ORIGINAL RESEARCH

Candida dubliniensis: The New Culprit on the Block Causing Denture Stomatitis? An *In Vivo* Study

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Abstract

Aim: The present study aimed to carry out DNA extraction and polymerase chain reaction (PCR) amplification to isolate the physiological and phenotypic traits of *Candida albicans* and *Candida dubliniensis* in denture wearer patients with and without denture-induced stomatitis.

Materials and methods: A total sample size of 160 participants were divided into two equal groups (80 each), patients in the study group having 40 males and 40 females with Newton type II denture stomatitis, and in the control group, healthy 40 males and 40 females those who wear complete denture were selected.

All the samples were collected from the hard palate with a sterile swab and inoculated on CHROM agar plate; samples that displayed dark green colored colonies were selected for DNA extraction. DNA isolation was done on agarose gel using electrophoresis. Biorad gene identification was used. Strands depicting the presence of DNA in particular samples were identified, and further standardization of the procedure was done. PCR amplification was done using *Candida* species-specific primer, preset to the hyphal wall of the protein 1 gene with the CRR forward and reverse primers, under strict standard conditions with reverse transcriptase technique.

Results: Results showed that prevalence of *C. albicans* was more in females with denture stomatitis which was 67.50% than in males, i.e., 52.50%, and prevalence of *C. dubliniensis* was found in one female and in one male who were having denture stomatitis and it was not isolated from patients without denture stomatitis. Statistical analyses were performed using the Chi-square test.

Conclusion: Denture stomatitis is the most common problem faced by long-term denture wearers, with *C. albicans* as one of the causative organisms. However, recent findings show an emerging pathogenic yeast species, *C. dubliniensis*, which was isolated from denture-induced stomatitis candidates in the present study, which is closely related to the *C. albicans* species. The identification of candidal strains causing denture stomatitis with DNA extraction and PCR amplification and its management by determination of its susceptibility to antifungals may improve the treatment outcome of the same.

Clinical significance: Candidiasis is the most frequently seen mucocutaneous infection of the oral cavity especially in denture wearers. It is caused mainly by the genus *Candida*. *C. dubliniensis* is phenotypically similar but genotypically different from *C. albicans*. This affects the treatment outcome drastically as there is enough literature suggesting resistance to the common antifungal drugs. Hence, drugs like fungus-specific calcineurin inhibitors should also be considered in resistant patients. Therefore, DNA identification of *Candida* genus plays a major role in deciding the treatment outcome.

Keywords: *Candida albicans, Candida dubliniensis*, DNA extraction, PCR amplification.

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Introduction

Candida or oral candidiasis is a frequently presenting mucocutaneous pathology of the mouth, produced by *Candida* species (occurring almost 53% times in the general population as a common oral commensal).[1] One hundred and fifty different species have been found in the oral cavity, 80% of which correspond to *Candida albicans*, which colonize the oral cavity solo or in conjugation with other species.[1]

Candida dubliniensis is phenotypically similar to but genotypically distinct from *C. albicans*.[2] It is found as a minor constituent of the oral microflora. Previously, *C. dubliniensis* was misdiagnosed for *C. albicans*, as both the species produce chlamydospores and are germ-tube positive.[2]

C. dubliniensis was mainly isolated from the individuals who were immunocompromised or from the mouths of 18% of the patients with diabetes and who use insulin.[3] A study done by Christian, Oscar, and Mosca has isolated *C. dubliniensis* from a teenager wearing an orthopedic oral prosthesis without any underlying medical history.[4]

A study done by Loster JE, Wieczorek A, and Loster BW concluded that males were less prone to infection than females. Strong, intermittent, and frequent growth of yeast occurred most frequently in the youngest group of females.[5,6]

A study done by Gasparoto et al. also has shown a higher incidence of *C. albicans* (89.2%) from denture stomatitis patients whereas *C. dubliniensis* prevalence in low rates i.e., 5.3% in patients

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without denture stomatitis and 10.3% with denture stomatitis patients. *C. dubliniensis* and *C. albicans* occurred in 10% of denture-related stomatitis patients.[7]

C. dubliniensis shows similar phenotypic and physiological traits like *C. albicans* which forms a vital aid for its virulence.[8-11]

The association of *C. dubliniensis* and denture-related stomatitis is suggestive of its role in the institution and persistence of denture-related stomatitis.[12,13]

Denture insertion causes the formation of plaque, increasing the population of potentially pathogenic *Candida* species and bacteria.[14-16]

Biofilms are formed over denture surfaces by *Candida* species and, therefore, are found more frequently in denture plaque than in dental plaque.[17-19]

Microbes from the oral cavity colonize the denture surface to form a sticky biofilm which is dependent on the species of *Candida*, denture surface, and oral hygiene of the individual.[20,21]

Dentures made from synthetic materials like PMMA are porous in nature and, hence, cause *Candida* to readily stick and colonize. Additionally, different host factors such as food, immune response, surface characteristics, denture cleansers, cleaning modalities, saliva with food, age, hormonal imbalance, and other risk factors facilitate the adherence and colonization of the organism.[22]

Plaque, stain, and calculus accumulate on dentures in a similar manner to natural teeth.[23] The subsequent proliferation of oral microbes and the formation of plaque on nonshading surface may initiate pathogenesis from the oral to systemic front.[24]

The association of *C. dubliniensis* and denture-related stomatitis suggests that it may play a major role in causing denture-related stomatitis.[6-8] Hence, this particular study was undertaken to isolate *C. albicans* and *C. dubliniensis* from denture stomatitis patients and healthy denture wearers.

Materials and Methods

A thorough and detailed presentation of the study was presented to the institutional ethics committee and after approval, a total of 160 study participants were selected over a period of 3 years, based on the sample size calculation done by using the basic sample size estimation formula with the level of significance at 0.05, from the Department of Prosthodontics and crown and bridge, KLE Vishwanath Katti Institute of Dental Sciences, Belgaum, Karnataka, India, who were completely edentulous and wearing complete heat cure acrylic resin dentures. A thorough intraoral clinical examination was done, including relative past medical and dental history.

Inclusion Criteria

- *Study group*—Total of 80 participants with age-group of 44 to 94 years including 40 males and 40 females having Newton type II denture stomatitis who were wearing dentures for past 2 years and at least 12 hours a day, were selected.
- *Control group*—Total of 80 participants with age-group of 44 to 94 years including 40 males and 40 females who were healthy and wearing heat cure acrylic resin complete dentures without denture stomatitis were selected.

Exclusion Criteria

- History of smoking and alcoholism
- Type 1 and Type 2 diabetes
- Systemic diseases
- Any diseases and medications that cause immunosuppressant
- Autoimmune diseases
- Recent history of broad-spectrum antibiotics
- Corticosteroids and antifungal therapy

After all study participants signed informed consent, samples were collected from the hard palate of all participants with a sterile swab, and a microbiological study was carried out accordingly. Samples were inoculated on CHROMagar plates to identify candida species. Only the samples that displayed light or dark green colored colonies were selected.[9,10]

Extraction of DNA

C. albicans and *C. dubliniensis* were differentiated using the technique put forth by Romeo et al.[11] CHROMagar plates were used to grow yeast bacteria to form green colonies to maintain over the Sabouraud's dextrose agar for performing DNA purification, at room temperature, as shown in Figure 1.[11] Washing of the formed yeast was carried out at four degrees Celsius for 5 minutes at 10,000 rpm. 0.1 mL DNA/RNA free water was used to suspend the pellet and incubated at 56°C for 30 minutes. Boiling of the samples was then carried out for 10 minutes followed by centrifugation at 4°C for 5 minutes at 13,000 rpm.[12] To perform the analysis of the DNA of *C. albicans* or *C. dubliniensis*, supernatants were obtained accordingly.

The supernatant was then mixed with ethidium bromide dye and DNA isolation on agarose gel using electrophoresis, as shown in Figure 2. Bio-Rad gene identification was used. Strands depicting the presence of DNA, in particular, samples were identified and further standardization of the procedure was done.[11]

Amplification of *C. albicans* and *C. dubliniensis* Species Using PCR

Samples were amplified using the reverse transcriptase technique under standard conditions using CRR forward primer, 59-GTTTTTGCAACTTCTCTTTGTTTA-39 and CRR reverse primer, 59-ACAGTTGTATCATGTTCAGT-39. A total volume of 25 mL of the polymerase chain reaction (PCR) mixture containing 1.5 mM $MgCl_2$, 2.5 U GoTaq polymerase, 0.2 mM each deoxynucleoside triphosphate (IDT primer), 3 mL genomic DNA template, and 0.4 mM each primer. Denaturation was carried out at 95°C for 5 minutes after amplification, following which denaturation for 34 cycles was carried out at for 45 seconds at 94°C, followed by primer annealing

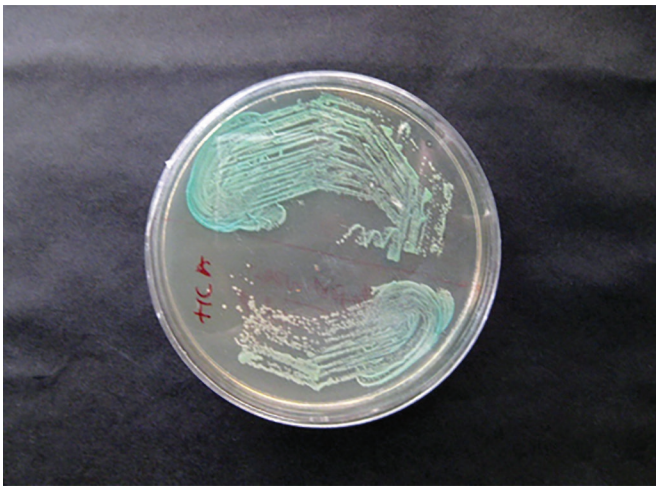

Fig. 1: Green color colonies on CHROMagar plates

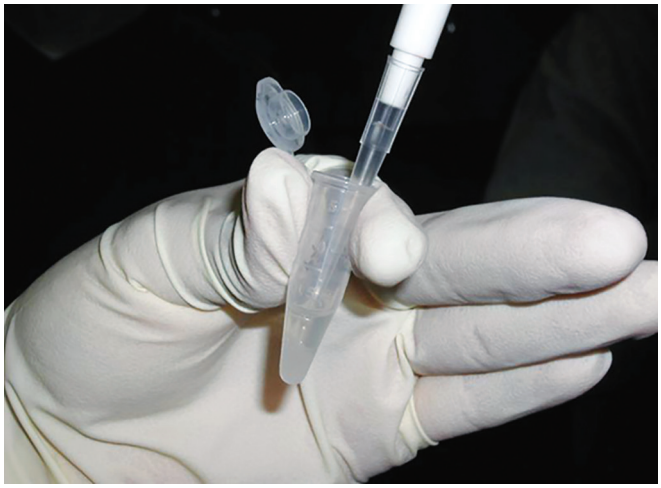

Fig. 2: Supernatant obtained for analysis of *Candida albicans* or *Candida dubliniensis* DNA

for 60 seconds at 50°C, which is extended for 90 seconds at 72°C, with a final extension for 10 minutes at 72°C in a thermocycler (Progene; Techne). Separation of PCR products was carried out on a 1.5% (w/v) agarose gel, followed by staining with 0.5 mg mL ethidium bromide and comparison with DNA size marker (100 Base-Pair Ladder; Amersham Biosciences) with a transilluminator (Sigma T2202). The expected amplicons for *C. albicans* was 1180 bp and for *C. dubliniensis,* it was 930 bp.

Results

The data collected was tabulated, and statistical analysis was carried out in SPSS 25 software using the Chi-square test.

When compared the study group to control group, the presence of *C. albicans* was more i.e., 60% in the study group and it was 30% in the control group; whereas the presence of *C. dubliniensis* was 2.50% in the study group and it was null in the control group as shown in Tables 1 and Table 2

Table 1: Comparison of study group and control group with respect to status of presence of *Candida albicans*

C. albicans	*Study group*	*%*	*Control group*	*%*	*Total*
Present	48	60.00	24	30.00	72
Absent	32	40.00	56	70.00	88
Total	80	100.00	80	100.00	160

Chi-square = 14.5451, $p = 0.0001$*
*$p < 0.05$

Table 3: Comparison of study group and control group in male samples with respect to the status of presence of *Candida albicans*

C. albicans	*Study group (male)*	*%*	*Control group (male)*	*%*	*Total*
Present	21	52.50	9	22.50	30
Absent	19	47.50	31	77.50	50
Total	40	100.00	40	100.00	80

Chi-square = 7.6801 $p = 0.0060$*
*$p < 0.05$

When comparison was done between males of study group and control group, it was 52.50 and 22.50%, respectively, found positive for *C. albicans*. Here, it suggests that a patient with denture stomatitis was found more for *C. albicans*. In comparison between males of both the groups, the study group showed 2.50% positive for *C. dubliniensis* and null for the control group; i.e., it was found in one male having denture stomatitis as shown in Tables 3 and Table 4. In comparison between females for the occurrence of *C. albicans*, it showed 67.50% prevalence in study group which was higher than control group, i.e., 37.50% and as compared to males also it was higher in prevalence as shown in Table 5.

In comparison between females of both the groups for occurrence of *C. dubliniensis*, it showed 2.50% prevalence and it was null in control group as shown in Table 6. Here, it suggests that *C. dubliniensis* was found in one female with denture stomatitis.

Hence, results showed that the prevalence of *C. albicans* was more in females with denture stomatitis which was 67.50% than in males i.e., 52.50% and prevalence of *C. dubliniensis* was found in one female and in one male who were having denture stomatitis and it was not isolated from patients without denture stomatitis.

Discussion

The oral cavity is an amalgamation of various strains and colonies of *Candida* species, which are dynamic in nature and keep changing with time and age. *C. albicans* species have been the most predominant strains known to cause infection in elderly individuals wearing dentures due to various factors like type of denture base materials, surface roughness, surface energy, etc.[12,13] However, the literature also shows other strains of *Candida* like *C. dubliniensis*, *Candida glabrata,* and *Candida tropicalis* to play a role in the occurrence of denture-induced stomatitis.[15,17,18] Few studies have identified *C. dubliniensis* from the palatal mucosa and samples taken from dentures from patients with denture-related stomatitis, whereas these micro-organisms were not found in individuals without denture-related stomatitis.[14,20,21,23] On the contrary, in the present study, few strains of *C. dubliniensis* were present in study participants with denture-related stomatitis.

Table 2: Comparison of study group and control group with respect to the status of presence of *Candida dubliniensis*

C. dubliniensis	*Study group*	*%*	*Control group*	*%*	*Total*
Present	2	2.50	0	0.00	2
Absent	78	97.50	80	100.00	158
Total	80	100.00	80	100.00	160

Chi-square with Yates's correction = 0.5061 $p = 0.4772$
*$p < 0.05$

Table 4: Comparison of study group and control group in male samples with respect to the status of presence of *Candida dubliniensis*

C. dubliniensis	*Study group (male)*	*%*	*Control group (male)*	*%*	*Total*
Present	1	2.50	0	0.00	1
Absent	39	97.50	40	100.00	79
Total	40	100.00	40	100.00	80

Chi-square with Yates's correction = 0.0000 $p = 1.000$
*$p < 0.05$

Table 5: Comparison of study group and control group in female samples with respect to the status of presence of *Candida albicans*

C. albicans	*Study group (female)*	*%*	*Control group (female)*	*%*	*Total*
Present	27	67.50	15	37.50	42
Absent	13	32.50	25	62.50	38
Total	40	100.00	40	100.00	80

Chi-square = 7.2182 $p = 0.0071$*
*$p < 0.05$

Table 6: Comparison of study and control groups in female samples with respect to the status of presence of *Candida dubliniensis*

C. dubliniensis	*Study group (female)*	*%*	*Control group (female)*	*%*	*Total*
Present	1	2.50	0	0.00	1
Absent	39	97.50	40	100.00	79
Total	40	100.00	40	100.00	80

Chi-square with Yates's correction = 0.0000 $p = 1.000$
*$p < 0.05$

C. dubliniensis has been recovered from various anatomical oral sites from healthy individuals but has also been found to be mainly associated with HIV-infected individuals. The relation between *C. dubliniensis* and denture stomatitis shows that this microorganism may be important in causing denture stomatitis, as seen in the present study. In recent times, rapid and reliable identification of *C. dubliniensis* in clinical samples is carried out by the use of a number of phenotype based molecular tests.

In the present study, the most predominant species i.e., 58.9% were *C. albicans*, *C. tropicalis* (15.1%), *Candida guillermondi* (13.7%), *C. glabrata* (9.6%), and *Candida parapsilosis* (2.7%) in participants without denture stomatitis. In study participants with denture stomatitis, *C. albicans* was found in (73.4%), *C. glabrata* (8.9%), *C. tropicalis* (8.9%), and one isolate for *C. dubliniensis*. Present study shows isolation of *C. dubliniensis* only from the patient having denture stomatitis which is in contrast with the study done by Newton,[15] which shows the presence of *C. dubliniensis* in complete maxillary denture wearers with/without denture-related stomatitis and has denied its role in the occurrence of denture-related stomatitis; therefore, more research is needed to discern the influence of *C. dubliniensis* in the oral environment of the systemically healthy individuals including those wearing dentures.

Conclusion

Denture stomatitis is a trivial problem of the denture wearers, the cause of which includes infection, trauma, and probably a breach in the host immune response. Current ideology suggests the interplay of most of these factors in the occurrence of the disease. The extent of the interplay of these factors is still a question. *C. albicans* has been suspected to be the causative organism. However, from recent research, it is arguable if it is the only causative organism. Recently, strains resistant to antifungal treatments have been recorded. In such cases, other microorganisms have also been found. The treatment of oral candidiasis is carried out using antifungal agents like nystatin and amphotericin B, and other most commonly used drugs like itraconazole, fluconazole, and miconazole. However, some *Candida* species, such as *Candida krusei* and *C. glabrata*, are less effective to the antifungal agents and can acquire resistance.

C. dubliniensis is an evolving pathogenic yeast similar to *C. albicans* known to frequently cause and colonize the oral cavity of patients with HIV/AIDS. The use of antifungal drugs which do not cause any drug resistance is of prime importance against the *Candida* species. The FK506, an inhibitor for calcineurin shows a significant antifungal effect to *C. albicans, Cryptococcus neoformans* and *Aspergillus fumigatus*, when used with echinocandins or azoles. Thus, a combination therapy using azoles and echinocandins can be tried and tested against the emerging *C. dubliniensis* infections. The identification of candidal strains with DNA extraction and PCR amplification and its effectiveness to antifungals may improve the treatment outcome of denture stomatitis caused due to *Candida* species.

Limitations of the Study

Although a lot of studies have shown the interaction of *Candida* species in the formation of biofilms, little or no evidence is present to show the interaction of *C. dubliniensis* and non-*Candida* species. So, further research is required to determine the same. Also, other factors like the type of denture base material used, surface roughness, surface energy, etc. were not considered in the present study, which might play a role in the colonization of the *Candida* species.

Clinical Significance

Although *C. dubliniensis* is genotypically different from *C. albicans*, its prevalence has been underestimated because of similar physiological and phenotypic traits. Both organisms show light green to dark green colonies on CHROMagar. With limited evidence on the occurrence and severity of its effects, it becomes prudent to conduct further research to delineate the effects of *C. dubliniensis* in the oral cavity.

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