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Assessing Antifungal Resistance and Biofilm-Forming Capacity of *Candida parapsilosis* Isolates from Makeup Sponges

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Abstract: *Candida parapsilosis* is an opportunistic pathogen that can colonize human mucous membranes and skin and develop resistance to antifungal treatments due to its ability to form biofilms. This study investigated the biofilm formation capacities and antifungal resistance of *Candida parapsilosis* isolates obtained from makeup sponges. The biofilm formation capacities among 46 *Candida parapsilosis* isolates were determined using the crystal violet quantitative method. Amphotericin B resistance in the isolates was evaluated with the agar disk diffusion method. The antifungal resistances of biofilm-producing species were assessed using the gradient test method. The crystal violet analysis revealed that most isolates were weak biofilm producers, with 17 exhibiting moderate biofilm production. It was determined that the isolates gave inhibition zones between 19.73 ± 1.54 mm and 23.71 ± 0.70 mm against amphotericin B by the agar disk diffusion method. It was concluded that 17 isolates with moderate biofilm production were susceptible to the antifungal agents clotrimazole, fluconazole, itraconazole, amphotericin B, and nystatin. The study emphasizes that makeup sponges may serve as reservoirs for *Candida parapsilosis* and highlights the importance of evaluating the antifungal susceptibility of biofilm-forming isolates. These findings are significant for the hygienic use of personal care products and implementing infection control measures.

Keywords: *Candida parapsilosis*, Makeup sponge, Antifungal resistance, Biofilm.

Makyaj Süngerlerinden İzole Edilen *Candida parapsilosis* İzolatlarının Antifungal Dirençliliği ve Biyofilm Oluşturma Kapasitelerinin Belirlenmesi

Öz: *Candida parapsilosis*, insan derisini ve mukoza zarlarını kolonize edebilen, biyofilm oluşturma yeteneği nedeniyle antifungal tedavilere direnç geliştirebilen fırsatçı bir patojendir. Bu çalışmada makyaj süngerlerinden izole edilen *Candida parapsilosis* izolatlarının antifungal dirençlilikleri ve biyofilm oluşturma kapasiteleri araştırılmıştır. 46 *Candida parapsilosis* izolatı arasındaki biyofilm oluşturma kapasiteleri kristal viyole kantitatif yöntemi kullanılarak belirlenmiştir. İzolatlardaki amfoterisin B direnci agar disk difüzyon yöntemi kullanılarak değerlendirilmiştir. Biyofilm üreten türlerin antifungal dirençlilikleri ise gradyan test yöntemi ile değerlendirilmiştir. Kristal viyole analizi sonucunda, izolatların çoğunun zayıf biyofilm üreticisi olduğu 17 izolatın ise orta düzeyde biyofilm üreticisi olduğu belirlendi. Agar disk difüzyon yöntemiyle amfoterisin B'ye karşı izolatların 19,73 ± 1,54 mm ile 23,71 ± 0,70 mm arasında inhibisyon zonu verdiği tespit edildi. Orta düzeyde biyofilm üreticisi olan 17 izolatın ise flukonazol,

itrakonazol, klotrimazol, amfoterisin B ve nistatin antifungallerine duyarlı olduğu belirlendi. Çalışmada, makyaj süngerlerinin *Candida parapsilosis* için rezervuar görevi görebileceğini ve biyofilm oluşturan izolatların antifungal duyarlılığının değerlendirilmesinin önemini vurgulamaktadır. Bu bulgular, kişisel bakım ürünlerinin hijyenik kullanımı ve enfeksiyon kontrol önlemlerinin uygulanması açısından önemlidir.

Anahtar Kelimeler: *Candida parapsilosis*, Makyaj süngeri, Antifungal direnç, Biyofilm

Introduction

The genus *Candida* is a natural component of the human microbiota and is commonly found in the gastrointestinal tract, mucosal membranes, and skin. However, these organisms are opportunistic pathogens, and the infections they cause are called candidiasis (Branco et al., 2023). *Candida* species can lead to mucosal, cutaneous, and deep-seated organ infections, as well as nosocomial fungal systemic bloodstream infections. Although *Candida albicans* is the most frequent cause of diseases, the incidence of invasive infections caused by non-*Candida albicans* (NAC) species, including *Candida auris, Candida parapsilosis*, *Candida tropicalis*, *Candida krusei, and Candida glabrata* has steadily increased over the past two decades (Tóth et al., 2019; Branco et al., 2023; Govrins and Lass-Flörl, 2024).

Candida parapsilosis is a member of the human microbiota that effectively colonizes human skin and mucous membranes. However, it has been isolated from various non-human sources, including domestic animals, insects, marine, and soil environments, and is widely found in nature (Branco et al., 2023). *Candida parapsilosis, Candida metapsilosis*, and *Candida orthopsilosis* are part of the *Candida parapsilosis* complex, also referred to as *Candida parapsilosis* sensu lato. Species within *Candida parapsilosis* sensu lato can be rapidly and reliably identified using MALDI-TOF MS (Matrix-assisted laser desorption ionization-time of flight mass spectrometry) (Govrins and Lass-Flörl, 2024).

Biofilms, which are mixed communities of microorganisms formed by the adhesion of microorganisms to surfaces through an extracellular matrix, are linked to high levels of antifungal resistance (Silva et al., 2017). The biofilm-forming ability of the *Candida parapsilosis* contributes to the microorganism's persistence on host surfaces and medical devices and its resistance to antifungal agents (Branco et al., 2023). In recent years, the capacity of the *Candida parapsilosis* strain to develop resistance to antifungal drugs and its biofilm-forming ability have emerged as significant research topics in infection control (Branco et al., 2023; Govrins and Lass-Flörl, 2024).

Recent studies (Bashir and Lamber, 2020; Agi et al., 2023; Ogbonna et al., 2024), including experiments conducted on social media, have shown that personal care products and cosmetics are susceptible to microbial contamination. Makeup sponges, which are made from various materials with differing pore structures, can create an ideal environment for the growth of microorganisms due to a combination of cosmetic products, user practices, and environmental factors. As these applicators are used repeatedly daily, makeup sponges foster conditions conducive to the adhesion and spread of microorganisms. However, only two studies in the literature address the antifungal resistance profiles of microorganisms found in these sponges (Akgül and Bakan, 2021; Ogbonna et al., 2024), and no studies have examined their ability to form biofilms. Therefore, this study aimed to evaluate the antifungal susceptibility profiles and biofilm formation capabilities of *Candida parapsilosis* strains isolated from makeup sponges.

Material and Metod

Isolates

In the study, isolates obtained from an investigation into microbial contamination of makeup sponges, supported by the code ÇOMÜ BAP-FLÖAP-2022-3934 and with permission from the ÇOMÜ LEE Ethics Committee were used. These sponges were collected from 26 female researchers aged 18-26, literate, using make-up, residing in Çanakkale, who agreed to participate in the study between 01/03/2022 and 31/12/2022. This study used 46 *Candida parapsilosis* isolates isolated from makeup sponges and identified by MALDI-TOF MS. The isolates, stored in Sabouraud Dextrose Agar (SDA) (Biolife, Italy) at +4°C, were first revived in SDA medium. Subsequently, the purity of the isolates was verified in HiCrome™ Candida Differential Agar (Himedia, India) medium before initiating the analyses. A standard culture of *Candida parapsilosis* (ATCC 22019) was used as a control for the antifungal sensitivity tests.

Determination of Biofilm Formation Potential of Isolates

Quantitative assessment of biofilm formation was conducted using the crystal violet method described by Özcan Ateş and Otkun (2023). *Candida parapsilosis* isolates were revived overnight at 37°C in Sabouraud Dextrose Broth (SDB) (Biolife, Italy). The density of these

cultures was then adjusted to OD_{600} = 1.0 in SDB medium containing 8% glucose. 200 µL of the cell suspension was added to the wells of 96-well flat-bottom microplates. After 48 h incubation at 37°C, the wells were washed three times with SPS (Sterile Physiological Saline, 0.85% NaCl w/v) to remove planktonic cells. Then, 99% methanol (Merck, Germany) was added to fix the cells and waited for 15 minutes. Afterward, 1% (v/v) crystal violet dye was added to make the fixed biofilm visible. Waited for 15 minutes, excess dye was washed with sterile distilled water. Then microplates were dried. Finally, 33% acetic acid (Merck, Germany) was added to each well and measurements were made at a wavelength of 570 nm using a microplate (Thermo Multiscan FC) reader.

Biofilm formation was assessed based on the following criteria (Stepanovic et al., 2007):

- No biofilm production: If the optical density (OD_s) is equal to or less than that of the negative control ($OD_s \leq OD_{nc}$).
- Weak biofilm production: If the ODs are greater than but no more than twice the negative control ($OD_{nc} < OD_s \leq 2\times OD_{nc}$).
- Moderate biofilm production: If the ODs are greater than but no more than four times the negative control ($2\times ODnc < OD_s \leq 4\times OD_{nc}$).
- Strong biofilm production: If the ODs exceed four times the negative control ($4\times OD_{nc} < OD_s$).

Antifungal Susceptibility Testing

The resistance of the all isolates to amphotericin B (20 IU, 21.19 mcg) antifungal was evaluated using the CLSI M44-A agar disk diffusion method (CLSI, 2004). The isolates revived in SDA were initially adjusted to a 0.5 McFarland standard with SPS. After adjusting the inoculum suspension, it was inoculated evenly onto the 2% Glucose + 0.5 µg/ml Methylene Blue containing Mueller-Hinton Agar (M1825, Himedia, India) medium with a cotton swab. Following incubation (at 37°C for 24-48 hours), zone diameters were measured using a digital caliper. The study was conducted twice (Özcan Ateş and Otkun, 2023).

In determining the MIC values of antifungals, 17 isolates that were intermediate level biofilm producers were selected. MIC values of the isolates against five antifungals (amphotericin B (0.002-32 mcg/mL), clotrimazole (0.002-32 mcg/mL), fluconazole (0.016-256 mcg/mL), nystatin (0.002-32 mcg/mL and itraconazole (0.002-32 mcg/mL) (Himedia, India) were determined using the gradient test method specified in CLSI M27-A2 (CLSI, 2002). RPMI 1640 agar (M1972, Himedia, India), containing 2% glucose and 0.165 M MOPS, was utilized in this study. After adjusting the cell suspension to 0.5 McFarland with SPS, it was evenly distributed on the surface of the medium, and gradient test strips were placed on the agar surface. The plates were then incubated at 37°C for 24-48 hours. This study was conducted twice (Özcan Ateş and Otkun, 2023).

Statistical Analysis

The biofilm study was conducted with three repetitions, and mean values were calculated for each data set. Inhibition zones were determined as mean (M) ± standard deviation (SD) using the SPSS Package Program (v23.0, IBM Corp).

Results

Crystal violet staining, a quantitative method, was employed to assess the biofilm formation potential of *Candida parapsilosis* isolates obtained from makeup sponges. Based on the absorbance values determined in the microplate reader for 46 isolates, the difference between the highest and lowest OD_s/OD_{nc} was determined at 2.61 (Table 1). Seventeen isolates were identified as moderate biofilm producers, while the remaining isolates were classified as weak biofilm producers (Figure 1).

In the initial stage of assessing antifungal resistance, the agar disk diffusion method was employed to evaluate the resistance of the isolates against the antifungal amphotericin B. The results indicated that the isolates displayed a minimum inhibition zone of 19.73 ± 1.54 mm and a maximum of 23.71 ± 0.70 mm for amphotericin B (Table 1). The inhibition zone for the standard *Candida parapsilosis* ATCC 22019 culture was measured at 15.10 ± 0.39 mm..

Table 1. Biofilm formation potential and amphotericin B inhibition zones of isolates

Isolate No	ODs/ODnc	Biofim Production	*Inhibition zone (amphotericin B) (M±sd)*
1	1.84	Weak	22.63 ± 2.47
2	1.55	Weak	21.32 ±2.09
3	1.41	Weak	22.35 ± 2.07
4	1.21	Weak	22.13 ±1.25
5	1.48	Weak	20.77 ±1.82
6	1.85	Weak	21.42 ±1.73

7	2.50	Moderate	21.31 ±0.63
8	2.41	Moderate	21.87 ±0.09
9	2.02	Moderate	23.71 ± 0.70
10	1.78	Weak	22.63 ± 0.47
11	1.57	Weak	21.78 ± 0.33
12	1.68	Weak	21.22 ± 0.62
13	2.78	Moderate	22.15 ± 0.53
14	3.18	Moderate	21.33 ± 2.10
15	2.72	Moderate	22.83 ± 0.30
16	1.84	Weak	20.77 ± 0.87
17	1.49	Weak	22.11 ± 1.29
18	2.18	Moderate	20.87 ± 1.89
19	3.62	Moderate	22.31 ± 1.12
20	3.13	Moderate	21.72 ± 1.19
21	2.99	Moderate	21.70 ± 0.28
22	2.07	Moderate	21.15 ± 0.69
23	1.63	Weak	22.61 ± 1.01
24	2.15	Moderate	20.66 ± 1.27
25	2.55	Moderate	22.58 ± 0.23
26	3.06	Moderate	22.60 ± 1.32
27	2.71	Moderate	22.48 ± 1.52
28	1.99	Weak	22.67 ± 0.46
29	1.88	Weak	21.18 ± 0.49
30	1.75	Weak	22.68 ± 0.90
31	2.26	Moderate	22.66 ± 1.37
32	3.17	Moderate	22.10 ± 0.78
33	1.41	Weak	22.22 ± 1.20
34	1.47	Weak	20.91 ± 0.26
35	1.76	Weak	21.64 ± 0.86
36	1.98	Weak	20.74 ± 0.62
37	1.74	Weak	20.69 ± 0.70
38	1.85	Weak	22.18 ± 0.54
39	1.01	Weak	22.69 ± 0.42
40	1.61	Weak	22.33 ± 1.67
41	1.44	Weak	22.36 ± 0.28
42	1.56	Weak	22.33 ± 0.84
43	1.37	Weak	21.69 ± 0.43
44	1.46	Weak	21.59 ± 2.56
45	1.53	Weak	21.16 ± 0.45
46	1.50	Weak	19.73 ± 1.54
***Candida parapsilosis* ATCC 22019**	-	-	15.10 ± 0.39

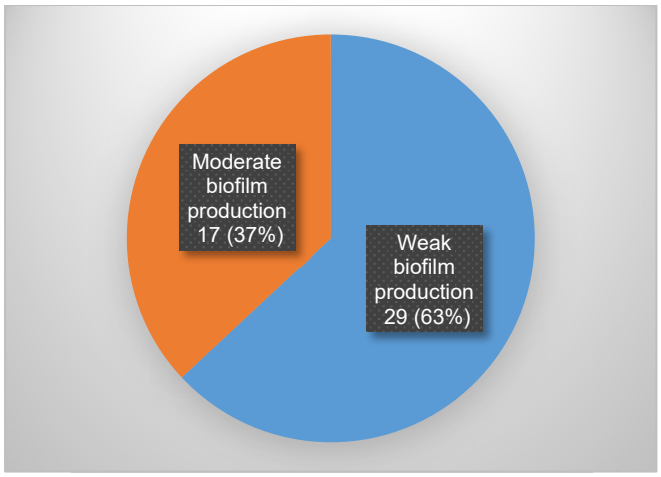

Figure 1. Biofilm formation capacity *Candida parapsilosis* isolates

In the susceptibility tests, 17 isolates determined as moderate biofilm producers were found to be susceptible to amphotericin b, clotrimazole, fluconazole, nystatin. and itraconazole (Table 2).

Table 2. MIC values of isolates that are moderate biofilm producers (in µg/ml)

Isolates No	Amphotericin B	Clotrimazole	Fluconazole	Nystatin	Itraconazole
7	1	0.032	0.50	2	0.25
8	1	0.032	0.50	2	0.25
9	1	0.032	0.50	2	0.25
13	1	0.032	0.50	2	0.25
14	1	0.032	0.50	2	0.25
15	0.75	0.012	0.50	2	0.25
18	0.75	0.012	0.50	2	0.25
19	0.75	0.016	0.75	2	0.125
20	1	0.012	0.50	2	0.125
21	0.75	0.012	0.50	2	0.125
22	0.75	0.012	0.50	2	0.125
24	0.75	0.012	0.50	2	0.125
25	0.75	0.012	0.50	2	0.125
26	0.75	0.012	0.50	2	0.125
27	0.75	0.012	0.50	2	0.125
31	0.75	0.012	0.50	2	0.125
32	0.75	0.012	0.50	2	0.125
***C. parapsilosis* ATCC 22019**	2	0.125	0.25	6	0.19

Discussions

In this study, we evaluated the biofilm formation capacity and antifungal susceptibility profiles of *Candida parapsilosis* isolates obtained from makeup sponges. Our findings revealed that the biofilm formation ability of these isolates varied, with the majority being weak biofilm producers. Similarly, a study conducted by Melo et al. (2011) reported that the biofilm formation ability of *Candida parapsilosis* isolates differed among strains. However, other studies indicated that these isolates were generally strong biofilm producers, although biofilm production varied according to the strain (Treviño-Rangel et al., 2015; Modiri et al., 2019). Biofilm formation occurs when microorganisms adhere to surfaces and are surrounded by an extracellular matrix, posing a significant challenge in treating infections (Silva et al., 2017). The

biofilms formed by *Candida parapsilosis* are clinically significant due to their potential to develop resistance to antifungal agents (Govrins and Lass-Flörl, 2024). In our study, we found that 17 isolates formed moderate biofilms.

Antifungal susceptibility tests revealed that the isolates were responsive to amphotericin B, fluconazole, itraconazole, clotrimazole, and nystatin. The Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) did not determine the inhibition zone diameter to evaluate the susceptibility of *Candida parapsilosis* isolates to amphotericin B. Regarding the inhibition zone, the manufacturer of the antifungal disk has reported the diameter between 10 and 17 mm for *Candida parapsilosis* ATCC 22019. In both CLSI and EUCAST, MIC values for amphotericin B are reported as Susceptible (S): MIC ≤ 1 mg/L and Resistant (R): MIC > 2 mg/L. S or R breakpoints for nystatin are not specified. The MIC values of the isolates against amphotericin B ranged from 0.75–1 µg/ml and are susceptible to polyene antifungals. This result suggests that environmentally sourced isolates may be susceptible to polyenes. However, previous studies have shown that biofilm production may reduce the efficacy of polyene antifungals (Tumbarello et al., 2007; Ramage et al., 2002). Additionally, there are (MIC) breakpoints determined by CLSI and EUCAST in the susceptibility assessment of *Candida* species for fluconazole and itraconazole. For fluconazole, CLSI reported S: MIC ≤ 2 µg/mL, Susceptibility-Dose Dependent (SDD): MIC = 4 µg/mL, and R: MIC ≥ 8 µg/mL, while EUCAST reported S: MIC ≤ 2 mg/L and R: MIC > 2 mg/L. The constant MIC value observed for fluconazole in all isolates (0.50 µg/ml) is consistent with the ranges generally reported for *Candida parapsilosis* (0.25–2 µg/ml) (Pfaller et al., 2010). However, it is also known that fluconazole resistance is increasing in some regions, and that this resistance is mostly associated with mutations in the ERG11 gene (Grossman et al., 2015). Although no resistance was detected in this study, molecular monitoring of isolates is important since resistance development may be observed in areas where fluconazole is used intensively. For itraconazole, according to CLSI, S: MIC ≤ 0.12 µg/mL, SDD: MIC = 0.25-0.5 µg/mL and R: MIC ≥ 1 µg/mL were reported, while according to EUCAST, S: MIC ≤ 0.06 mg/L and R: MIC > 0.06 mg/L were reported. Also, for clotrimazole, S or R breakpoints have not been specified. Particularly, clotrimazole and itraconazole from the azole group of agents attracted attention with their very low MIC values. This finding suggests that these drugs have high efficacy against non-clinical *Candida parapsilosis* isolates. Similarly, in the literature, it has been reported that the development of resistance against azoles is lower in environmental isolates compared to clinical isolates (Colombo et al., 2017; Arastehfar et al., 2020). The results obtained in the study are compatible with the susceptibility limits determined by CLSI and EUCAST. The literature has reported that some *Candida parapsilosis* strains showed reduced susceptibility to antifungals such as fluconazole and itraconazole (Tóth et al., 2019). However, no resistant isolate was detected in this study. Although many studies have examined the antifungal resistance of *Candida parapsilosis* in clinical samples (Miranda-Cadena et al., 2018; Daneshnia et al., 2023; Önal et al., 2024) and hospital environments (Sabino et al., 2011; de Paula Menezes et al., 2020), specific data on antifungal resistance in *Candida parapsilosis* isolates isolated from non-clinical, i.e., environmental and other environments, are very limited. Nevertheless, it should be kept in mind that such isolates may also develop resistance mechanisms and spread in various environments.

Previous studies also support that makeup sponges provide a suitable growth environment for microorganisms (Bashir & Lambert, 2020; Agi et al., 2023). While two studies (Akgül & Bakan, 2021; Ogbonna et al., 2024) have been identified in the literature regarding the antibiotic resistance of bacteria isolated from makeup products, no research has been found on the isolation of *Candida* species from makeup sponges, nor on their biofilm formation capacity and antifungal resistance. Thus, this study significantly contributes to filling this gap in literature.

This study has some limitations. First, biofilm formation was assessed using only a quantitative method, crystal violet staining. Future studies should incorporate qualitative analyses, in-depth investigations into how biofilm structure is formed, and examinations of genetic mechanisms in assessing biofilm formation. Additionally, increasing the sample size and evaluating more isolates may provide more comprehensive results regarding biofilm formation profiles and antifungal resistance.

This study has demonstrated that microbial contamination in makeup sponges poses a risk to public health and that *Candida parapsilosis* can spread through cosmetic products. Contaminated cosmetic products can lead to mucosal, eye, and skin infections, particularly in individuals with weakened immune systems. Furthermore, antimicrobial resistance in these microorganisms can complicate treatment and pose serious health risks. Consequently, the hygienic use of makeup products, the significance of personal hygiene, and regular cleaning of makeup products are crucial to minimizing infection risks. In conclusion, these findings may help establish hygiene recommendations to minimize microbial contamination of makeup sponges,

develop antimicrobial products, and determine strategies against microorganisms' antimicrobial resistance.

Author contributions

1. Author: Designed the study, performed laboratory analyses, interpreted the results, wrote and edited the article.
2.Author: Conducted laboratory analyses and prepared the draft of the article.

Conflicts of interest

The authors declare no competing interests.

Ethical Statement: It is declared that scientific and ethical principles have been followed while carrying out and writing this study and that all the sources used have been properly cited (Gülçin ÖZCAN ATEŞ, Tuğba HANEDAN)

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