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Araştırma/ Research Article

THE EVALUATION OF THE COLONIZATION AND BIOFILM FORMATION CAPACITY OF THE CANDIDA SPECIES ISOLATED FROM DENTURE WEARER PATIENTS

HAREKETLİ PROTEZ KULLANAN HASTALARDA KANDİDA TÜRLERİNİN KOLONİZASYONUNUN VE BİYOFİLM OLUŞTURMA KAPASİTELERİNİN

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ABSTRACT

Aim: Candida species, although being a member of the oral flora, may exhibit pathological features under certain conditions. The aim of this study was to determine the distribution and biofilm production of Candida species that were isolated from denture related stomatitis (DRS) patients and to compare with healthy subjects.

Materials and Methods: The 56 non-smoker, systemically healthy, maxillary removable denture wearer subjects included in this study and diagnosed as DRS(N=27) and healthy(N=29). Samples from the palatal mucosal surface of patients were collected by sterile swabs during 20 seconds. Cultivation and selective isolation of Candida species were performed on CHROMagar (CHROMagar® Candida, CHROMagar, Paris, France) at 37ºC for 2 days. Isolated Candida species were identified with API ID 32 C (bioMérieux®, France). Biofilm formation by Candida species were determined by a visual tube method.

Results: The prevalence of the yeasts in the healthy group was found to be 37.94%, while in the DRS group it was 100% (p<0.001). The isolation rate of *C. albicans* in patients with DRS significantly higher (p<0.001). Biofilm formation was observed in a total of 37 oral yeast isolates, 9 isolates from healthy den- ture wearers and 28 isolates from DRS patients. The number of *C. albicans* and *C. glabrata* isolates showing biofilm formation ability in the DRS group was significantly higher than the healthy group (p<0.05).

Conclusions: Within the limitations of this study, our results suggest that Candida species play a major role on the development of DRS. While *C. albicans* was the most isolated species in DRS, *C. glabrata* was as important as *C. albicans* on the pathogenesis of DRS.

Keywords: Biofilms, Candida, Denture stomatitis, Mycology, Oral pathology

ÖZ

Amaç: Candida türleri, oral floranın doğal üyesi ol- makla birlikte, belirli koşullar altında patolojik özellikler sergileyebilir. Bu çalışmanın amacı, protez stomatiti (PS) olan hastalardan ve protez kullanan sağlıklı bireylerden elde edilen Candida türlerinin dağılımını ve biyofilm üretime kapasitelerini karşılaştırmaktır.

Gereç ve Yöntem: Sigara içmeyen, sistemik olarak sağlıklı, maksiller tam damak protez kullanan 56 kişi bu çalışmaya dahil edildi. Katılımcılar PS (N = 27) ve sağlıklı (N = 29) olmak üzere gruplandırıldı. Hastaların palatal mukoza yüzeyinde 20 saniye boyunca steril *swab*lar gezdirilerek sürüntü örnekleri elde edildi. Candida türlerinin kültivasyon ve seçici izolasyonu 2 gün boyunca 37°C'de CHROMagar (CHROMagar® Candida, CHROMagar, Paris, Fransa) besiyerinde ger- çekleştirildi. İzole edilen Candida türlerinin identifikas- yonu API ID 32 C (bioMérieux®, Fransa) ile gerçekleş- tirildi. Candida türlerinin biyofilm oluşturma potansi- yelleri görsel tüp yöntemi ile saptandı.

Bulgular: Sağlıklı gruptaki mayaların prevalansı % 37.94 iken, PS grubunda % 100'dü (p <0.001). PS'li hastalarda *C. albicans*'ın izolasyon oranı anlamlı dere- cede daha yüksekti (p <0.001). Sağlıklı katılımcılardaki 9 PS katılımcılardaki 28 izolatta biyofilm oluşumu gözlendi. PS grubunda biyofilm oluşumunu gösteren *C. albicans* ve *C. glabrata* izolatlarının sayısı sağlıklı gruba göre anlamlı olarak yüksekti (p <0.05).

Sonuç: Bu çalışmanın sınırları dahilinde, sonuçlarımız Candida türlerinin PS'nin gelişiminde önemli bir rol oynadığını göstermektedir. *C. albicans*, PS'de en sık izole edilen tür olsa da *C. glabrata* da PS'nin patoge- nezinde *C. albicans* kadar önemlidir.

Anahtar Kelimeler: Biyofilm, Kandida, Protez stomatitisi, Mikoloji, Oral patoloji

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INTRODUCTION

Denture-related stomatitis (DRS) is a common erythematous inflammatory response that is generally seen on the oral mucosa underlying removable dentures.[1-3] Various factors may play role in the aetiology of DRS which can be listed as age, systemic diseases that affect immune response such as diabetes mellitus and HIV infection etc, salivary flow rate, prolonged usage of dentures, unhygienic and old dentures.[4-7] Furthermore, *Candida* species, as members of the flora on skin and mucosa, were shown to be as causative factors in DRS.[8] Polymethyl metacrylate resin is preferred to fabricate the denture base generally. And due to porous structure of this material fungal colonization can be easier.

Although *Candida albicans* is the most commonly isolated yeast from the DRS patients[9], non-albicans species such as *Candida glabrata*, *Candida tropicalis*, *Candida kefyr*, *Candida parapsilosis, Candida krusei* and *Candida dubliensis* may be present in the oral cavity of these patients.[1,8,10] Virulence factors of *Candida* species may play a significant role in this diversity. Phospholipase and proteinase activities are the well-known virulence factors of the *Candida* species.[1,8] Besides these hidrolytic enzymes, Candida species could obtain the ferritine from the host due to their hemolytic potentialsand metabolize it for own homeostasis.

Another pathogenic capability of *Candida* species is the biofilm formation capacity.[11] Biofilm is described as surface-attached microbial community and mutualistic interactions occur between the members of this community.[12] Interactions among the members of a biofilm is called as "quorum sensing" and this plays a crucial role for the maintenance of nutritional chain within biofilm. Slime layer, the exterior layers of *Candida* cells is crucial for adherence to host surfaces and has an important role in the formation of biofilm structure. Mutualistic life form in the biofilms provides environmental protection, access to nutrients, metabolic co-operations and sharing of genetic traits to their members. The ability of the biofilm structure to reduce the success of antifungal therapies can be shown an example to this phenomenon.[13] Studies showed that *Candida* biofilms are not innocent formations, they act like a reservoir for the infections ranging from superficial to invasive systemic candidiasis such as gastrointestinal tract infections, pneumonia and intravascular device-related infections.[14-16] *Candida albicans* and its pathogenicity have been reported many times in literature[1,17], but the effects of non-albicans species in the pathogenesis of DRS are still not fully clarified.

The objective of this study was to evaluate and compare the colonization and biofilm formation capacity of the *Candida* species isolated from denture wearer patients with DRS and healthy controls**.**

MATERIALS and METHODS

Subjects and Clinical Examination

The protocol of this cross-sectional study was approved by XXX with the reference number B.30.2.AYD.0.00.00-480.2/193. This research was conducted according to the principles outlined in the Declaration of Helsinki on experimentation involving human subjects.

The 56 maxillary removable denture wearer subjects (21 male/ 35 female) included in this study were recruited from Periodontology and Oral and Maxillofacial Radiology Departments of Faculty of Dentistry, Istanbul Aydin University. A detailed medical and dental history was obtained from all participants. Clinical parameters including number of teeth, plaque index (PI)[18], bleeding on probing (BoP), probing depth (PD) and clinical attachment level (CAL) were recorded at baseline. While PD was defined as the distance from the free gingival margin to the bottom of the periodontal pocket, the distance from the cementoenamel junction to the bottom of the periodontal pocket was recorded as CAL. All clinical examinations were carried out by a single examiner (S.E.M.). To achieve the intra-examiner calibration, 3 non-study subjects were selected and full mouth PD scores of them were recorded twice within 5 days. The intra-examiner correlation was calculated as 96.2% reproducibility.

The sample size was calculated on the basis of a similarly designed study.[1] Assuming that a sample of 25 participants per group would provide 90% power to detect a true difference between the groups.

While 27 of these subjects were diagnosed as having Type II DRS according to Newton's classification[1], 29 did not have any DRS symptoms. All

subjects were using their dentures for at least 12 months. Criteria for exclusion from the study were as follows: (a) any systemic disease (i.e. diabetes mellitus, HIV infection, anaemia) and (b) use of prolonged antibiotics or steroids that might promote oral candidiasis, (c) history of candidiasis and antifungal medications, (d) smoking, (e) any physical limitations or restrictions that might preclude normal oral hygiene procedures. Informed consent was obtained from all individual participants included in the study.

Obtaining Oral Samples and Cultivating

To ensure the standardization of sampling, all samples were carried out early in the morning at least two hours after food and beverage consuming, or any oral hygiene procedures.[1] Oral swab samples were obtained from the normal palatal mucosa of healthy individuals whereas from the affected mucosa in patients with DRS by rubbing a sterile cotton swab several times along the surface for 20 seconds (Figure 1). All samples were cultivated on CHROMagar (CHROMagar® Candida, CHROMagar, Paris, France) for mycological examination (Figure 2). Following the incubation of plates at 37°C for 48 hours, identification of the isolates were performed by morphological procedures such as chlamydospore production on corn meal agar supplemented with 1% Tween 80 and carbohydrate assimilation pattern via commercially available API ID 32°C system (Biomerieux, Marcy I'Etoile, Paris, France)

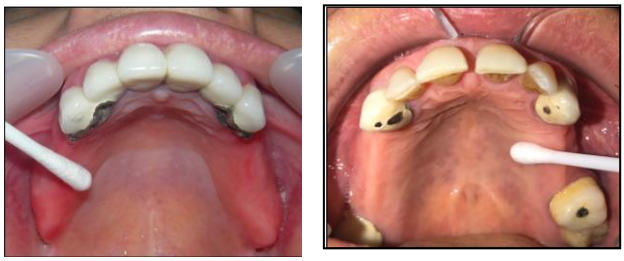

Figure 1. Obtaining of samples from the palate of patients in each group via sterile swab

Figure 2. Candida colonies on chromogenic Candida agar

Determination of the Biofilm Formation

Visual methods were used to determine the biofilm formation (Figure 3). Isolates grown on the Sabouraud's dextrose agar plate were inoculated into a polystyrene tube contained 10 ml of glucose supplemented with Sabouraud-dextrose broth that the final concentration was 8%. Following the incubation period of 48 h at 35°C, the broth was gently taken out from the tubes and the tubes were washed with distilled water for two times. Additionally, 2% safranin was applied to stain the tube for 10 min to examine the presence of the adherent layer. Biofilm formation capacity was scored as negative (-), weak (+), moderate (++) and strong (+++). As a positive control, the biofilm producer, Staphyloccoccus epidermidis ATCC35984 was used.[19]

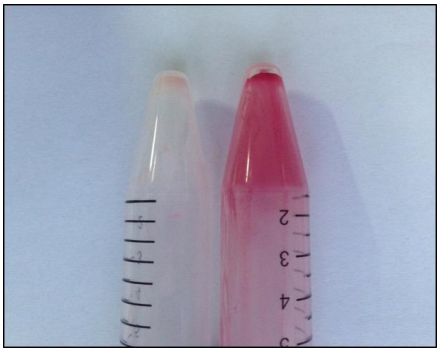

Figure 3. Biofilm formation by Candida species

Statistical analyses

The statistical analysis was performed using a computer-run statistical programme, SPSS 20 (SPSS Corporation, Chicago, USA). Mean and percentages were given for the distribution of the *Candida* species.

Chi-square test was used to determine the differences in biofilm formation capacity of the isolates. Results were calculated within 95% confidence intervals with the significance level set at p<0.05.

RESULTS

A total of 56 denture wearer subjects were included in this study and grouped according to the clinical signs of DRS as healthy (n=29) or not (n=27). Mean ages of healthy and DRS groups were 65.25±7.87 and 65.13±7.74, respectively (p>0.05). While female/male ratio of the healthy group was 2,37, female/male ratio of DRS group was 1,23 (p<0.05). The mean duration of denture use in the DRS group (10.11±6.41years) was significantly higher in comparision to the healthy group (6.11±4.17 years) (p<0.05).

Table 1 displays findings about clinical parameters at baseline. No difference was observed between the groups regarding any clinical parameters (p>0.05).

Table 1. Data about clinical parameters

	Healthy Group (Mean±Sd)	DRS Group (Mean±Sd)	p*
Number of teeth	11.24±1.22	10.12±1.32	NS
PI	1.25±0.73	1.54±0.88	NS
BoP (%)	54.45±10.72	60.05±11.02	NS
PD (mm)	4.25±1.94	5.05±1.08	NS
CAL(mm)	5.33±0.98	6.01±0.78	NS

Mean±Sd:Mean Mean±Standard deviation DRS: denture-related stomatitis, PI:Plaque index, BoP:Bleeding on probing, PD: Probin depth, CAL: clinical attachment level, mm:milimeter, *Mann Whitney U test, NS: nonsignificant, p<0.05

The prevalence of the yeasts in the healthy group was found to be 37.94%, while in the DRS group it was 100% (p<0.001). *C. albicans* was the most frequently isolated species in both groups with the isolation rate of 51.85% in patients with DRS and 17.24% in the healthy group (p<0.001). The other yeast species identified were *C. glabrata*, *C. tropicalis*, *C. krusei*, and *C. dubliniensis*. The distribution of the isolated *Candida* species were listed in Table 2. Additionally, mixed isolation rate was determined to be higher in the DRS group (22.21%) in comparision to healthy group (10.32%). *C. albicans* and *C. glabrata* were the species most frequently isolated together and isolation of these two species in DRS group was significantly higher than in the healthy group (18.51% and 6.88%, respectively) (p<0.05).

Biofilm formation was observed in a total of 37 oral yeast isolates, nine isolates from healthy denture wearers and 28 isolates from denture wearers with DRS as presented on Table 3. Of the 14 isolates from the healthy group, nine (64.2%) were biofilm positive while out of 33 isolates from the DRS group 28 (84.8%) were biofilm positive. Biofilm formation abilities of the species are shown in Table 2. Out of the 27 tested isolates of *C. albicans*, 20 (74.0%) were biofilm positive. Only 1 (5.0%) of the *C. albicans* strains was strongly positive, 14 (70.0%) strains were moderately positive, and five (25.0%) strains were weakly positive. Among the 20 non-albicans *Candida* strains, 17 (85%) were biofilm positive while three (15%) did not produce biofilms. All of the *C. tropicalis*, *C. kefyr*, and *C. dubliniensis* isolates tested were found to be biofilm positive. Comparison between the two groups revealed that, the number of *C. albicans* isolates showing biofilm formation ability in the DRS group (16 of 19, 7 of 9, respectively) was significantly higher than the number of isolates from patients in the healthy group (4 of 8) (p<0.001). Moreover, similarly, the number of *C. glabrata* isolates showing biofilm formation ability in the DRS group was more pronounced than the healthy group (p<0.05).

Table 2. Intragroup analyses of the distrubution of the Candida species.

***Candida* species**	**Healthy Group (n=29)**		**DRS Group (n=27)**		**p***
	n	%	n	%	
No growth	18	62.06	0	0	**0.001**
C. albicans	5	17.24	14	51.85	**0.001**
C. glabrata	0	0	3	11.11	**0.029**
C. tropicalis	1	3.44	1	3.70	NS
C. krusei	0	0	1	3.70	NS
C. kefyr	2	6.88	1	3.70	NS
C. dubliniensis	0	0	1	3.70	NS
C. albicans+C. glabrata	2	6.88	5	18.51	**0.037**
C. albicans+C. dubliniensis	1	3.44	0	0	NS
C. glabrata+C. tropicalis	0	0	1	3.70	NS

n: Number of subjects, DRS: denture-related stomatitis, *Chi-square test, NS: nonsignificant, p<0.05

Table 3. Biofilm formation capacity of the candida species

***Candida* species**	**Healthy Group (n=14)**		**DRS Group (n=33)**		**p***
	Negative Isolates	Positive Isolates (+,++,+++)	Negative Isolates	Positive Isolates (+,++,+++)	
C. albicans	4	4 (3,1,0)	3	16$^{\mu}$ (2,13,1)	**0.001**
C. glabrata	1	1 (1,0,0)	2	7 (2,5,0)	**0.043**
C. tropicalis	0	1 (0,1,0)	0	2(1,1,0)	NS
C. krusei	0	0	0	1 (0,1,0)	NS
C. kefyr	0	2 (1,1,0)	0	1 (0,1,0)	NS
C. dubliniensis	0	1 (0,1,0)	0	1 (1,0,0)	NS

n: Number of isolated *Candida* species, DRS: denture-related stomatitis *Comparision between groups, Chi-square test
$^{\mu}$Intragroup analyses, Chi-square test, NS: nonsignificant p<0.05.

DISCUSSION

Oral candidiasis emerges as a result of excessive increase of *Candida* species that is a member of the oral flora.[20] Some predisposing factors have been reported such as old age, systemic diseases, declined salivary flow rate and denture usage.[21,22] Phospholipase and proteinase production of *Candida* species facilitates the adherence to the mucosal surface, invasion to deep layers and causing inflammation.[23] Another virulence factor of the *Candida* species is the biofilm formation capacity with their slime layer.

Candida species were reported to have a very high occurrence rate in the oral cavity of healthy individuals, and *C. albicans* is the most frequently isolated species. Wearing partial or complete dentures causes this rate to be higher. In several studies, the overall prevalence of *Candida* species was reported to be 52-55.2% and 28-89% in the oral cavities of patients without and with DRS, respectively.[1,8-10,24-26] In the present study, *Candida* species were isolated from the oral cavities of 37.4% of healthy people while they were isolated from all of the patients with DRS.

Due to the better adherence capacity of *C. albicans* to the mucosal surface, it has higher prevalence of isolation from the oral candidal infections.[24,27] In the other studies, *C. albicans* was reported to be the most frequently isolated species with a rate of 52.9-78%.[1,8,10,24,25]Similar to the other studies, the findings of the present study indicated that *C. albicans* was the most commonly isolated *Candida* species in both groups.[28,30] Additionally, the prevalence of *C. albicans* in DRS group was higher than in the healthy group (51.8% and 17.2, respectively).

Although *C. albicans* is the predominant yeast in DRS, mycological ecology contributed by species diversity plays an important role in inflammation.[25] In agreement with the other studies, *C. glabrata* was the most common non-albicans species in both groups. Coco et al. showed that *C. glabrata* was isolated from 31% of the DRS patients and speculated that this yeast has a synergistic relationship that causes enhanced pathogenic features with *C. albicans.*[25] In the present study, *C glabrata* appeared to co-colonize more frequently with *C albicans*.

The biofilm formation ability of *Candida* species is considered to be an important virulance factor for causing infection. Biofilm formation provides these microorganisms a protection against salivary flow and other mechanical forces. The prevalence of *Candida* isolates effects the ability of biofilm formation. In the present study, *C. albicans* strains were 74% positive, while non-albicans strains were 85% positive for biofilm production. Yigit et al. reported that 88.0% *C. albicans* strains and 51.6% of the non-albicans strains were biofilm positive.[10]

CONCLUSION

As a conclusion, our results show that the presence of yeasts is probably linked to extensive inflammation. *C. albicans* was the most frequently isolated species, and *C. glabrata* was the most frequently isolated non-albicans species. Our results also indicate the biofilm production of non-albicans species as important as *C. albicans*.

Further investigations of the fungal virulence factors and the factors such as denture hygiene, systemic diseases and immune system deficiencies that may be involved in DRS can contribute to the development of new strategies for DRS management.

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