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Research Article

Biofilm Formation and Antifungal Susceptibility of *Candida* Isolates from Oral Cavity of Denture Wearer and Free Denture Individuals

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Abstract

Aims: The aim of the current study was to examine the differences, if any, in the capabilities of forming biofilms to isolate types of *Candida* from dental wearers compared to individuals who have natural teeth, as this attribute of oral *Candida* species isolates from oral cavities of denture wearer and normal people has not been examined before. Also determine the species distribution and anti-fungal sensitivity to isolated *Candida*.

Materials and Methods: The study group consisted of 104 denture wearers patients and 108 individuals without dental prosthesis. Salivary samples were collected using the oral rinse technique. Then they were cultured and identified by standard methods. After that the isolated *Candida* species were tested for biofilm production by the phenotypic method i.e. Tissue culture palate methods (TCPM). Finally, antibiogram susceptibility pattern of oral *Candida* species was done by Kirby-Bauer disc diffusion method for amphotericin B, ketoconazole, and fluconazole. .

Results: The most common yeast was *C. albicans* (37.7%), followed by *C. krusei* (18.9%), while *C. tropicalis* was 9.4% and *C. glabrata* was 1.88%. Also, there was an increase in the percentage of *Candida albicans* among the dentist (65.4%) compared to the members of the free dentures (11.1%).There were significant quantitative differences in biofilm formation between Candida species isolates from denture patients compared to isolates from denture-free individuals (54.2% versus 19.2%) (p = 0.001). The rate of formation of biofilms was 47.9% for all types of *Candida* and it was found that biofilm formation occurs more frequently among *C. tropicals* (70%) than *C. albicans* (48.75%). All *Candida* species isolates were sensitive to amphotericin B and ketoconazole while resistance to fluconazole was found in 25% of *C. krusei* and *C. tropicalis* and only in 5% of *C. albicans* isolates.

Conclusion: The present study proved that *C. albicans* is still the major isolate from oral cavity, but non-albicans spp. colonization is raised; denture was factor for oral colonization of *Candida* species, and biofilm formation. The *C. tropicalis* were more biofilm - producers compared to *C. albicans*. The species isolated in the current study are less susceptible to fluconazole and drug resistant factor in the *Candida* species isolates was found to be associated with *Candidal biofilm* formation.

Keywords: *Candida Species; Candida albicans; Non-albicans spp.; Biofilm Formation; Antifungal Resistance; Oral Cavity; Denture*

Introduction

Oral yeast infection occurs from members of the genus *Candida*. Candidiasis is an opportunistic infection due to pathological changes in the surface of the lumen oral mucosa [1-5]. Candidiasis patients may exhibit various symptoms including burning, painful sensation, difficulty swallowing and changing taste, but most often they are asymptomatic [6]. Infection is generally treated with antifungal medications, but in immunocompromised patients and patients who perform internal devices such as catheters, dentures etc. the return of infections can be a problem [1,4,7]. In the past twenty years, some species of *Candida*, including *C. tropicalis*, *C. glabrate, C. parapsilosis* and *C. krusei*, have been isolated, with an increased frequency in cases of candidiasis [5,8-11]. Furthermore, there are reports of several cases describing persistent infections and colonization of denture patients and FOA patients [4,11,12].

A relatively small number of antifungal drugs are available when compared to the group of antibiotics that have been produced, which may reflect both the relatively recent recognition of the importance of fungal infection in humans and the difficulty involved in developing an agent that has activity against an eukaryotic cell type without problems from the associated host cell toxicity with it [13]. It has been found that each type of *Candida* differs in the production of recognized virulence factors and sensitivity to anti-fungal agents. Therefore, isolation and recognition of *Candida* is useful in choosing the correct treatment, because some types may be resistant to certain groups of anti-fungal drugs [13-18]. The infection caused by the *non-Candida albicans Candida* (NCAC), such as *C. glabrate, C. tropicalis* and *C. krusei* was less responsive to fluconazole [18,19]. Newer triazoles, including posaconazole, echinocandins, voriconazole, micafungin, anidulafungin, and caspofungin are anti-fungal medications that show strong activity against *Candida*. On the other hand, echinocandins appear to be less effective against some species, such as *C. guilliermondii* and *C. parapsilosis* [10,13]. Also, *C. dubliniensis* is extremely similar to *C. albicans* and it has been reported to have low sensitivity to azole drugs [13].

Wearing dentures is associated with the excessive growth of *Candida* species, due to the formation of biofilms, which leads to stomatitis. Studies to determine the types of *Candida* species in patients with stomatitis have yielded contradictory results for the association between denture, biofilm formation and the occurrence of stomatitis [4,11,12,20]. Several studies have suggested that the important factors that contribute to the virulence of *Candida* species are the formation of surface-related microbial communities known as "biofilms" [20-22]. Biofilms are attached to a surface and coated in a matrix of exopolymeric material. The typical laboratory fungal model for biofilm formation includes three practical steps: (a) adhesion, (b) growth of biofilms, (c) maturity [21]. Biofilm formation helps the organism avoid host defenses, is present as a permanent source of infection and develops resistance against anti-fungal agents. *Candida* species are frequently found in the natural microbial flora of the oral cavity and other sites of the human body, making it easier to counter them through cultivated biomaterials such as dentures, etc.; and host surfaces [22]. The resistance of biofilm forming *Candida* species to antifungal agents acts for a major challenge particularly in the plan of therapeutic and prophylactic strategies [23]. The objectives of the present study was therefore to examine the differences, if any, in the biofilm-forming abilities of *Candida species* isolates from denture wearers comparing with free-denture individuals, as this attribute of oral *Candida* species isolates from oral cavities of denture wearer and normal people has not been examined before. Also determine the species distribution, and antifungal susceptibility of oral *Candida* isolates.

Subjects and Laboratory Methods

Subject selection

This study included two hundred and twelve people, 104 of whom were denture wear patients while 108 others with natural teeth, were randomly chosen from Al-Thawrah hospital, Al-Gumhory hospital and Dental Centers in Sana'a. City, Yemen. The duration of the study was six months, beginning in August 2017 and ending in February 2018. The inclusion criteria included the selection of healthy people who had no clinical signs of candidiasis and no systemic diseases. Additionally, individuals who smoke, currently taking antifungals, steroids, antibiotics, or immunosuppressive drugs in the past six months; have been excluded.

Collection and identification of samples

Saliva samples were collected using the mouth rinse technique. In summary, each person was asked to rinse the mouth for 60 seconds with 10 ml of phosphate sterile saline (PBS, 0.01m of phosphate buffered saline, pH 7.2) and flush out the wash into a sterile 15 ml sterile container [25]. Individuals with removable dentures were asked to take out the dentures before collecting the samples. The samples were immediately transported on ice to the microbiology laboratory. Each oral rinse was centrifuged at 3,500 rpm for 10 minutes, and then the supernatant was removed. Pellet was re-suspended in 1 ml sterile PBS. One hundred µl of the concentrated oral rinse was inoculated onto Sabouraud's dextrose agar and incubated at 37°C for 48 hours. The lasting samples were stored at -20°C. If *Candida* colonies appeared on the Sabouraud dextrose agar, then chromogenic *Candida* agar was inoculated using 100 µl of the oral rinse supernatant and incubated for 48 hours for colonies study. *Candida* species were identified by the color of the colonies using the color reference guide supplied by the manufacturer. When color identification was unclear, fermentation assay of sucrose, maltose, glucose, lactose and galactose was done. *Candida* species have also been identified through the ability to produce chlamydia spores in glutinous rice agar [26].

Antifungal susceptibility testing

The *in vitro* activity of antifungal agents (amphotericin B, ketoconazole and fluconazole) was measured by disk diffusion method according to the procedure described in the clinical and laboratory standard institute [27]. The plates were incubated at 35°C, and inhibition zone diameters (dz)were measured after 24 and 48 h particularly for *C. glabrata*. The interpretive criteria for the disk test were as follow: amphotericin B: dz ≥ 15 mm, susceptible; 14 ≥ dz ≥ 10 mm, susceptible dose dependent and dz ≤ 9 mm, resistant. Fluconazole: dz ≥ 19 mm, susceptible; 15 ≤ dz ≤ 18 mm, susceptible dose dependent and dz ≤ 14 mm, resistant. As for ketoconazole: dz ≥ 20 mm, susceptible; 10 < dz susceptible dose dependent and dz ≤ 10 mm, resistant [28].

Biofilm production detection

The detection of biofilm was done by tissue culture method/microtiter plate method (TCA) [29,30]. The yeast isolates from fresh agar plates were inoculated in 2 ml of BHI broth and incubated for 24h at 37°C. The cultures were then diluted 1:40 with fresh medium (BHI broth supplemented with 1% glucose); 200 µl of the sample was dispensed in the individual microtitration plate and incubated further 24h at 37°C. With a gentle tapping, the content was removed further with a subsequent washing with phosphate buffer saline (pH 7.2) three times to remove free floating sessile *Candida*. The adherent yeast, biofilm producer, were fixed with sodium acetate (2%) and stained with crystal violet (0.1% w/v) for 10 - 15 min. The unbound crystal violet solution was removed with a triplicate washing with PBS, and the plate, then, was kept for drying. Finally, all wells were filled with 200 µl ethanol (95%) to release dye from the well and Optical Density (OD) was taken at the wavelength of 630 nm. OD value of each test strain and negative control were calculated, and OD cutoff values (ODc) were assessed as described previously [30].

Data analysis

The results were expressed as percentages for the description of *Candida* isolates according to species and various clinical samples. Data were statistically analyzed using the chi-squared test. A value of $p < 0.05$ was considered significant.

Ethical approval

We obtained written consent from all cases. Assent was taken from participants before collecting the specimens. The study proposal was evaluated and approved by the Ethics Committee of Faculty of Medicine and Health Sciences, Sana'a University.

Results

There was a significant oral carriage rate of *Candida albicans*, *C. krusei* and *C. tropicalis* among denture wearers equivalent to 65.4%, 30.8% and 15.4%, respectively compared with only 11.1%, 7.4% and 3.7% among normal teeth individuals, respectively. Out of 144 *Candida* species tested, 69 (47.9%) were found to be biofilm producers. Maximum biofilm production was observed in the current study in *C. tropicalis* where 12 out of 20 isolates (60%) showed biofilm production followed by *Candida albicans* (48.75%) and *C. krusei* (40%). In our study the degree of biofilm was divided from high and moderate to non or weak; *C. tropicalis* showed 55% ability to produce a high level of biofilm formation, while only 15% of *C. albicans* showed that (Table 2). Positive biofilms were more observed with denture patients 64/104 (54.2%) versus 19.2% in non-denture wearer isolated strains. The association (odds ratio) between denture wear and biofilm formation was 4.97, with 95% CI= 1.7-14, and significant p value (p = 0.001) (Table 3). *In vitro* antifungal susceptibilities of various *Candida* species; showed in our study that all isolates were susceptible to amphotericin B and ketoconazole. Fluconazole resistance was found in 4% of *Candida albicans*, 25% in *C. krusei* and *C. tropicalis and 0% in C. glabrata* (Table 4). Biofilm strains showed relatively high resistance against Fluconazole14/69 (20.3%) compared to non-producing biofilm strains 5/75 (6.7%) (Table 5).

Organisms	Denture wearers (No. = 104)		Normal teeth (No. = 108)		Total N = 212	
	No.	%	No.	%	No	%
Candida albicans	68	65.4	12	11.1	80	37.7
C. krusei	32	30.8	8	7.4	40	18.9
C. tropicalis	16	15.4	4	3.7	20	9.4
C. glabrata	2	1.9	2	1.85	4	1.88

Table 1: *The yeast distribution in the denture wearer and non denture wearer groups of the study populations.*

Candia species	Biofilm detection by TCP						Total biofilm positive	
	High*		Moderate*		Non/weak*			
	No.	%	No.	%	No.	%	No.	%
Candida albicans n = 80	12	15	27	33.7	41	51.3	39	48.75
C. krusei n = 40	6	15	10	25	24	60	16	40
C. tropicalis n = 20	11	55	2	10	7	35	12	60
C. glabrata n = 4	0	0	1	25	3	75	1	25
Total n = 144	29	20.1	40	27.8	75	52.1	69	47.9

Table 2: *Biofilm detection by TCP method for different oral Candida species isolates.*

*TCP-*High-O.D (> 0.240), *Moderate-O.D (0.120 - 0.240), *Weak/Non-O.D (< 0.120).*

	Biofilm positive n = 69		OR	CI	X^2	p
	No	%				
Denture wearer n = 104 *Candida* isolates n = 118	64	54.2	4.97	1.7 - 14	10.5	0.001
Non denture wearer n = 108 *Candida* isolates n = 26	5	19.2	0.2	0.07 - 0.56	10.5	0.001
Total n = 144	69	47.9				

Table 3: *The association between denture wearing and biofilm formation of oral Candida species.*

Organisms	Fluconazole		Ketoconazole		Amphotericin B	
	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)
Candida albicans n = 80	76 (95)	4 (5)	80 (100)	0 (0)	100 (100)	0 (0)
C. krusei n = 40	30 (75)	10 (25)	40(100)	0 (0)	40 (100)	0 (0)
C. tropicalis n = 20	15 (75)	5 (25)	20(100)	0(0)	20 (100)	0 (0)
C. glabrata n = 4	4 (100)	0(0)	4(100)	0(0)	4 (100)	0 (0)
Total n = 144	125 (86.8)	19 (13.2)				

Table 4: *In-vitro antifungal susceptibility of oral Candida species isolated from denture wearer and non denture wearer.*

R= Resistant, S= Sensitive.

Antimicrobial agents	Biofilm producing *Candida* species n = 69	Non-biofilm producing *Candida* species n = 75	P value
Fluconazole	14 (20.3%)	5 (6.7%)	0.002
Ketoconazole	0 (0%)	0 (0%)	1.0
Amphotericin B	0 (0%)	0 (0%)	1.0

Table 5: *Antifungal resistance pattern of Candida species.*

Discussion

It is noted that, to date, most studies of *Candida* species have been carried out on suspension cultures; however, the medical effect of *Candida* species (such as that of many other microorganisms) depends on its ability to form surface-associated communities called biofilms [31,32]. Biofilms are recognized for their composition on many implanted medical devices, including catheters, pacemakers, heart valves, dentures, and artificial joints, which provide a surface and safe haven for the growth of biofilms [11-13,33]. The human health consequences of device -related infection can be severe and very life-threatening [32].

In the present study, there was a significant oral carriage rate for *Candida albicans*, *C. krusei* and *C. tropicalis* among denture wearers was 65.4%, 30.8% and 15.4%, respectively compared to only 11.1%, 7.4% and 3.7% among normal teeth individuals, respectively. Also, out of 144 *Candida* species 69 (47.9%) were found to be biofilm producers. This high rate of colonization and biofilm production of *Candida* species may lead to oral infections in our individuals or move to the respiratory and digestive systems. This suggestion can be confirmed by NHI analysis that indicates that biofilms in general (including bacterial and fungal biofilms) are responsible for more than 80% of all microbial infections [34]. For structural and physiological reasons, the biofilms are inherently resistant to antimicrobial therapy and immune defenses of the host. Biofilms cause many infections, ranging from infections of the superficial mucosa to severe, diffuse bloodstream infections. These infections are most frequently started from biofilms formed on mucosal surfaces or implanted medical devices, such as dentures.

Our study showed that among the *non-albicans* species, the biofilm positivity occurred most frequently among isolates of *C. tropicalis* (60%), also *C. tropicalis* showed the highest score of biofilm intensity 11/20 (55%). This result is similar to several published studies in which *C. tropicalis* was recognized as strong slime producers [35-37]. However, Kuhn., *et al.* [38] showed that *C. albicans* produces quantitatively more biofilm than other *Candida* species, but in that study the assessment of biofilm was based on quantitation and fluorescent microscopic examination proving that the biofilm formed by pathogenic *C. albicans* was a complex phenomenon composed of blastospore layer coved by a thick biphasic matrix, consisting of a dense extracellular component comprised of cell wall-like compounds and abundant hyphal elements composed of polysaccharide elements [38].

In the current study, *in vitro* antifungal sensitivity to various *Candida* species showed that all isolates were sensitive to amphotericin B and ketoconazole. However, resistance to fluconazole was found in 4% of *Candida albicans*, 25% in *C. krusei* and *C. tropicalis*; and 0% in *C. glabrata* (Table 4). Also, biofilm strains displayed relatively high resistance against tested fluconazole14/69 (20.3%) than non biofilm producers 5/75 (6.7%) (Table 5). This result can be explained by the facts that *Candida* biofilms are resistant to standard antifungal medications due to the availability of biofilms that are considered physical protection of fungi from medications, as well as cells in biofilms become essentially resistant to drugs due to their altered metabolic states and their constitutive up regulation of drug pumps [34]. *C. albicans* biofilm development *in vitro* can be divided into four phases: [39-44] (1) attachment and colonization of round yeast cells to a surface; (2) growth and proliferation of yeast cells creating a basal layer of anchoring cells; (3) growth of pseudohyphae (oval yeast cells joined end to end) and hyphae (long cylindrical cells) accompanying the production of the extracellular matrix and; (4) dispersal of cells

from the biofilm to find new sites to colonize. Recent studies suggest that these characteristics of biofilm formation also apply *in vivo*. For example, in *C. albicans* biofilms from denture stomatitis patients, yeast cells, hyphae and extracellular matrix were observed [45].

Our study showed that *C. albicans* was the predominant species recovered from oral cavity of both denture wearers and non-denture wearers. These findings are consistent with those previously reported by other researchers [2-5]. In a recent studies *C. albicans* was reported as the major agents of stomatitis [4,5]. Positive biofilms were more observed with denture patients 64/104 (54.2%) versus 19.2% in non-denture wearer isolated strains. The association (odds ratio) between denture wear and biofilm formation was 4.97, with 95% CI = 1.7 - 14 and significant p value (p = 0.001) (Table 3). Our data provide evidence that the majority of *Candida* species recovered from the dentures (biomaterials) (54.2%) have higher capacity to produce biofilm. Similar results were obtained by other studies [23,46]. Kuhn., *et al*. [38] reported that invasive *C. albicans* isolates form more biofilm than noninvasive isolates [38]. *Candida* species are frequently found in the normal microbial flora of humans, which facilitates their encounter through implanted biomaterials and host surfaces [22]. The devices become colonized by *Candida* which forms biofilm, the detachment of which can result in infections. Dentures therefore, represent a major risk factor associated with oral *Candida* infections [2-5].

In this study the resistance of all the isolated *Candida* species to fluconazole was 13.2%. The study by Nemati., *et al.* [47] and Mohamed and Al-Ahmadey [48] reported that the rate of resistance to fluconazole among *Candida* species ranged from null to the 15% [47,48]. Furthermore, our data on the fluconazole against *C. albicans*, revealed that 95% of tested strains were susceptible. This sensitivity rate is more or less comparable with those rates of 95%, 87.5% and 89.5% previously reported by Mohamed and Al-Ahmadey [48], Citak., *et al.* [49] and Badiee and Alborzi [50], respectively.

In agreement with the study of Mohamed and Al-Ahmadey [48] and Sabatelli., *et al.* [51], most of the detected resistant strains belong to *non-albicans* species (25%), emphasizing, its greatest potential to acquire resistance to fluconazole. Also, in agreement with the finding of Ng., *et al.* [28] who reported, amphotericin B and ketoconazole susceptibility data and showed that all yeast isolates were susceptible. The possibility of increase in the percentage of the resistance to antifungal agents among *Candida* species might be due to widespread use of antifungal drugs, long-term use of suppressive azoles, and the use of short courses of antifungal drugs [28].

Conclusion

The present study proved that *C. albicans* is still the major isolate from oral cavity, but non-*albicans* species colonization is raised; denture was factor for oral colonization of *Candida* species, and biofilm formation. The *C. tropicalis* were more biofilm - producers compared to *C. albicans*. The species isolated in the current study are less susceptible to fluconazole and drug resistant factor in the *Candida* species isolates was found to be associated with Candidal biofilm formation.

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Conflict of Interest

No conflict of interest associated with this work.

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