplified using universal primers ITS1 and ITS4 which produced a single PCR product of 540 bp in both species. Restriction enzyme analysis of the PCR products was done by *Bln*I (*Avr*II) producing two strong bands in *C. dubliniensis* (200 bp, 340 bp) and only one in *C. albicans* (540 bp) [9].

For differentiating between *C. albicans* and *C. dubliniensis* by PCR-RFLP, some authors

have used three [15] while others have used two enzymes [8,16] utilizing different targets; however use of additional enzymes adds to the cost of the test. Single enzyme has been preferred by most of the researchers who have used different combination of targets and enzymes [9,17-19]. ITS region as a target for PCR is popular in most of the laboratories because it facilitates the identification of most medically important fungal species including *Candida*. In addition, this region offers distinct advantages over other molecular targets of increased sensitivity due to the existence of approximately 100 copies per genome [20]. Also these general primers are available in most molecular mycology laboratories.

The approach of PCR-RFLP used in our study for differentiating between *C. albicans* and *C. dubliniensis* has been reported as accurate, simple and rapid by Mirhendi SH et al. [9] and Shokohi T et al. [12] Our results were stable after both repeated sub-culturing and storing the isolates for one year in contrast to six months as reported by Williams et al. [12]. In our study PCR-RFLP could be completed in 8 h as against 7 h reported by Vijayakumar R et al. [11]. However the whole process of recovering DNA from the growth followed by PCR-RFLP took two work days which was similar to most of our phenotypic methods. The time to identification can further be reduced by faster extraction of DNA from isolates using commercial kits, [21] and by other DNA amplification techniques like multiplex PCR [22], and RAPD methodologies [23].

Of the 186 test isolates recovered from oral cavity of HIV-negative cancer patients, none was found to be *C. dubliniensis* by PCR-RFLP. Zero prevalence of *C. dubliniensis* by PCR-RFLP observed in our study is similar to that reported by many authors. Shokohi et al. (2010, Iran) [12] after reevaluating 138 *C. albicans* isolated from lip, throat and tongue of cancer patients found no *C. dubliniensis*. Similarly, no *C. dubliniensis* was found by Tekeli A et al. (2002, Ankara) [24] who investigated the colonization rates of fungal species in the oropharyngeal samples from cancer patients. Lattif AA et al. (2004, New Delhi, India) [25] did not isolate any *C. dubliniensis* from 125 HIV-positive patients screened for oropharyngeal candidiasis. Wadhwa A et al., (2007, North India) [26] studied AIDS-related opportunistic mycoses seen in a tertiary care hospital but none of the isolates was *C. dubliniensis*. None of 140 clinical isolates from skin and nail of patients suspected of superficial and cutaneous mycosis were identified as *C. dubliniensis* by Mirhendi SH et al (2005, Iran) [9]. Other authors who reported no *C. dubliniensis* from various specimens and different patient populations include Thierry K. et al. (2014, Cameroon) [27] Zaini F et al. (2006, Iran) [28] Shan Y et al. (2014, China) [29] and Jain S et al. (2014, Western India) [30].

There can be many explanations for absence of *C. dubliniensis* in our study. *C. dubliniensis* has been reported more frequently among HIV-positive patients, patients with recurrent oropharyngeal infection and patients who have already received antifungal treatment. The different composition of our study material which included HIV-negative patients, and excluded patients who had taken antifungal drugs in the past 4 weeks and who were screened once could be one of the factors. The probability of finding *C. dubliniensis* is more in azole resistant *C. albicans* but all the test isolates in our study were sensitive to both fluconazole and voriconazole. In our hospital, overall resistance of *C. albicans* isolated from different clinical samples to azoles is low.

There have been reports of mixtures of *C. albicans* and *C. dubliniensis* from oral cavity of head and neck cancers [31]. Since routinely we incubate HiCrome only for 48h, when both species produce light blue green coloured colonies, the possibility of mixtures having been overlooked at the time of initial isolation of our test strains from clinical samples cannot be ruled out.

Determination of accurate measurements of the incidence of this *Candida* species and its precise role in disease has intrigued researchers in the last decade who have reported varied prevalence from different geographical locations and patient populations. In these studies different methodologies have been adopted for identification of *C. dubliniensis*. Determination of accurate prevalence of *C. dubliniensis* is crucial for the actual epidemiology of this organism which is possible only by identification methods that are 100% accurate, PCR-RFLP being one of them.

5. CONCLUSION

Our results of not finding *C. dubliniensis* in this subset of patients support the need for further investigations into the prevalence of this species among other clinical samples and other susceptible patient populations in Kashmir.

ETHICAL APPROVAL

The study was approved by the Institutional Ethics Committee of Sher-i-Kashmir Institute of Medical Sciences, Srinagar, Kashmir, India.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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