

Investigating Biofilm Production, Coagulase and Hemolytic Activity in *Candida* Species Isolated From Denture Stomatitis Patients

Protez Stomatitli Hastalardan İzole Edilen Candida Türlerinde Biofilm Üretimi, Koagülaz ve Hemolitik Aktivite Araştırılması

Nimet Yigit¹, Esin Aktas², Saadetin Dagistan³, Ahmet Ayyildiz²

¹ Department of Medical Laboratory, Health Services Vocational Training School, Atatürk University, Erzurum, Turkey

² Department of Microbiology and Clinical Microbiology, Faculty of Medicine, Atatürk University, Erzurum, Turkey

³ Department of Radiology, Faculty of Dentistry, Atatürk University, Erzurum, Turkey

Abstract

Objective: Oral candidiasis, in the form of *Candida*-associated denture stomatitis, represents a common disease in a large percentage of denture wearers, and *Candida albicans* remains the most commonly isolated species. In this study, we aimed to evaluate biofilm production, coagulase and hemolytic activity of *Candida* species isolated from denture stomatitis patients.

Materials and Methods: This study included 70 patients (31 female, 39 male). Forty-eight of the patients were found to have a positive culture. A total of 48 *Candida* isolates representing five species, *C. albicans* (n=17), *C. glabrata* (n=10), *C. krusei* (n=9), *C. kefyr* (n=7) and *C. parapsilosis* (n=5), were tested. Their coagulase activities were evaluated by a classical tube coagulase test with rabbit plasma. A blood plate assay on 3% enriched sheep blood Sabouraud-dextrose agar (SDA) was used to determine their in vitro hemolytic activities. Biofilm production was determined by a visual tube method.

Results: Twenty-one *Candida* isolates exhibited coagulase activity, and the coagulase activities of the *C. albicans* (64.7%) isolates were higher than other species. *C. albicans*, *C. glabrata*, *C. kefyr* and *C. krusei* species demonstrated beta hemolysis. *C. parapsilosis* strains failed to demonstrate any hemolytic activities. Fifteen (88.0%) of the *C. albicans* strains were biofilm positive. Six (35.2%) of these strains were strongly positive, 8 (47.0%) *C. albicans* strains were moderately positive and 1 (5.8%) *C. albicans* strain was weakly positive. Sixteen (51.6%) of the non-*albicans* *Candida* strains were biofilm positive while 15 (48.3%) did not produce biofilms.

Conclusion: The results of this present study indicate coagulase, hemolytic activity and biofilm production by *Candida spp.* isolated from patients with denture stomatitis. Investigations of these virulence factors might be helpful in gaining information about the possible virulence of oral *Candida* species related to denture stomatitis.

Key Words: *Candida* species, Coagulase activity, Denture stomatitis

Özet

Amaç: Oral kandidiyazisin *Candida* ile ilişkili olarak gelişen protez stomatiti şekli, protez kullananların büyük bir kısmında görülen yaygın bir hastalıktır ve *Candida albicans* en çok izole edilen türdür. Bu çalışmada, protez stomatitli hastalardan izole edilen *Candida* türlerinde biofilm üretimi, koagülaz ve hemolitik aktivite araştırılması amaçlanmıştır.

Gereç ve Yöntem: Bu çalışma 31 kadın, 39 erkek toplam 70 hasta üzerinde yürütüldü. Hastaların 48'inde kültür pozitif bulundu ve izole edilen 48 *Candida* suşu 5 tür olarak tiplendirildi [(*C. albicans* (n=17), *C. glabrata* (n=10), *C. krusei* (n=9), *C. kefyr* (n=7), *C. parapsilosis* (n=5)]. Suşların koagülaz aktivitesi tavşan plazması kullanılarak klasik tüp koagülaz testi ile hemolitik aktiviteleri ise %3 glikoz ile zenginleştirilmiş koyun kanlı Sabouraud-dextrose agar (SDA) kullanılarak araştırılmıştır. Biofilm üretimi tüp adherans testi ile belirlenmiştir.

Bulgular: Yirmibir *Candida* izolatinde koagülaz üretimi belirlenmiş ve koagülaz aktivitesi *Candida albicans* (%64.7) suşlarından diğerlerinden daha yüksek bulunmuştur. *Candida albicans*, *Candida glabrata*, *Candida kefyr* ve *Candida krusei* suşları beta hemoliz oluşturmuştur. *Candida parapsilosis* suşları hemolitik aktivite göstermemiştir. On beş (%88.0) *C. albicans* suşu biofilm oluşturmuş, bunların altısında (%35.2) kuvvetli, sekizinde (%47.0) orta dereceli ve birinde (%5.8) zayıf biofilm oluşumu belirlenmiştir. On altı (%51.6) non-*albicans* *Candida* türünde biofilm oluşumu pozitif bulunurken, 15'inde (%48.3) biofilm üretimi belirlenmemiştir.

Sonuç: Bu çalışma sonucunda protez stomatitli hastalardan izole edilen *Candida* türlerinde koagülaz, hemolitik aktivite ve biofilm üretimi belirlenmiştir. Bu faktörlerin araştırılması protez stomatit ilişkili *Candida* türlerinin muhtemel virulansları hakkında bilgi vermesi açısından faydalı olabilir.

Anahtar Kelimeler: *Candida* türleri, Koagülaz aktivitesi, Protez stomatiti

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Correspondence to: Esin Aktas, Department of Microbiology and Clinical Microbiology, Faculty of Medicine, Atatürk University, Erzurum, Turkey

Phone: +90 442 231 65 81 Fax: +90 442 236 09 68 e-mail: aeaktas@yahoo.co.uk

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Introduction

Candida is a commensal fungus that harmlessly inhabits various niches of the human body, including the oral cavity, gastrointestinal tract, vagina and skin of healthy individuals [1]. *Candida* has been recognized as a part of the normal oral flora that has no harmful effects on the host. There are 300 to 400 species of microorganisms in the oral cavity, including 20 species of *Candida*. The fungus is frequently isolated from various oral sites, including the tongue, cheek, palatal mucosa, dentures, dental plaque, dental caries and subgingival flora [2]. It is estimated that the oral cavity is colonized by *Candida* in 40 to 60% of the population. Changes in the oral environment, which can be affected by tooth loss or denture wearing, can cause changes in the oral microflora [3]. When considering only those who wear dentures, the proportion of the population colonized by *Candida* increase to 60-100%. *C. albicans* is the most common species, and its various strains account for almost 70% of *Candida* isolates. In addition to *C. albicans*, other species including *C. tropicalis*, *C. glabrata*, *C. kefyr*, *C. krusei* and *C. parapsilosis* are also typically isolated from denture and non-denture wearers [4-8].

Denture stomatitis is a term used to describe certain pathological changes of the mucosa under complete or partially removable dentures. These changes are characterized by erythema and usually localized to both jaws, less frequently found in the mandible, and are commonly associated with angular cheilitis and median rhomboid glossitis [6, 9-11]. It is more common in females than males. Deficient oral and denture hygiene is an important predisposing factor because poor hygiene facilitates both the presence of yeast and bacteria in saliva, and their colonization of the oral mucosa and denture surface. Other factors such as reduced salivary flow, roughness and micropores on the acrylic surface, trauma, pH alterations in the denture plaque, diabetes, long-term use of steroids and immunological impairment may also predispose an individual to the development of denture-induced stomatitis. Although the dominant etiological factor at present appears to be a fungal infection, other factors must also be considered. *Candida* species have been identified in most or all patients [4, 8, 11-14], and the involvement of *Candida* as a potential causative agent in denture-induced stomatitis ("rubber sore mouth") was first described in 1936 [11]. *C. albicans* remains the most frequently isolated yeast in the oral cavity. However, other species have been isolated and are involved in the disease [4-8, 11].

An important co-factor associated with the pathogenesis of oral candidiasis appears to be virulence of the infecting organism. Several factors, such as adherence, persistence, dimorphism and/or germ tube formation, phenotypic switching, interference with host defense systems, synergism with

bacteria and the production of hydrolases (lipases and proteases such as the secreted aspartyl proteinase, phospholipase, esterases, and phosphatases), have been proposed to be *Candida* spp. virulence factors [15-19].

One of the major factors contributing to the virulence of *Candida* is its versatility. It adapts to a variety of different habitats for growth and can form surface-attached microbial communities known as biofilms. Biofilm formation is thought to be one of *Candida*'s most important growth adaptations because biofilms allow it the possibility to colonize oral surfaces. The possibility that these biofilms could serve as a reservoir for disseminated infections, such as aspiration pneumonia and gastrointestinal infection, has been pointed out earlier [1, 2].

The capacity of *Candida* spp. to colonize host tissue and cause tissue invasion has been associated with its ability to produce extracellular enzymes. Research on *Candida* pathogenicity has been focused on hydrolytic enzymes, such as proteinases, phospholipases and lipases. Little information concerning *Candida* coagulase and hemolytic activities was found in a recent literature search [17, 20-22]. Hemolytic and coagulase activities in *Candida* spp. are virulence factors less well-studied, and their importance to pathogenicity require new and more rigorous studies.

The present study investigated biofilm production, coagulase and hemolytic activities of *Candida* spp. isolated from denture stomatitis patients. To our knowledge, this is the first study analyzing coagulase activity among *Candida* spp. isolated from denture stomatitis patients.

Materials and Methods

Subjects

This study included 70 (39 male and 31 female) complete denture wearers that were recruited from the Oral Diagnosis and Radiology Department of the Faculty of Dentistry. Denture stomatitis was assessed according to Newton's classification [6].

Mycological examination

Oral swab samples were obtained from the affected palatal mucosal site under the denture by passing a sterile cotton swab. Each swab was then transferred into 1 ml sterile phosphate-buffered saline and rinsed by vortexing to remove the yeast cells from the swab. From the diluted samples, 0.1 ml was used to inoculate Sabouraud dextrose agar (SDA) (Merck/Germany) supplemented with 1% chloramphenicol. Plates were incubated at 37°C for 48 h. After incubation, the isolates were identified by standard taxonomic procedures, which included germ tube production, typical microscopic appearances on cornmeal agar with Tween-80, production

of chlamydospores, colony morphology and pigment production on chromogenic medium. Identifications were confirmed by the API 32 C AUX (bioMerieux, Marcy-l'Etoile, France) identification system for yeasts.

Determination of biofilm formation

Biofilm production was determined by visual methods. Colonies from the surface of SDA plate were inoculated into a polystyrene tube (Falcon conical tube with screw cap) containing 10 ml of Sabouraud-dextrose broth (SDB) supplemented with glucose (final concentration 8%). After incubation at 35°C for 48 h, the broth in the tubes was gently aspirated. The tubes were washed with distilled water twice and then stained with 2% safranin for 10 min. They were then examined for the presence of an adherent layer. Biofilm production was scored as negative (-), weak (+), moderate (++) or strong (+++). The biofilm producer *Staphylococcus epidermidis* ATCC 35984 was used as a positive control [23, 24].

Assessment of hemolytic activity

Hemolytic activity was evaluated with a blood plate assay [20, 25]. Sheep blood SDA were prepared by adding 7 ml of fresh sheep blood to 100 ml of SDA supplemented with 3% glucose. Yeast strains were streaked onto SDA and incubated at 37°C for 18 h. Suspensions equal to McFarland 2 turbidity from the pure culture of the yeast colonies on SDA were prepared. Ten microliters of this suspension were spotted on sheep blood SDA (with 3% glucose). Plates were incubated at 37°C for 48 h. The presence of a distinctive translucent halo around the inoculum site indicated positive hemolytic activity. The ratio obtained by dividing the diameter of the colony by the total diameter of the colony plus the translucent halo was used as a hemolytic index (Hi) representing the intensity of the hemolysin production by the *Candida* species.

Statistical analysis

Statistical analyses were performed using SPSS version 13.0. To identify significant differences, the data were analyzed by analysis of variance using the general linear model, which permits the analysis of samples with different numbers of replicates. The mean values were separated by Duncan's multiple range tests.

Assessment of coagulase activity

A total of 48 *Candida* isolates were tested for coagulase activity using EDTA-rabbit plasma by a classical tube test. Approximately 0.1 ml of an overnight culture of each test strain in Sabouraud-dextrose broth was inoculated into a tube containing 500 ml of EDTA-rabbit plasma. The tubes were incubated at 35°C and observed for clot formation at 2, 4, 6, and 24 h [12]. The type strains *Staphylococcus aureus*

ATCC 25923 and *S. epidermidis* ATCC 14990 were used as positive and negative controls, respectively, for coagulase expression [26].

Results

Yeast strains

Forty-eight patients had positive cultures. A total of 48 *Candida* isolates representing five species, *C. albicans* (n=17), *C. glabrata* (n=10), *C. krusei* (n=9), *C. kefyr* (n=7) and *C. parapsilosis* (n=5), were identified.

Biofilm production by *Candida* spp.

Fifteen (88.0%) *C. albicans* strains were biofilm positive. Six (35.2%) of these strains were strongly positive, 8 (47.0%) strains were moderately positive and 1 (5.8%) strain was weakly positive (Table 1). Sixteen (51.6%) of the non-*albicans* *Candida* strains were biofilm positive while 15 (48.3%) did not produce biofilms. The distribution of strongly, moderately and weakly positive biofilm producers according to non-*albicans* *Candida* spp. are shown in Table 1.

Hemolytic activities of *Candida* spp.

C. albicans (n=17), *C. glabrata* (n=10), *C. krusei* (n=9) and *C. kefyr* (n=7) species exhibited beta hemolysis on sheep blood SDA (supplemented with 3% glucose) at 48 h post-inoculation. All tested *C. parapsilosis* (n=5) isolates exhibited neither alpha nor beta hemolysis despite 48 h of incubation (Table 2). The quantitative data indicated that the beta-hemolytic activities of *C. albicans*, *C. glabrata* and *C. krusei* showed significantly higher beta-hemolytic activities than *C. kefyr* ($p < 0.01$) (Table 2). Further, there were no significant intraspecies differences in the beta-hemolytic activities among isolates belonging to *C. albicans*, *C. glabrata* and *C. krusei* (Table 2).

Coagulase activities of *Candida* spp.

The coagulase activities of 48 *Candida* isolates are shown in Table 3. Eleven (64.7%) *C. albicans* strains induced clot formation in the tube coagulase test with rabbit plasma. Coagulase activities were detected among other *Candida* species in tube coagulase tests with rabbit plasma. Three (30.0%) *C. glabrata*, 2 (22.2%) *C. krusei*, 3 (42.8%) *C. kefyr* strains and 2 (40.0%) *C. parapsilosis* strains were coagulase positive (Table 3).

Discussion

The oral cavity provides a diverse number of surfaces including soft shedding non-keratinized buccal mucosal epithelia, the keratinized mucosa of the gums, the highly papil-

Table 1. Biofilm production by *Candida* species isolated from individuals with denture stomatitis

Species	Biofilm			
	(-)	(+)	(++)	(+++)
<i>C. albicans</i> (n=17)	2 (11.7%)	1 (5.8%)	8 (47.0%)	6 (35.2%)
<i>C. glabrata</i> (n=10)	4 (40.0%)	-	4 (40.0%)	2 (20.0%)
<i>C. krusei</i> (n=9)	5 (55.5%)	2 (22.2)	1 (11.1%)	1 (11.1%)
<i>C. kefyr</i> (n=7)	3 (42.8%)	2 (28.5%)	2 (28.5%)	-
<i>C. parapsilosis</i> (n=5)	3 (60.0%)	1 (20.0%)	1 (20.0%)	-
Total (n=48)	17 (35.4%)	6 (12.5%)	16 (33.3%)	9 (18.7%)

Table 2. Hemolytic activities of *Candida* species isolated from individuals with denture stomatitis

Species	No. of isolates with hemolysis patterns (hemolysis index, mean±SD)		
	alpha	beta	None (gamma)
<i>C. albicans</i> (n=17)	-	(2.32±0.51) ^a	-
<i>C. glabrata</i> (n=10)	-	(2.08±0.54) ^a	-
<i>C. krusei</i> (n=9)	-	(2.14±0.50) ^a	-
<i>C. kefyr</i> (n=7)	-	(1.18±0.46) ^b	-
<i>C. parapsilosis</i> (n=5)	-	-	5

–: No activity.
^a: Within columns, the statistical difference between two means is given by the letters a and b. If the letters are different, the difference is significant (P < 0.01) by Duncan tests

Table 3. Coagulase activities of *Candida* species isolated from individuals with denture stomatitis

Species	Total no. of strains	No. of positive strains
<i>C. albicans</i>	17	11 (64.7%)
<i>C. glabrata</i>	10	3 (30.0%)
<i>C. krusei</i>	9	2 (22.2%)
<i>C. kefyr</i>	7	3 (42.8%)
<i>C. parapsilosis</i>	5	2 (40.0%)
Total	48	21 (43.7%)

lated mucosa of the tongue and the hard non-shedding surface of the teeth. In comparison with the dentate individual, the mouth of the denture wearer presents additional hard non-shedding areas and new environments (tissue-fitting

surfaces) to support the growth of microorganisms and the development of plaque [9, 27].

Oral candidiasis, in the form of *Candida*-associated denture stomatitis, represents a common disease in a large percentage of denture wearers, and *C. albicans* remains the most commonly isolated species in denture stomatitis [11]. In this study, *Candida* spp. were isolated from the palatal mucosal surfaces of 48 (68.5%) cases. *C. albicans* 17 (35.4%) was the most commonly isolated species followed by *C. glabrata* 10 (20.8%), *C. krusei* 9 (18.7%), *C. kefyr* 7 (14.5%) and *C. parapsilosis* 5 (10.4%). Twenty-two (31.4%) patients had negative fungal cultures. In the present study, denture-induced stomatitis was more common among males (55.7%). The average male age was 62.8 (41-82), and the average female age was 55.6 (36-77). None of the patients had diabetes or an immune system-related illness, and none were using corticosteroid drugs for lengthy periods.

In the oral cavity, most colonizing and infecting microorganisms are not found as single-living cells but rather as complex structured microbial communities, which are often encapsulated within a matrix of exopolymeric material and attached to a biotic or abiotic surface. These communities are referred to as "biofilms". Yeasts cells adhere and colonize oral surfaces including mucosa and acrylic dentures and have the ability to co-aggregate with oral bacteria. The attachment of *Candida* to dental prostheses seems to be a critical event in the initiation of colonization and infection. This process may be especially important in the development of oral candidiasis and denture-related stomatitis, where *Candida* can adhere to the acrylic to form a reservoir for the chronic dissemination of fungal cells [9, 27-29]. In addition, *Candida* cells in a biofilm exhibit distinct properties. The most significant property is resistance to several antimicrobial drugs, notably the popular azole drugs [7, 10, 30-32].

In this study, we determined biofilm production in *Candida* strains isolated from denture stomatitis by tube adherence methods. The *C. albicans* strains were 88.2% positive for biofilm production. In contrast, *C. glabrata*, *C. krusei*, *C. kefyr* and *C. parapsilosis* were 60.0%, 44.4%, 57.1% and 40%, respectively, positive (Table 1). *C. albicans* exhibits a wide range of adhesion capabilities, and germ tube production may play a key role in the adhesion process [28, 30].

Candida spp. isolated from the oral cavity and patients with oral candidiasis had slightly stronger adhesion ability to buccal epithelial cells and dentures. Adherence is considered an essential virulence factor in *Candida* spp. [6, 7, 11, 29, 30]. Prior studies have shown a correlation between enzyme production and cellular adherence [6, 7, 10, 30, 33-35].

Hemolysins are known to be putative virulence factors contributing to *Candida* pathogenesis, particularly facilitating hyphal invasion in disseminated candidiasis [36]. The

hemolytic activities of medically important yeasts like genera *Candida* and *Cryptococcus* have been scarcely explored [37]. A complement-mediated hemolysis induced by *C. albicans* has been reported by Manns *et al.* [25]. Watanabe *et al.* [38] reported that *C. albicans* secretes a hemolytic factor that causes the release of hemoglobin, which is then used as an iron source by the organism. Luo *et al.* [20] studied 80 *Candida* isolates representing 14 species. These authors reported that *C. albicans* and others showed alpha and beta hemolysis. This study was the first to demonstrate variable hemolysin expression profiles by different *Candida* species [20]. Recently, we reported that *Candida* species, including *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. kefyr* and *C. krusei*, exhibit varying abilities to produce hemolysins on human, rabbit and sheep blood SDA supplemented with 3% glucose mediums [22].

Studies on hemolysin activities from *Candida* spp. isolated from oral isolates are limited. The first study on a hemolytic activity from an oral cavity isolate of *C. albicans* was carried out by Tsang *et al.* [39]. Tsang *et al.* [39] reported that the hemolytic activity of an oral *C. albicans* isolated from type 2 diabetes mellitus patients was significantly higher than those isolated from controls (a hemolysis index of 0.764 ± 0.08 in the non-diabetic group vs. 0.673 ± 0.06 in the diabetic group). In another study, Maia *et al.* [7] detected beta-hemolytic activities in *C. albicans* (2.08 ± 0.389), *C. glabrata* (2.29 ± 0.36) and *C. krusei* (1.97 ± 0.33) strains isolated from the oral cavities of healthy elderly individuals.

In this study, the *in vitro* hemolytic activities of *Candida* species isolated from oral cavities were investigated. *C. albicans* (n=17), *C. glabrata* (n=10), *C. krusei* (n=9) and *C. kefyr* (n=7) species exhibited beta hemolysis on sheep blood SDA supplemented with 3% glucose at 48 h post-inoculation. All tested *C. parapsilosis* isolates (n=5) exhibited neither alpha nor beta hemolysis despite 48 h of incubation (Table 2). The quantitative data indicated that the beta-hemolytic activities of *C. albicans* (2.32 ± 0.51), *C. glabrata* (2.08 ± 0.54) and *C. krusei* (2.14 ± 0.50) showed significantly higher beta-hemolytic activities compared to *C. kefyr* (1.18 ± 0.46) ($p < 0.01$) (Table 2). Further, there were no significant intraspecies differences in the beta-hemolytic activities among isolates belonging to *C. albicans*, *C. glabrata* and *C. krusei* (Table 2). The possibility that species-specific hemolysins may exist should still be considered. These hemolysins may vary in molecular size and, thus, have different diffusion rates [20, 36].

Extensive research in *Candida* spp. has been focused on proteinases such as secreted aspartyl proteinases, phospholipases and hemolysins. Nevertheless, systematic studies on plasma coagulase are rare and, to the best of our knowledge, there are no studies on the coagulase activities of *Candida* spp. isolated from individuals with denture stomatitis.

In a previous study, Rodrigues *et al.* [17] detected coagulase activity in a large number of isolates from various clinical specimens, *C. albicans* (88.5%) and *C. tropicalis* (82.6%), and lower activities for other species using a rabbit plasma coagulase test after incubation for 24 h. Positive latex test results were obtained with 68.9% of the *C. albicans* strains, 56.5% of the *C. tropicalis* strains and 6.9% of the *C. parapsilosis* strains. None of the *C. glabrata*, *C. guilliermondi* or *C. krusei* strains had positive latex tests in the Rodrigues study [17]. In another study, Becker *et al.* [40] observed that 40 (26.7%) of 150 yeast isolates had positive agglutination results from the Pastorex Staph-Plus LAT [24]. In a previous study, we demonstrated coagulase activities for 125 *Candida* strains that represented 8 species isolated from different clinical specimens [21].

In the present study, the coagulase activities of 48 *Candida* isolates are shown in Table 3. Eleven (64.7%) *C. albicans* strains induced clot formation in a tube coagulase test with rabbit plasma. Coagulase activities were detected among other *Candida* species using a tube coagulase test with rabbit plasma. Three (30.0%) *C. glabrata*, 2 (22.2%) *C. krusei*, 3 (42.8%) *C. kefyr* and 2 (40.0%) *C. parapsilosis* strains were coagulase positive using a tube coagulase test with rabbit plasma (Table 3).

Our results also indicate the biofilm production of *C. albicans* was higher, and the beta hemolytic activities of *C. albicans*, *C. glabrata* and *C. krusei* were higher compared to *C. kefyr*. In addition, we report here for the first time the coagulase activity of *Candida* species isolated from denture stomatitis and that the coagulase activities of *C. albicans* strains were higher than those of non-*albicans* strains. Further studies on the simultaneous expression of candidal extracellular enzymes are urgently needed to understand the natural history and host-pathogen relationships associated with oral candidal infections.

Conflict of interest statement: The authors declare that they have no conflict of interest to the publication of this article.

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