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Original Research Article

BIOFILM FORMATION AND ANTIFUNGAL SUSCEPTIBILITY OF *CANDIDA* ISOLATES FROM ORAL CAVITY AFTER THE INTRODUCTION OF FIXED ORTHODONTIC APPLIANCES

ABSTRACT

Background and aims: Orthodontic appliances serve as different impact zones and modify microbial adherence and colonization, acting as foreign reserves and possible sources of infection. This study was conducted to investigate the effect of fixed orthodontic appliances introduction on the growth and adherence (biofilm formation) of *Candida* species. And also determine the species distribution, and antifungal sensitivity to isolated *Candida*. **Material and methods**: The investigational group was selected from orthodontic patients whom were examined clinically as soon as to get baseline information before active treatment. The cluster included 210 patients; 45 males, 165 females (mean age 21.6 ± 4.5 years). Clinical, demographic data and risk factors were collected in standard questionnaire then each individual was directed to carry out oral wash by a phosphate-buffered saline solution, which was expectorated and processed intended for the isolation and identification of *Candida* species 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 colonized oral cavity after the introduction of FOA was *C. albicans* (72.5%), followed by *C. glabrata* and *C. tropicalis* (12.5%) , while *Candida parapsilosis only* was 2.5%. The rate of formation of biofilms was 52.5% for all types of *Candida*, and it was found that biofilm formation occurs more frequently among *C.tropicals and C. glabrata* (60%) than *C. albicans* (48.3%). All *candida* species isolates were sensitive to amphotericin B and ketoconazole while resistance to fluconazole was found in 40% of *C. tropicalis* and 20% in *C. glabrata* and 13.8% in *C. albicans*. **Conclusion:** The present study proved that *C. albicans* is still the major isolate from oral cavity after the introduction of FOA, but non-*albicans* species colonization is raised and FOA might be factor for biofilm formation. The *C. tropicalis* and *C. glabrata* 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* species; biofilm formation; antifungl resistance, oral cavity, fixed orthodontic appliances, FOA

INTRODUCTION

For a long time, the conventional orthodontic patient was considered a low-risk patient and the orthodontic process was regarded as non-invasive[1]. Nevertheless, these devices can be associated with difficulty cleaning. During treatment, festive areas are created that favor the accumulation of biofilms and bacterial and fungal growth [2]. One of the biggest challenges in orthodontics is to maintain proper oral hygiene during treatment. , bands and other accessories further exacerbate these conditions by retaining the dental plaque, which can lead to gingivitis and enamel demineralization, causing white spots, caries and *candida* stomatitis [3-6]. Microbiological studies have proven that after setting a fixed orthodontic appliance, the number of microorganisms increases significantly, especially *streptococci*, *lactobacilli* and *Candida*, exposing the oral environment to an imbalance and allowing the emergence of diseases [2-6].

Even though dental biofilms are made up of many types of microorganisms, yeast and bacteria are believed to be involved in the early development of oral and dental lesions[4-7]. Therefore, the success of orthodontic treatment lies in correcting the occlusion in the best possible way, in spite of this, without affecting the health of the teeth and the supporting tissues; unless, the benefits of treatment can be questioned[4,5,8]. The practice of orthodontics is undergoing continuous progress using new techniques and materials that benefit both patients and practitioners[9]. Emphasis has been placed on attempts to prevent the development of carious lesions or oral infections in orthodontic patients on controlling bacterial and yeast biofilms around the arcs [3-6]. During treatment, orthodontists are also responsible for preventing caries and preventing other mouth infections [4-6,8].

The orthodontic appliance acts as a different site for the formation of biofilms[10]. In a study by Eliades and others [11], stainless steel feet above critical surface tension can be expected to have a higher plaque retaining capacity. Also, metal orthodontic brackets have been found to stimulate specific changes in the oral environment such as

low pH levels and the affinity of microorganisms to a metal surface due to electrostatic reactions [12], increased plaque buildup, and colonization of organisms increased. However, other studies on possible differences in initial convergence and adherence to microorganisms on metal, ceramic and plastic brackets over time were inconclusive[4,5,13,14]. Thus, it is difficult to make a clear assessment that metal braces have less carcinogenic effect on the teeth or reduce colonization of the mucous membrane than plastic or ceramic braces. Inserting the orthodontic wire tends to create new surfaces available to form plaques and thereby increase the level of microorganisms in the oral cavity. It has long been suggested that orthodontic bands and wires lead to increased plaque buildup and elevated levels of *streptococcus*, *lactobacilli* and yeast. Additionally, orthodontic patients with fixed devices often offer an abundance of *S. mutans* per plaque compared to untreated orthodontic patients [15]. As a result, preventing microbial attachment to orthodontic wires is a major concern for orthodontists.

Candida species are most often recovered in the mouth, equal to 50% in young adults [5,16,17]. *Candida albicans* is the common species; on the other hand other species such as *C. parapsilosis, C. dubliniensis, C. krusei , C. tropicalis*, and *C. glabrata* have increased in occurrence with restricted drugs sensitive to them including allylamines, polyenes, azoles and echinocandins classes due to the development of drug resistance promptly to *Candida* species[4,5]. The yeast of the genus *Candida, albicans* species, has been analyzed because it is most common in oral mucosa. It has been proven that this yeast colonizes cement, enamel and dentine, and serves as a reservoir for the spread of infection[18]. However, the ability of yeast to remain on inactive surfaces needs further clarification in order to understand its virulence and dissemination routes[19,20]. This study was conducted to investigate the effect of fixed orthodontic appliances introduction on the growth and adherence (biofilm formation) of *Candida* species. And also determine the species distribution, and antifungal sensitivity to isolated *Candida*.

SUBJECTS AND LABORATORY METHODS

Subject Selection

A total of two hundred and ten people were included, during FOA treatment, who were randomly selected from Al-Thawra Hospital, Al-Gomhoria Hospital, Faculty of dentistry Sana'a and IBB University clinics and Dental Centers in Sana'a and IBB; including Alhasani clinic and Dental Center in IBB City, Yemen. The duration of the study was six months period, started in August 2019 and ended in February 2020. Inclusion criteria for subject selection were healthy individuals with no clinical signs of *Candida* infection and no systemic disease. In addition, individuals who currently taking antifungal, steroids, antibiotics, or immunosuppressive drugs in the past 6 months were excluded.

Collection and identification of samples: Saliva samples were collected using the oral rinse technique. In summary, each subject was required to rinse the mouth for 60 seconds using 10 mL of a phosphate sterile saline (PBS, 0.01M phosphate buffered saline, pH 7.2) and eject the rinse into a sterile 15 mL container[21]. The samples were immediately transported on ice to the microbiology laboratory. Each oral rinse was centrifuged at 3500 rpm for 10 minutes, and then the supernatant was discarded. The pellet was re-suspended in 1ml sterile PBS. One hundred µl of the concentrated oral rinse was inoculated onto Sabouraud's dextrose agar and incubated at 37 ^{o}C for 48 hours. The lasting samples were stored at -20o C. If *Candida* colonies appeared on the Sabouraud's dextrose agar, then chromogenic *Candida* agar was inoculated using 100µl 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. The *Candida* species were also identified by the ability to produce chlamydo-spores on glutinous rice agar[22].

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 [22]. 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 ≥15mm, susceptible; 14≥dz≥10mm, susceptible dose dependent and dz≤9mm, resistant. Fluconazole: dz ≥19mm, susceptible; 15≤dz≤18mm, susceptible dose dependent and dz≤14mm, resistant. As for ketoconazole: dz≥20mm, susceptible; 10<dz susceptible dose dependent and dz≤10mm, resistant [23].

Biofilm production detection

The detection of biofilm was done by tissue culture method/microtiter plate method (TCA)[24,25]. The yeast isolates from fresh agar plates were inoculated in 2 ml of BHI broth and incubated for 24 h 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 24 h 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[25].

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. The study proposal was evaluated and approved by the Ethics Committee of Faculty of Medicine and Health Sciences, Sana'a University.

RESULTS

Table 1 shows the age and gender distribution of patients with fixed orthodontics at a selected dental clinic in Sana'a. 78.6% of the participants are female and only 21.4% are male. The age average ± SD for participants was 21.6 ± 4.5 years. Most of the subjects covered were in the age group 21-25 years (55.7%) followed by 16-20 years (29%). Table 2 shows the distribution of different types of *Candida* species among Fixed Orthodontic patients. The predominant isolated *Candida* species were *C. albicans* with a significantly improved OCAC rate of 13.8% after the introduction of FOA. Also the rate of *Candida glabrata* and *Candida tropicalis* was 2.4% after the introduction of FOA; and *Candida parapsilosis* isolated from 1 patient (0.5%).

Out of 40 *Candida* species tested, 21 (52.5%) were found to be biofilm producers. Maximum biofilm production was observed in the current study in *C. tropicalis and Candida glabrata* where 3 out of 5 isolates (60%) showed biofilm production followed by *Candida albicans* (48.3%). In the present study the degree of biofilm was divided from high and moderate to non or weak; *C. tropicalis* showed 40% ability to produce a high level of biofilm formation, while only 17.2% of *C. albicans* showed that (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 13.8% of *candida albicans*, 40% in *C. tropicalis and 20% in C. glabrata* (Table 4). Biofilm strains showed relatively high resistance against Fluconazole 19% compared to non-producing biofilm strains 5.2% (Table 5).

DISCUSSION

The current study, explored OCAC rate through fixed orthodontic therapy, indicates that the wearing of such appliances leads to enhanced carriage and extensive changes in the oral microorganism population, probably due to the appliance-induced ecological alterations within the oral cavity. The OCAC primary absence of the baseline patient cluster was not unexpected, as applicants were requested to establish good oral hygiene prior to the trial. However, after the introduction of FOA, a 13.8 percent increase in the OCAC rate was observed in the test group. The incidence of orthodontic attachments on the labial and lingual surfaces of these teeth is likely to be the cause for this observation, as they interfere with thorough brushing of the gingival area. Similar changes in OCAC rate during orthodontic treatment with removable and fixed appliances have been reported by several authors[4-6]. Furthermore, the presence of rough-surfaced bonding material in FOA or dentures acting as a *candida albicans* trap and a gingival irritation [4,16,17,26,27] may have played a causative role. Thus, a significant increase in the OCAC rate after the introduction of FOA in the current study may be partly due to the patient's attitude and behavior, in addition to the presence of FOA which made it difficult to maintain dental hygiene. Knowing the growth and adhesion of cariogenic *streptococci* and *Candida albicans* to orthodontics will highlight a better way to prevent the enamel demineralization and the formation of white spots [28]. 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 [18, 29-31]. 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*, among FOA patients (13.8%). Also, out of 40 *Candida* species 21 (52.5%) were found to be biofilm producers. This high rate of colonization and biofilm production of *Candida* species in FOA patients 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[33]. 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 FOA.

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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 40%. This result is similar to several published studies in which *C. tropicalis* was recognized as strong slime producers [34-36]. However, Kuhn *et al,* [37] showed that *C. albicans* produces quantitatively more biofilm than other *Candida* species, but in that study the assessment of biofilm was based on quantization 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 [37].

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 13.8% of *Candida albicans*, 40% in *C. tropicalis*; and 20% in *C. glabrata* (Table 4). Also, biofilm strains displayed relatively high resistance against tested fluconazole 19% than non biofilm producers 5.2% (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 [33]. *C. albicans* biofilm development in vitro can be divided into four phases:[38-41] (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.

Our study showed that *C. albicans* was the predominant species recovered from oral cavity of FOA patients. These findings are consistent with those previously reported by other researchers [4-6,44]. In a recent studies *C. albicans* was reported as the major agents of stomatitis [5,6]. Our data provide evidence that the majority of *Candida* species recovered from the FOA (biomaterials) have higher capacity to produce biofilm. Similar results were obtained by other studies[45,46]. Kuhn *et al*, [37] reported that invasive *C. albicans* isolates form more biofilm than noninvasive isolates [37]. *Candida* species are frequently found in the normal microbial flora of humans, which facilitates their encounter through implanted biomaterials and host surfaces [47].

In this study the resistance of all the isolated *Candida* species to fluconazole was 17.5%. The study by Nemati *et al* [48], and Mohamed and Al-Ahmadey [49] reported that the rate of resistance to fluconazole among *Candida* species ranged from null to the 15% [48,49]. 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 [49], Citak *et al*, [50] and Badiee and Alborzi [51], respectively. In agreement with the study of Mohamed and Al-Ahmadey [49] and Sabatelli *et al.* [52], 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* [23] 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 [23].

CONCLUSION

The present study proved that *C. albicans* is still the major isolate from oral cavity of after introducing FOA, but non-*albicans* species colonization is raised; FOA 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.

AUTHOR'S CONTRIBUTION

This research work is part of a Master's thesis. The candidate is Manal Ahmad Saleh AL-amri to conduct laboratory, field work and thesis. Corresponding author (HAA), first author (AHA), second author (AAA), third author (HSA), and last author (MAA) supervised the work, revised and edited the thesis draft and the manuscript.

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CONFLICT OF INTEREST

"No conflict of interest associated with this work".

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Table 1: The age and sex distribution of patients with fixed orthodontics at a selected dental clinic in the city of Sana'a and Ibb.

characters	**number**	**percentage**
Sex		
Male	45	21.4
female	165	78.6
Age groups		
≤15 years	12	5.7
16-20 years	61	29
21-25 years	117	55.7
>25 years	20	9.5
Total	210	100
Mean age	21.6 years	
SD	4.5 years	
Median	21 years	
Mode	21 years	
Min	13 years	
Max	25 years	

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Table 2: Distribution of different types of *Candida* species among Fixed Orthodontic patients.

***Candida* species**	**Number**	**Rate from Total n=210**	**Rate from total isolates n=40**
Candida albicans	29	13.8	72.5
Candida glabrata	5	2.4	12.5
Candida tropicalis	5	2.4	12.5
Candida parapsilosis	1	0.5	2.5
Total Candida species	40	19	100

Table 3: Biofilm detection by TCP method for different oral *Candida* species isolates.

Candia species	**Biofilm detection by TCP**						**Total biofilm positive**	
	High*		**Moderate***		**Non/weak***			
	No.	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
Candida albicans n=29	5	17.2	9	31	15	51.7	14	48.3
Candida glabrata n=5	1	20	2	40	2	40	3	60
Candida tropicalis n=5	2	40	1	20	2	40	3	60
Candida parapsilosis n=1	0	0	1	100	0	0	1	100
Total n=40	8	20	13	32.5	19	47.5	21	52.5

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

Table 4: In-vitro antifungal susceptibility of oral *Candida* species isolated from Fixed Orthodontic patients.

Organisms	**Fluconazole**		**Ketoconazole**		**Amphotracin B**	
	S (%)	**R (%)**	**S (%)**	**R (%)**	**S (%)**	**R (%)**
Candida albicans n=29	25 (86.2)	4 (13.8)	29 (100)	0 (0)	29(100)	0 (0)
Candida glabrata n=5	4 (80)	1 (20)	5 (100)	0 (0)	4(100)	0 (0)
Candida tropicalis n=5	3(60)	2 (40)	5 (100)	0(0)	5 (100)	0 (0)
Candida parapsilosis n=1	1 (100)	0(0)	1 (100)	0(0)	1 (100)	0 (0)
Total n=40	33 (82.5)	7 (17.5)	40 (100)	0 (0)	40 (100)	0 (0)

R= resistant, S= sensitive

Table 5: Antifungal resistance pattern of *Candida* species associated with biofilm formation in oral *Candida* species isolated from FOA patients

Antimicrobial agents	**Biofilm producing Candida species n=21**	**Non-biofilm producing Candida species n=19**	***P* value**
Fluconazole	4 (19%)	1 (5.2%)	0.01
Ketoconazole	0 (0%)	0 (0%)	1.0
Amphotracin B	0 (0%)	0 (0%)	1.0