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**RESEARCH ARTICLE**

# ANTIFUNGAL SUSCEPTIBILITY AND BIOFILM PRODUCTION OF *CANDIDA* SPP. ISOLATED FROM THE ORAL MUCOSA OF DENTURE PATIENTS AND ORTHODONTIC PATIENTS COMPARED TO HEALTHY CONTROLS

**Abeer Hasan Sharafuddin[1], Ebtihal Mohamed Madar[2,3], Khaled A AL-Haddad[4], Mohammed Mohammed Ali Al-Najhi[4,5], Taghreed Ahmed M Al-Kibsi[6], Hassan Abdulwahab Al-Shamahy[2,3]**

[1]*Oral Medicine,Oral Diagnosis, Periodontology and Oral Rediology Departement, Faculty of Dentistry, Sana'a University.*
[2]*Department of Basic Sciences, Faculty of Dentistry, Sana'a University, Republic of Yemen.*
[3]*Medical Microbiology and Clinical Immunology Department, Faculty of Medicine and Health Sciences, Sana'a University.*
[4]*Orthodontics, Pedodontics and Prevention Department Faculty of Dentistry, Sana'a University, Yemen.*
[5]*Orthodontics, Pedodontics and Prevention Department Faculty of Dentistry, Genius University for Sciences & Technology, Dhamar city, Republic of Yemen.*
[6]*Department of Oral and Maxillo-Facial Surgery, Faculty of Dentistry, Sana'a University, Republic of Yemen.*

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***Address for Correspondence:**
**Dr. Hassan Abdulwahab Al-Shamahy**, Department of Basic Sciences, Faculty of Dentistry, Sana'a University. Medical Microbiology and Clinical Immunology Department, Faculty of Medicine and Health Sciences, Sana'a University, Republic of Yemen. Tel: +967-1-239551.
E-mail: *shmahe@yemen.net.ye*

**Abstract**

**Background and objectives:** The intensity of the infection, the host's immune system, and the selected antifungal drug all affect how a *Candida* infection is treated. The aim of this investigation was to ascertain the prevalence of antifungal resistance in 148 isolates of oral *Candida* species and to explore any potential association between the formation of oral *Candida* biofilms and the rate of antifungal resistance in oral *Candida* isolates.
**Methods**: The 310 study participants, whose mean age±SD was equivalent to 37.01±20.9 years old, were split into 3 groups: 104 with dentures, 104 with orthodontic abaratus, and 102 controls without dental prosthesis. Of them, 58.1 percent were women and 41.9% were men. Next, the biofilm development of 148 isolates of *Candida* species was assessed using the Tissue Culture Palate Methods (TCPM) phenotypic technique. The E-test was subsequently used to ascertain the antibifungal susceptibility pattern of the 148 isolates for different medications.
**Results:** Seventeen isolates (11.5%) shown a high biofilm formation capacity, 45.4% a moderate biofilm formation capacity and the remaining isolates exhibited little or weak biofilm formation capacity when the isolates were submitted to the TCP method for biofilm detection. All of the examined isolates of *Candida* species were successfully combatted by amphotericin B, anidulafungin, and capsaicin. *Candida* species isolates showed varying resistance rates to fluconazole, voriconazole, itraconazole, posaconazole, and ketoconazole: 25%, 15.5%, 27%, 10.8%, and 16.2%, respectively.
**Conclusions:** Our findings indicate that azole-resistant *Candida* species are more common in Yemen. Therefore, a strategy to reduce the overuse and unnecessary side effects of antifungal drugs is urgently required. Fungal culture and antifungal susceptibility testing will be useful tools for resistance surveillance and patient care.
**Keywords**: Adult, anti-fungal resistance, biofilm formation, buccal mucosa, *Candida* species, oral cavity.

## INTRODUCTION

Since biofilms contain over 95% of all bacteria in nature, researchers can now study bacteria and fungus in their native environments thanks to recent technological advancements[1]. *Candida* species are the primary source of oral infections in people wearing prosthetics and are found as normal flora in healthy individuals. They are known to induce opportunistic infections with high fatality rates, particularly in immunocompromised individuals[2-8]. The highest percentage of non-*Candida albicans* isolation and the fast spreading resistance of *Candida* species are the most difficult clinical issues. Systemic diseases are the

fourth most common cause of nosocomial bloodstream infections in modern hospitals, and they are caused by *Candida* spp[9-11]. Of the several species of *Candida*, *Candida albicans* is the most common, causing infections that can be superficial or systemic. *Candida tropicalis*, *C. glabrata*, *C. parapsilosis*, and *C. krusei* are other pathogenic species that cause candidiasis; they account for 25%, 8%, 7%, and 4% of cases, respectively[6,12]. The development of virulence factors such as germ tube formation, adhesions, phenotypic switching, biofilm formation, and the generation of hydrolytic enzymes is crucial to the pathogenesis of candidiasis[9,10,13]. Biofilm development is the primary cause of most illnesses caused by *Candida* spp. Microorganisms known as biofilms are those that are embedded in extracellular matrix (ECM) and develop intricate three-dimensional structures on both biotic and abiotic surfaces[14]. Both mucosal surfaces and the plastic surfaces of indwelling devices have the potential to develop biofilms. Amphotericin B (AMB) and fluconazole (FLU) are two antifungal medicines to which biofilms are genetically resistant. Depending on the species of *Candida*, different biofilms form[15]. *Candida albicans* is the species that causes pathogenic consequences the most often, with other species contributing less[7,8,16]. As a result of widespread and prolonged use of azole, *Candida* exhibits resistance to it[17]. Recurrences of candidiasis are possible. Long-term antifungal medication prescriptions from some medical professionals might result in drug-resistant candidiasis, which is more challenging to cure. Consequently, early detection of *Candida* species and tracking of their susceptibility to antifungals aid in the course of treatment. Due to the fact that Yemen has produced relatively few studies on drug resistance and biofilm formation, the present study is undertaken to isolate *Candida* species from buccal mucausa of denture patients, OFA patients, and normal healthy individuals, detect biofilm formation, and study their antifungal susceptibility pattern and its association with biofilm development.

## MATERIALS AND METHODS

One hundred forty-eight oral *Candida* isolates from 310 patients who visited both private and Sana'a University Faculty of Dentistry dental clinics were tested for the capacity to form biofilms. Subsequently, the isolated *Candida* was phenotypically identified using established techniques in accordance with the 2015 recommendations (CLSI) of the Clinical and Laboratory Standards Institute[18].

**Biofilm production detection**

Biofilm was identified using the tissue culture/ microtiter plate technique (TCA)[19,20]. Yeast isolates on fresh agar plates were covered with two milliliters of Brain Heart Infusion (BHI) broth, and the plates were then incubated at 37°C for the entire day. Each microtitration plate received 200 µl of the sample that had been diluted 1:40 times with fresh medium (BHI broth supplemented with 1% glucose). The plates were then incubated for a further 24 hours at 37°C. Free floating sessile *Candida* was removed by repeatedly rinsing it with phosphate buffer saline (pH 7.2) after gently tapping the contents. The yeast was maintained with 2% sodium acetate after attaching to the surface and creating biofilms, and it was then colored with 0.1% w/v crystal violet for ten to fifteen minutes. The plate was allowed to dry after the unbound crystal violet solution was removed using three different PBS washes. After releasing the dye in each well with 200 µl of 95% ethanol, an Optical Density (OD) measurement was made at 630 nm. Each test strain's and the negative control's OD values were computed, and the OD cutoff values (ODc) were evaluated in accordance with the previously mentioned reasons[20,21].

**Antifungal sensitivity testing (Epsilometer test)**

Amphotericin B, voriconazole, caspofungin, fluconazole, ketoconazole, itraconazole, posaconazole, and anidulafungin (bioMérieux, France) were used in antifungal susceptibility studies.

**Etest strips**: In compliance with the manufacturer's instructions, the test was conducted. The agar plates were made with RPMI-1640 medium (Sigma, USA), which was supplemented with 1.5% agar and 2% glucose. Additionally, 0.165 mol L-1 MOPS (3-[N-morpholino] propanesulfonic acid) (Sigma, USA) was used to buffer the medium to a pH of 7.0. After suspending yeast colonies in saline, the final inoculum's turbidity was adjusted to 0.5 McFarland. Using a sterile swab, inoculate the agar plates by dipping it into the suspension and swabbing the surface in three different directions. Using sterile forceps, test strips were placed to the agar surface of the plates after they had been left to dry for fifteen minutes in a safety cabinet. The plates were incubated for 24 to 48 hours at 35°C or in ambient air. The drug concentration was recorded at the point where the ellipse intersected the MIC scale on the Etest strip, which was the lowest inhibitory concentration (MIC), which was found to be 80% inhibition for the azoles and echinocandins and 100% inhibition for amphotericin B. The Clinical and Laboratory Standards Institute (CLSI) M27-S4 document's species-specific breakpoints were utilized to assess an isolate's susceptibility to caspofungin, anidulafungin, voriconazole, fluconazole, and itraconazole[18]. Table 5 presents these breakpoints. The CLSI M27-S4 paper lacks interpretation criteria for amphotericin B, ketoconazole, and posaconazole. Thus, MIC breakpoints suggested by earlier researchers were applied to amphotericin B and ketoconazole[22-25], whereas voriconazole breakpoints were used to posaconazole. For amphotericin B, isolates having minimum inhibitory concentrations (MICs) of less than 1 µg/mL, ketoconazole ≤ 0.125 µg/mL, and posaconazole ≤ 0.125 µg/mL were deemed sensitive. It was determined that isolates exhibiting MICs ranging from 0.25 µg/mL to 0.5 µg/mL for ketoconazole were sensitive in a dose-dependent manner. It was determined that isolates exhibiting MICs ≤ 0.25 - 0.5 µg/mL for posaconazole demonstrated intermediate resistance. Reactions having minimum inhibitory concentrations (MICs) of at least 2 µg/mL for amphotericin B, at least 1 µg/mL for ketoconazole, or at least 1 µg/mL for posaconazole were deemed resistant.

**Statistical Analysis**: Epi-Info Statistics version 7 was utilized to examine the information. A statistical study was performed to account for the degree of drug resistance of 148 *Candida* isolates with varying degrees of biofilm development. This was done by calculating the difference, 95*% CI*, and *p*-value of the antifungal resistance for each tested antifungal with level of biofilm production.

**Ethical Consideration**: On August 21, 2022, the Medical Ethics and Research Committee granted the Sana'a University Faculty of Medicine and Health Sciences ethical authority for the Contract No. 217 project. The code of ethics established by the review committee was consistently followed. The selected participants gave their written and informed consent.

## RESULTS

The 310 participants in the study were divided into 3 groups 104 with dentures, 104 with orthodontic abaratus, and 102 controls without dental prostheses with a mean±SD of age equal to 37.01±20.9 years old. Of these, 41.9% were male and 58.1 were female. The age group of 21–30 years old comprised the majority of participants (25.8%), followed by ≥51 years old (23.9%) and 31–40 years old (22.3%). 34.8% (108/310) of the samples had *Candida* colonization (Table 1). For the first time, *Candida kefyr*, *Candida krusei*, *Candida famata*, *Candida africana*, and *Candida stellatoidea* were isolated from the oral cavities of Yemeni dental patients. Moreover, mixed cultures of two to three species of *Candida* were found in 44 cases (14.2%) out of 310 people.

**Table 1: General characteristics of participate in the study.**

| Characters | N (%) |
|---|---|
| Sex | |
| Male | 130 (41.9) |
| Female | 180 (58.1) |
| Ages (years) | |
| <21 years | 50 (16.1) |
| 21-30 | 80 (25.8) |
| 31-40 | 69 (22.3) |
| 41-50 | 40 (12.9) |
| ≥51 | 74 (23.9) |
| Mean age | 37.01Years |
| SD | 20.9 Years |
| Mode | 23 Years |
| Median | 26 Years |
| Min-Max | 9- 90 Years |
| Type of patients | |
| Denture | 104 (33.5) |
| orthodentic | 104 (33.5) |
| Normal | 102 (32.9) |
| Total | 310 (100) |

The final statistical analysis included 310 qualified research participants in total. 34.8% of the 108 individuals with OCC had a prevalence rate. Total 108 OCC patients had 148 oral *Candida* spp. identified. *C. albicans* (49.1%) was the most often isolated species throughout our study, followed by *C. glabrata* (35.2%) and *C. dubliniensis* (13%), as shown by the species distribution in Table 2.

**Table 2: Distribution of *Candida* strains (*n* = 108 patient) 148 isolated from denture, FOA and normal teeth individuals (*n* = 310).**

| Species | n (%) |
|---|---|
| *Candida albicans* | 53 (49.1) |
| *Candida glabrata* | 38 (35.2) |
| *Candida dubliniensis* | 14 (13) |
| *Candida tropicalis* | 15 (13.9) |
| *Candida famata* | 8 (7.4) |
| *Candida kefyr* | 8 (7.4) |
| *Candida krusei* | 4 (3.7) |
| *Candida parapsilosis* | 3 (2.8) |
| *Candida africana* | 3 (2.8) |
| *Candida stellatoidea* | 2 (1.9) |
| Single growth *Candida* isolates | 68/148 (45.9) |
| Mixed growth *Candida* isolates | 80 /148 (54.1) |
| Total *Candida* isolates | 148 |
| Mono-infection cases | 64/310 (20.6) |
| Co-infection cases | 44/310 (14.2) |
| Positive *Candida*iasis cases | 108/310 (34.8) |

The presence of non-*albicans* species was most frequently associated with co-infection with *Candida albicans* and/or *Candida glabrata*. Table 3 gives the interpretation of biofilm production by the tested *Candida* based on the average biofilm formation with an OD value obtained from the tissue culture plate method. Out of all the *Candida* that were studied, 42 species (36.5%) had a weak ability to produce biofilms (OD=0.17–0.34), and 32 species (21.6%) had a negative ability to do so (OD<0.17). 45.4% of the artificially isolated *Candida* showed moderate positive (OD=0.35-0.68), whereas only 17 (11.5%) of the examined *Candida* showed significant positivity for biofilm production (OD>0.68). Table 4 shows the correlation between antifungal resistance and *Candida* biofilm formation in isolates from patient buccal mucosa. For instance, the differential in fluconazole resistance was 26.5%, meaning that as compared to negative/weak strains, biofilm-producing bacteria (moderate/strong) have a 26.5% resistance to fluconazole. With $p<0.0001$, this result is extremely statistically significant, and the rate ranges between

13.9 and 39.3%. In conclusion, moderate/strong biofilm-producing strains of *Candida* had a higher rate of drug resistance against the isoniconazole, ketoconazole, voriconazole, and posaconazole studied than did negative/weak biofilm-producing strains. All *Candida* species were sensitive to amphotericin B, anidulafungin, and capsofungin, although there was no resistance to these drugs.

**Table 3: Interpretation of biofilm production by *Candida* isolates based on optical density values of tissue culture plate method Average value of OD* Biofilm production.**

| OD value | N (%) |
|---|---|
| <0.17, Negative | 32 (21.6) |
| 0.17-0.34, Weak positive | 54 (36.5) |
| 0.35-0.68, Moderate positive | 45 (30.4) |
| >0.68, Strong positive | 17 (11.5) |
| Total | 148 (100) |

## DISCUSSION

According to research, *Candida* species are commensal and need to disrupt the host's normal defensive mechanism in order to function as pathogens. A concerning opportunistic disease, candidiasis has been more prevalent due to an increase in patients who are immunocompromised, elderly, using antimicrobial and harsh cancer chemotherapy, or having invasive surgical procedures and organ transplantation[7,9,26]. Biofilms are ubiquitous, intricate, mutually reliant communities of microbes attached to surfaces that are encased in an exopolysaccharide matrix. They can be found on various surfaces, including those of medical devices[9,10]. *Candida* species pathogenicity is linked to their capacity to produce biofilms, which is a crucial factor in virulence during candidiasis[26]. Total 62% of *Candida* species produced biofilms, which is considered moderate or strong, according to the current study. This outcome is almost identical to what Kumar *et al.*,[27] reported.

The elevated frequency of *Candida* species colonization and biofilm formation in oral mucosa among the study participants may result in oral infections or spread to the respiratory and digestive systems. This theory is supported by NHI analysis, which shows that over 80% of all microbial illnesses are caused by biofilms, including bacterial and fungal biofilms[29]. The biofilms are intrinsically resistant to both the host's immune system and antimicrobial therapy due to structural and physiological factors. Numerous diseases, from serious, diffuse bloodstream infections to infections of the superficial mucosa, are brought on by biofilms. The most common source of these infections is biofilms that develop on mucosal surfaces or on implanted medical devices, including dentures and FOA[9,10].

**Table 4: Association of biofilm formation and Antifungal resistant of *Candida* species isolated from buccal mucosa of patients, n=148.**

| | Total n=148 | Biofilm Negative/weak N=86 | Biofilm Modrate/strong N=62 | DF (95% CI) | *p* value |
|---|---|---|---|---|---|
| Antifungal Agents | Resistance N (%) | Resistance N (%) | Resistance N (%) | | |
| Fluconazole | 37 (25) | 5 (5.8) | 20 (32.3) | 26.5 (13.9-39.3) | <0.0001 |
| Itraconazole | 23 (15.5) | 6 (7) | 17 (27.4) | 20.4 (8.3-33.1) | 0.0008 |
| Ketoconazole | 40 (27) | 15 (17.4) | 25 (40.3) | 22.9 (8.2-36.9) | 0.002 |
| Voriconazole | 16 (10.8) | 5 (5.8) | 11(17.7) | 11.9 (1.5-23.7) | 0.02 |
| Posaconazole | 24 (16.2) | 7 (8.1) | 17 (27.4) | 19.3 (7-32) | 0.0017 |
| Amphotericin B | 0 (0.0) | 0 (0.0) | 0 (0.0) | - | - |
| Anidulafungin | 0 (0.0) | 0 (0.0) | 0 (0.0) | - | - |
| Caspofungin | 0 (0.0) | 0 (0.0) | 0 (0.0) | - | - |

DF=difference

In the current study, moderate/strong biofilm-producing strains of *Candida* had a greater rate of medication resistance to the tested imitraconazole, ketoconazole, voriconazole, and posaconazole than did negative/weak biofilm-producing strains (Table 4). The following information can be used to explain this outcome: Changes in metabolic states and constitutive activation of drug pumps cause cells in biofilms to become effectively resistant to drugs[28]. Because biofilms are available, which are hypothesized to provide fungus physical protection against drugs, *Candida* biofilms are also resistant to traditional antifungal medications. Four stages comprise the development of albicans biofilm *in vitro*[29-32] (1) Round yeast cells attach to surfaces and begin to colonize them; (2) yeast cells grow and proliferate, forming a basal layer of anchoring cells; (3) yeast cells grow into long cylindrical cells called hyphae and pseudohyphae, which grow in tandem with the production of extracellular matrix; and (4) yeast cells disperse from the biofilm to find new sites to colonize. Antifungal resistance in *Candida* species is important to track because it can provide information about new, emerging dangers from resistant strains of the disease that can aid in empirical treatment. In our analysis, all 148 of the isolates of *Candida* were found to be amphotericin B susceptible, which is consistent with the findings published by Arora *et al.*[33]. According to Table 4, the resistance rates for fluconazole, itraconazole, ketoconazole, posaconazole, and voriconazole in the current investigation were, respectively, 25%, 15.5%, 27%, 16.2%, and 10.8%. However, Yenisehirli *et al.*, found that among *C. albicans*, fluconazole and voriconazole resistance rates

were 34% and 14%, respectively[34]. A hundred and five *Candida* isolates that were collected from various clinical specimens had a 34.3% fluconazole resistance rate, according to research published by Jayalakshmi *et al.*[35]. Fourty two out of 295 *Candida* isolates showed decreased fluconazole susceptibility, according to a research by Pelletier *et al.*[36]. Our fluconazole and voriconazole resistance rates are consistent with those seen in previous research. The study participants' extensive and prolonged usage of fluconazole and voriconazole may have contributed to their potential decreased susceptibility to those antifungals. Furthermore, our 25% resistance rate to fluconazole is lower than Fluconazole resistance was found in 55.2% of the *C. albicans* strains that were isolated from candiduria, according to research by Zarei Mahmoudabadi *et al.*[37].

**Table 5: CLSI Breakpoints (BP) for *C. albicans* (µg/mL).**

| *C. albicans* | Susceptible | Susceptible Dose-Dependent | Intermediate | Resistant |
|---|---|---|---|---|
| **Fluconazole** | | | | |
| M27-A3 BP | ≤ 8 | 16 - 32 | - | ≥ 64 |
| M27-S4 BP | ≤ 2 | 4 | - | ≥ 8 |
| **Voriconazole** | | | | |
| M27-A3 BP | ≤ 1 | - | 2 | ≥ 4 |
| M27-S4 BP | ≤ 0.12 | - | 0.25 - 0.5 | ≥ 1 |
| **Caspofungin** | | | | |
| M27-A3 BP | ≤ 2 | - | - | - |
| M27-S4 BP | ≤ 0.25 | - | 0.5 | ≥ 1 |
| **Anidulafungin** | | | | |
| M27-A3 BP | ≤ 2 | - | - | - |
| M27-S4 BP | ≤ 0.25 | - | 0.5 | ≥ 1 |

The same authors also found that *C. albicans* had a 59.2% fluconazole resistance rate in another investigation[38]. Prior research on fluconazole resistance rates has revealed modest rates, which is consistent with our findings[9,10,39-44]. Our study's resistance rates to fluconazole, itraconazole, and voriconazole were comparable to those found in a prior investigation carried out in an area west of Turkey[45]. Diverse breakpoint values, azole exposure in the past, and variations in the patient population could all contribute to variations in these resistance rates. It is crucial to highlight that in order to assess *C. albicans* strain susceptibility to fluconazole, itraconazole, and voriconazole, CLSI recently defined new species-specific MIC breakpoints.

Twenty five percent of the identified species of *Candida* in this investigation were resistant to fluconazole. According to studies by Mohamed and Al-Ahmadey[47] and Nemati *et al.*[46], the rate of fluconazole resistance in *Candida* species ranged from 0% to 15% [46,47]. Additionally, research on the effectiveness of fluconazole against *Candida* has shown that 75% of tested strains were sensitive. This sensitivity rate is not as comparable as the 95%, 87.5%, and 89.5% rates that were previously reported by Badiee and Alborzi[49], Citak *et al.*[48], and Mohamed and Al-Ahmadey[47]. In line with research by Mohamed and Al-Ahmadey[47] and Sabatelli *et al.*[50], the majority of resistant strains found (25%) are from non-albicans species, highlighting their highest potential for developing fluconazole resistance. Additionally, in line with the findings of Ng *et al.*[51], who published data on the sensitivity of all yeast isolates to ketoconazole and amphotericin B. Short courses of antifungal medication, long-term use of suppressive azoles, and widespread use of antifungal medicines may all contribute to a rise in the percentage of antifungal agent resistance among *Candida* species[51].

**Limatation of the study**

The biofilm development of the present study of *Candida* species was evaluated using the Tissue Culture Phenotyping Methods (TCPM) technique, and another sensitivity comparison technique should be performed to obtain a more accurate result for detecting the presence of biofilms in the examined fungi. The E test was also used to determine patterns of susceptibility to antifungals, and patterns of susceptibility to antifungals had to be determined and confirmed by genetic methods.

## CONCLUSIONS

Our findings suggest that the emergence of biofilms could play a role in the development of drug resistance. The majority of yeast, including *Candida*, are found in biofilm form, which is a significant issue for the medical community. Because *Candida* uses biofilms to survive, their innate immune response and anti-fungal properties make them very difficult to cure. Understanding the mechanism underlying the generation and regulation of oral *Candida* biofilms is essential for the development of anti-biofilm drugs and mouthwashes that prevent the formation of biofilms.

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## AUTHOR'S CONTRIBUTIONS

**Madar EM:** writing original draft, literature survey. **Sharafuddin AH:** methodology, conceptualization. **AL-Haddad KA:** formal analysis, review. **Al-Najhi MMA:** investigation, conceptualization. **Al-Kibsi TAM:** data curation, investigation. **Al-Shamahy HA:**

critical review, supervision. All authors revised the article and approved the final version.

## DATA AVILIABILITY

The empirical data used to support the findings of this study are available from the corresponding author upon request.

## CONFLICT OF INTEREST

None to declare.

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