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Influence of Orthodontic Appliances on Oral Candida albicans and Molecular Study of Their Virulence Factors

Nebras Dawood Al-Jubouri
Department of Microbiology, College of Dentistry, University of Babylon, Hilla, Iraq,
dr.nebrasdawood1991@gmail.com

Nebrass Nasir-Alla Al-Dapagh
Department of Microbiology, College of Dentistry, University of Babylon, Hilla, Iraq,
nebrassnasir@yahoo.com

Wisam Wahab Sahib Alhamadi
Department of Orthodontics, College of Dentistry, University of Babylon, Hilla, Iraq,
dent.wisam.wahab@uobabylon.edu.iq

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ORIGINAL STUDY

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Influence of Orthodontic Appliances on Oral *Candida albicans* and Molecular Study of Their Virulence Factors

Nebras Dawood Al-Jubouri [a,*], Nebrass Nasir-Alla Al-Dapagh [a], Wisam Wahab Sahib Alhamadi [b]

[a] Department of Microbiology, College of Dentistry, University of Babylon, Hilla, Iraq
[b] Department of Orthodontics, College of Dentistry, University of Babylon, Hilla, Iraq

Abstract

Background: *Candida albicans* is commonly found in the oral cavities of individuals with denture stomatitis, especially in middle-aged and elderly adults who use dentures and orthodontic appliances.

Objectives: This study aims to investigate the influence of orthodontic appliances on oral *Candida albicans* and demonstrate the isolation and identification of *C. albicans* from orthodontic patients.

Materials ans Methods: Eighty swab samples were collected for culture preparation. The swabs were cultured on various culture media (such as Influence of Orthodontic Appliances on Oral Candida albicans and Molecular Study of their virulence factors, SDA, and Chrom agar) and the culture is examined for microbial growth. Microscopic examination and PCR technique were also used for identifying the isolated yeasts.

Results: The current investigation showed an infection with opportunistic microorganisms as follows: 17% of *C. albicans* from two times (T1 and T2) are positive for Candida *albicans* in orthodontic patients and negative in (baseline T0). In orthodontic isolates, the (*ALS*1) gene was strongly found in *C. albicans*.

Conclusion: Oral *Candida albicans* was more prevalent in orthodontic patients. Among orthodontic patients, *Candida albicans* was more prevalent than other *Candida* species. Oral *Candida albicans* with the gene (ALS1) was more prevalent with biofilm formation.

Keywords: *Candida albicans*, Orthodontic appliances, PCR technique, Virulence factors

1. Introduction

Orthodontic appliance is an appliance-intensive endeavor, where an array of mechanical devices is used to bring about tooth movement by remaining in close proximity to the enamel, gingiva, and periodontal ligament intra-orally over a prolonged period of time, having a significant impact on the paradental tissues, oral environment, and oral microbiome, by acting as anchors for biofilm and plaque formation [1].

The presence of an orthodontic appliance within the oral cavity can alter the microbial balance and composition of dental plaque and physiological characteristics of the oral cavity [2]. Causing impaired hygiene, an increase in dental plaque accumulation, and a delay in spontaneous physiological processes of tooth cleaning that result in pathogenic colonization, leading to gingival inflammation, periodontal support degradation, and enamel surface alterations [3]. It can alter the composition and structure of dental plaque, metabolism, and lead to an increase in the

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* Corresponding author.
E-mail addresses: dr.nebrasdawood1991@gmail.com (N.D. Al-Jubouri), nebrassnasir@yahoo.com (N.N.-A. Al-Dapagh), dent.wisam.wahab@uobabylon.edu.iq (W.W.S. Alhamadi).

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Table 1. Universal primer pairs used in this study for the identification of Candida albicans.

Primers	DNA sequence (5-3)	Reference	Product size (bp)
SACAL-F	5′-TTTATCAACTTGTCACACCAGA-3′	[10]	~354 bp
SACAL-R	5′-GGTCAAAGTTTGAAGATATACGT-3′		

number of microorganisms [4]. Dental plaque is a contributing factor to oral cavity illnesses like caries, gingivitis, periodontitis, and peri-implantitis [5].

Using brackets for orthodontic therapy brackets is a factor that promote tooth biofilm preservation due to cleaning and plaque accumulation issues, and as a result, pH shifts and the development of caries and gingivitis being common [6]. Orthodontic appliances may alter the subgingival microbiota due to challenges in maintaining dental cleanliness. These accessories promote bacterial plaque retention, which in turn fosters the growth of pathogenic bacteria [7].

Candida albicans isolated from human orals contributing to *candidiasis* cases, systemic illness, and reduced immune function, causing alterations in the oral cavity environment [8]. *Candida albicans* is frequently seen in the oral cavities of individuals with denture stomatitis, especially in middle-aged and elderly adults who wear dentures and orthodontic appliances [9].

2. Materials and methods

2.1. Patients and sample collection

Eighty swab samples were collected from orthodontic patients, whose ages ranged (18–35 years) during the period from (from November 2023 to February 2024. All specimens were obtained from orthodontic patients (oral swabs from tooth surfaces) in Babylon city. Information from all patients was recorded using proprietary data.

2.2. Specimens processing

The specimens were transferred to the laboratory, Al-Ameen Center for Research and Advanced Biotechnologies, in AL-Najaf Governorate where specimens were cultured using the streaking method on culture media, Sabouraud's dextrose agar, and chromagar then incubated at 37°C for (1–7) days for visible growth of *Candida* colonies; other growth was discarded as negative.

2.3. Ethical approval

The study was conducted in accordance with the Helsinki Declaration's ethical guidelines. Before taking the samples, the patients' verbal and analytical consent was obtained. To obtain this permission, a local ethics committee evaluated and approved the study protocol, subject information, and consent form using document number 6275 on 12/12/2023.

3. Identification of C*andida albicans* using PCR technique

3.1. Preparing the primers

Two species-specific primer pairs derived from the internally transcribed spacer (ITS) region (comprising ITS-1, 5.8S rRNA and ITS-2) of ribosomal DNA (rDNA) were designed for differentiation of *C. albicans* strains by conventional PCRThe primers used in this study were received in a lyophilized form and prepared according to the manufacturer's instructions by adding deionized distilled water to achieve a concentration of 100 pmol/μl as a stock solution, primers the working solution was prepared by adding 10 μl of stock solution to 90 μl of deionized distilled water to reach a final concentration of working solution 10 pmol/μl [10] (Table 1).

3.2. Culturing of the isolates

The fungal strains were streaked in the Sabourd dextrose agar (SDA) plate and incubated at 37°C for (24 and 48) hours for C*andida albicans*. A single colony of fungal organism was transferred from SDA plate and cultured in Sabouraud's dextrose broth (SDB) for (24 and 48) hours before proceeding to the DNA extraction.

3.3. Fungal DNA extraction

Total DNA was extracted from the culture broth, and 1.5 ml of culture broth was pipetted into Eppendorf tubes. Afterward, it was centrifuged at 4.300×g for 5 min, and the supernatant was discarded. Subsequently, 200 ul of TE buffer was added, vortexed well, boiled for 10 min, and then placed on ice immediately for 1 min. This was centrifuged at 6.700×g for 10 min, and the supernatant was collected, which was used as the DNA template [11].

3.4. PCR assay

The PCR assay was conducted to amplify sequences for identifying C*andida albicans*. PCR reaction mixtures were listed in (Table 2).

Table 2. PCR mixture component used in the reaction.

PCR content mixture	Volume
Master mix	12.5 μl
DNA template	7 μl
Forward primer	2 μl
Reveres primer	2 μl
Nuclease free water	1.5 μl
Total volume	25 μl

3.5. Identification of virulence factors of C. albicans using PCR technique

The primer coding for *C. albicans* and Virulence Factors Target gene ALS_1, according to [12]. Primer sequences and DNA sizes are as follows: F: 5′GACTAGTGAACCAACAAATACCAGA-3′; R: 5′-CCAGAAGAAACAGCAGGTGA-3′, the amplicon DNA size is ∼318 bp

PCR amplification system was used with the following program: initial denaturation for 5 min at 94°C, then 1 min at 94°C, 1 min at 52°C, 1 min at 72°C, 35 cycles; 5 min at 72°C, and for 1 cycle. PCR products were separated by electrophoresis on 1.3% (w/v) agarose gel, and stained with ethidium bromide.

4. Results and discussion

4.1. Cultures and properties of growth on (SDA and Chrom) agar

The culture examination on the SDA medium of specimens from orthodontic patients showed that the fungus grows rapidly and matures within 24–48 hours. The colony's texture was smooth, glistening, or dry, colored creamy to yellow. The growth of yeast on Chrom agar is color-coded based on genus and species, aiding in the identification of *Candida* isolates. Table 3 illustrates the colors of *Candida albicans* and its identification. This color appearance was consistent with the findings of [11, 13], and [14].

4.2. Identification and detection of virulence factors in Candida albicans

4.2.1. Formation of germ tubes

The results show the ability of *Candida* species to produce germ tubes. Seventeen isolates tested positive for this trait. The growth of C. albicans on Chromogenic Agar and Congo Red Agar and its germ tube formation are illustrated in (Table 3). *Candida albicans* has the ability to produce short, slender tube-like structures called germ tubes when incubated in serum for (2–3) hours at 37°C [15]. The germ tube test is a crucial indicator for identifying *Candida albicans.*

Table 3. Candida species on (Chromogenic Agar and Congo Red Agar) and their germ tube formation.

Candida species	No.	Colored on Chromogenic and Congo red agars	Germ tube test
Candida albicans	17	green colony (Chromogenic Agar) and black dry to dark red colonies (Congo Red Agar)	+

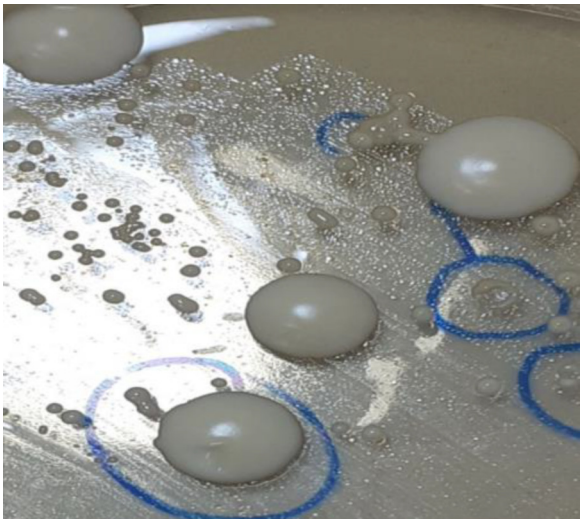

Fig. 1. Colonies of Candida albicans on SDA after incubation at 37°C for 48 hours.

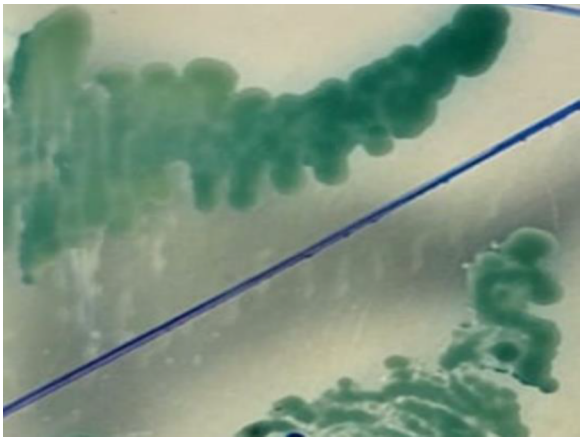

Fig. 2. The Candida albicans colonies on Chrome agar.

Positive testing for the germ tube formation of *C. albicans* was consistent with that reported by [16].

4.2.2. Biofilm formation

The results show that some species of *Candida* have the ability to form biofilms; the result was positive in the yeast *C. albicans,* which produced black, dry to dark red colonies. While isolates of weak biofilm producers remained pink, some darkening was observed

Table 4. Growth of Oral Candida albicans isolated from orthodontic patients and control.

Groups	Appliance	Oral *Candida albicans*	No.	%	p-value
Patients (N=40)	Fixed	Positive	11	100%	
		Negative	0	0%	0.683
	Removable	Positive	6	100%	
		Negative	0	0%	
Control (N=40)	–	Positive	0	0%	
		Negative	40	100%	

in the centers. Strains that do not form biofilms produced colonies that were either white or very light pink in color, consistent with the findings in [17].

The ability of *C. albicans* to form biofilms on abiotic or biotic surfaces is an important virulence factor, showing innate resistance to antibiotics, disinfectants, and clearance by host defense mechanisms. Biofilm formation is considered an important virulence factor in the pathogenesis of infections [18]. The Congo Red Agar method is qualitative, and this concurred with [19].

4.2.3. Prevalence of C. albicans in Orthodontic appliances

Orthodontic appliances are associated with retentive areas leading to the accumulation of food debris, which can favor the colonization of pathogens. Among them *Candida species;* orthodontic appliances, and particularly tooth movements, can induce the secretion of local inflammatory mediators, which may provide a favorable microenvironment for *Candida* colonization. *C. albicans* can actively modulate the host immune response and particularly potentiate cytokines secretion by releasing soluble factors [20, 21].

In the current investigation, 17(100%) of total isolates are *Candida albicans* positive in (fixed 11(100%) and 6 (100%) removable appliances) orthodontic patients (T1 and T2) and control negative (baseline T0), as shown in (Table 4). These results correspond with another study where *C.albicans* can be detected in the oral cavity. *C.albicans* was more prevalent followed by other *Candida* species [22, 23].

4.3. Identification and detection of Candida albicans by (PCR)

DNA amplification with universal fungal primers followed by detection using species-specific probes greatly improved the sensitivity of *Candida* detection [24]. Molecular techniques are targeted to detect *C. albicans* in a short period of time, with high sensitivity and specificity. For this purpose, several PCR methods have been developed, such as nested PCR, multiplex PCR, Taq-man PCR, Light-Cycler PCR and fluorescent PCR [25].

The phenotypic description of *Candida* may be insufficient and inadequate due to the similarities of morphological and biochemical characteristics with other *Candida* spp [26]. Therefore, in recent years, the identification of *Candida* has been done by modern genetic identification methods. In particular, PCR is the most common diagnostic method used for this purpose.

In this study, according to the identification of specimens that appear positive for the growth of *C.albicans* by PCR technique, we noticed that *C.albicans* appeared.

4.4. Detection of the virulence factor (ALS1 gene) of Candida albicans by (PCR)

Polymerase chain reaction (PCR) technology has a high level of sensitivity and specificity for detecting the presence of fungi, making it the most sensitive approach for detecting pathogen DNA in clinical samples [27]. Methods of cultivating fungi may occasionally yield conflicting results. The development of a reliable molecular approach for the identification and detection of *Candida albicans* based on the target amplicon's sequence variation [28].

PCR is a quick, sensitive, and accurate method for identifying and detecting *Candida albicans* by specific primers. Specimens from patients and controls showed positive results in oral specimens from all 17 (100%) of patients, with a higher incidence in orthodontic patients compared to controls. The incidence of oral *C. albicans* was observed to be higher in patients compared to controls; contributing factors include the patients' poor oral hygiene compared to controls [14, 29]. This current study agrees that orthodontic brackets greatly inhibit oral hygiene, predisposing to increased carriage of bacteria and yeasts. Brackets act as a reservoir of yeast and predispose to oral candidiasis, with predisposing factors leading to biofilm-forming activity [30]. However, the results of this study were incompatible with [31] how reported that the orthodontic appliances can consistently reduce Candida albicans colonization in the oral cavity.

The results showed *C. albicains* have the ability for formation biofilm, Identification of all specimens that tested positive for *Candida albicains* of patients by (PCR) technique revealed that 17% of the genes (ALS1) were detected in each 17% of these isolates, prevalence (biofilm formation) appeared from positive *C. albicains* growth, as shown in figures (4), this agrees with previous studies that showed (ALS) genes are associated with biofilm formation [32]. Factors including genes of the Agglutinin-like Sequence (ALS) protein that encodes large cell surface glycoproteins in *C. albicans* and provides adhesion to host surfaces,

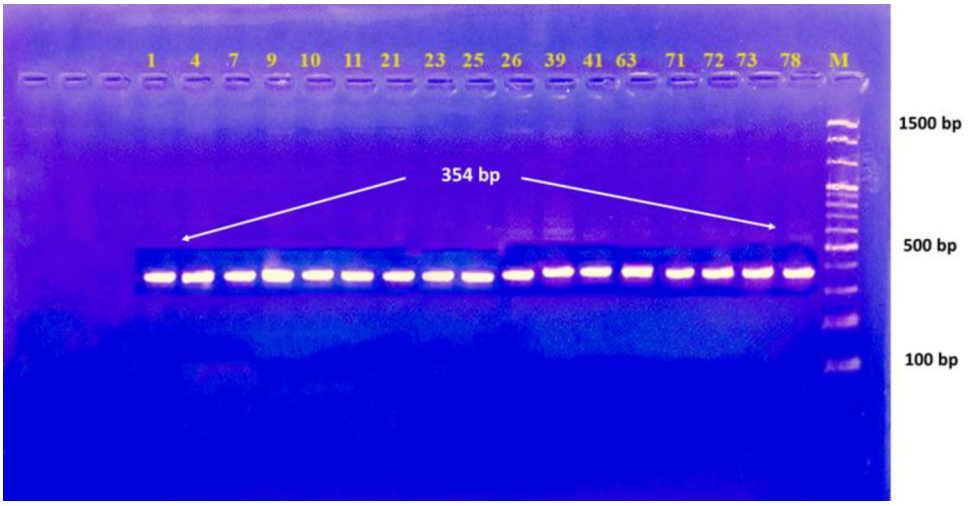

Fig. 3. Agarose gel electrophoresis (1.3% agarose gel, 100 volts for1 hour) of PCR product by specific primers SACAL for Candida albicans [M: Ladder, Isolates: 1,4,7,9,10,11,21,23,25,26,39,41,63,71,72,73,78, bp:354] Candida albicans isolated from oral orthodontic patients.

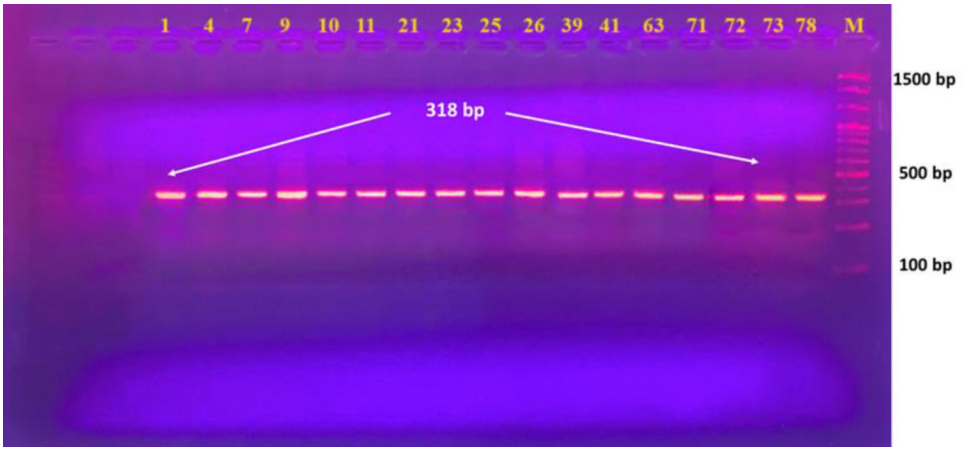

Fig. 4. Amplification of ALS1 gene (318 pb) by PCR technique of Candida albcains isolates [M: Ladder, Isolates: 1,4,7,9,10,11,21,23,25,26,39,41,63, 71,72,73,78].

these genes are the most important virulence factors [33, 34].

Virulence factors vary by microorganism according to its environment, living conditions, availability of nutrients, in addition to the most important element, which is host immunity, and yeast, like any microorganism, possesses weapons and factors that enable it to enter and penetrate host immunity, grow, reproduce, and activate the host's immunity through virulence factors such as adhesion and secretion. Some of these factors, like phospholipase, are considered as one of vital virulence factors as they lyse the cell membrane by hydrolyzing membrane lipids [35, 36]. Any enzyme able to cleave a specific ester bond an crucial for colonization and tissue penetration of host tissue, making phospholipase is extracellular hydrolytic enzyme similar to proteinase [37]. Also another factors including genes of the Agglutinin-like Sequence (ALS) protein that encodes large cell surface glycoproteins in *C. albicans* provides adhesion to host surfaces [14, 33].

These genes are the most important virulence factors of *C. albicans* [34]. According to this study, the identification of virulence factors of *C. albicans*. of specimen from orthodontic patients by PCR technique in orthodontic isolates. Our results from this study indicate that the capacity of the ALS1 gene was found strongly in *C. albicans*. This finding was in consistent with several authors worldwide [14, 33]. ALS proteins have been identified in *C. albicans* isolates. ALS1 proteins are responsible for adhesion and biofilm formation.

5. Conclusion

Orthodontic appliances, both fixed and removable, can increase the colonization and growth of *Candida albicans* in the oral cavity. The presence of *C. albicans*, significantly increases after the placement of orthodontic appliances. The reason for this that the appliances providing a favorable environment for *Candida* growth by trapping food debris and plaque, inhibiting mechanical removal by saliva, and allowing the yeast to adhere to the appliance surfaces. Patients with removable appliances seem particularly prone to increased oral *Candida* colonization. Overall, the evidence indicates that proper oral hygiene is essential for orthodontic patients to prevent the overgrowth of *Candida* species and potential oral infections.

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