ORIGINAL ARTICLE

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Detection of Biofilm Production in *Candida* Species from the Vagina by Two Different Methods

Vajenden Soyutlanan *Candida* Türlerinde Biyofilm Üretiminin İki Farklı Yöntemle Araştırılması

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ABSTRACT

Aim: The incidence of fungal infections has increased today, and antifungal resistance has increased in such infections. It is known that most infections produced by *Candida* species are associated with biofilm formation.

Materials and Methods: In this study, 192 patients diagnosed with yeast cytologically in cervico-vaginal smears between September 2015 and August 2020 were investigated.

Results: When all *Candida* species studied with Congo Red Agar were evaluated, biofilm positivity was found to be 61.4% in non-albicans *Candida* species and 38.6% in *C. albicans*. Biofilm positive non-albicans *Candida* species were identified as 11 (15.7%) *C. glabrata*, 11 (15.7%) *C. tropicalis*, 6 (8.6%), *C. guillermondii* and 6 (8.6%) *C. krusei*.

Conclusion: In fungal infections, the biofilm produced by the agent is directly proportional to antifungal resistance and invasion of the infection. Therefore, determining the biofilm production of the causative fungus is important in planning the treatment.

Keywords: Biofilm production, *Candida* species, *Candida* vaginitis

ÖZ

Amaç: Günümüzde mantar enfeksiyonlarının görülme sıklığı ve antifungal dirençleri artmıştır. *Candida* türleri tarafından üretilen enfeksiyonların çoğunun biyofilm oluşumu ile ilişkili olduğu bilinmektedir.

Gereç ve Yöntem: Bu çalışmada Eylül 2015 ile Ağustos 2020 tarihleri arasında Sitonet Sito-Patoloji Merkezi'nde serviko-vajinal smearlerinde sitolojik olarak maya tanısı saptanan 192 hasta araştırıldı.

Bulgular: Kongo Kırmızısı Agar ile çalışılan tüm *Candida* türleri değerlendirildiğinde biyofilm pozitifliği non-albicans *Candida* türlerinde %61,4 iken *C. albicans*'ta %38,6 olarak bulundu. Biyofilm pozitif olan non-albicans *Candida* türleri ise 11 (%15,7) *C. glabrata*, 11 (%15,7) *C. tropicalis*, 6 (%8,6), *C. guillermondii* ve 6 (%8,6) *C. krusei* olarak tanımlandı.

Sonuç: Mantar enfeksiyonlarında etkenin ürettiği biyofilm, antifungal direnç ve enfeksiyonun invazyonu ile doğru orantılıdır. Bu nedenle etken mantarın biyofilm üretiminin saptanması tedavinin planlanması açısından önem taşımaktadır.

Anahtar Kelimeler: Biofilm production, *Candida* species, *Candida* vaginitis

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INTRODUCTION

The incidence of fungal infections and also antifungal resistance in these types of infections have currently increased. *Candida* species are important fungal pathogens of humans that cause mucosal infections, especially in the vagina. *Candida* species are the most common pathogen seen in cervicovaginal smear. Ascomycola, Aspergillus, Sporidiobolaceae, Basidiomycota, Coprinellus, and Paracoccidioides are rarely found. Many pieces of evidence suggest that the majority of infections produced by *Candida* species are associated with biofilm growth. Biofilm formation is a major virulence factor in the pathogenicity of *Candida* species. Any biofilm or slime factor facilitates the colonization or invasive infections of *Candida* species in host cells, catheters, and prosthetic devices. Biofilm production is also associated with a high level of antifungal resistance in *Candida* species. When *Candida* species grow in vaginal samples, the detection of biofilm production as a criterion for antifungal resistance and virulence will guide the clinician for treatment. The method to be preferred for the detection of biofilm production should yield results in a short time, be easy to evaluate and be inexpensive. We, therefore, compared isolates from clinical vaginal discharge, representing different *Candida* species to each other for the capacity to form biofilms in glucose-containing medium such as 8% Sabouraud Dextrose Broth and Congo Red Agar (CRA).

MATERIALS AND METHODS

In our study, there were 192 routine patients who were examined in Sitonet Cyto-Pathology Center between September 2015 and August 2020. Two sets of examples were obtained using conventional smear cytology and wet smear (Figure 1). Conventional cervico-vaginal smears were scanned in Sitonet Cyto-Pathology and wet smears were studied in İstanbul Okan University Medical Faculty Hospital Microbiology Laboratory.

One hundred ninety-two *Candida* strain biofilm productions extracted from vaginal discharge samples were evaluated in the microbiology laboratory via two different methods. Vaginal samples were inoculated into Sabouraud Dextrose Agar (SDA) (Condalab, Spain) and then incubated for 24 hours at 37 °C. The suspected yeast colonies were subjected to Gram staining and then germ tube test. Subsequently, they were inoculated into Corn Meal Agar plate (Dalmau plate-Condalab, Spain) containing Tween-80 (Merck. Millipore, Germany). Isolates forming chlamydospore on germ tube and Corn Meal Agar plate were defined as *C. albicans*. Yeast samples from other non-albicans *Candida* (NAC) were identified with the VITEK 2 (BioMérieux, France) YST automated identification system.

Biofilm production in *Candida* strains was investigated using the tube adherence method (Sabouraud broth with 8% glucose/Sabouraud Dextrose Broth-SDB-) and CRA with glucose[1,2]. SDB (Condalab, Spain) was prepared in accordance with the manufacturer's recommended method by adding 60 g of glucose per liter (glucose density 80 g/L or 8%) and each sample was incubated at 35 °C for 24 hours at SDA. Then the grown yeast colonies were suspended in sterile saline, the concentration of $3x10^7$ CFU/mL was added to 9 mL of methylene blue and SDB suspension in 10 mL glass tubes and diluted to $3x10^6$ CFU/mL, incubated at 35 °C without shaking. Specimens were observed macroscopically at 24th and 48th hours. The specimens stained with methylene blue at the tube rim and the bottom were considered "positive" for biofilm production. In CRA, after 48 hours of incubation at 35 °C, the presence of black colonies and visually biofilm production was detected[1-5].

RESULTS

In the study, 70 of 192 *Candida* strains were identified as *C. albicans* (36.5%) and 122 as NAC (63.5%). Of the NAC species, 51 (41.8%) *C. glabrata,* 35 (28.7%) *C. tropicalis*, 18 (14.7%) *C. pseudotropicalis*, 9 (7%) 4) *C. guillermondii* and 9 (7.4%) were identified as *C. krusei*.

Fifty-eight (30.2%) biofilm positivity was detected at the 24th hour in all *Candida* species in SDB and 109 (56.8%) at the 48th hour (Figure 2). In CRA, 97 (50.05%) biofilm-positive *Candida* species were detected (Figure 3). Biofilm-positive *Candida* strain was found to be 88 (45.8%) and biofilm-negative *Candida* strain was found to be 74 (38.6%) with both methods (Table 1, 2, 3).

Of the 4 *Candida* strains that were positive at the 48th hour in SDB and negative in CRA, 22 were found to be negative at the 24th hour and weakly positive (+1 positive) at the 48th hour. No *Candida* strain that was positive in CRA but negative in SDB was found.

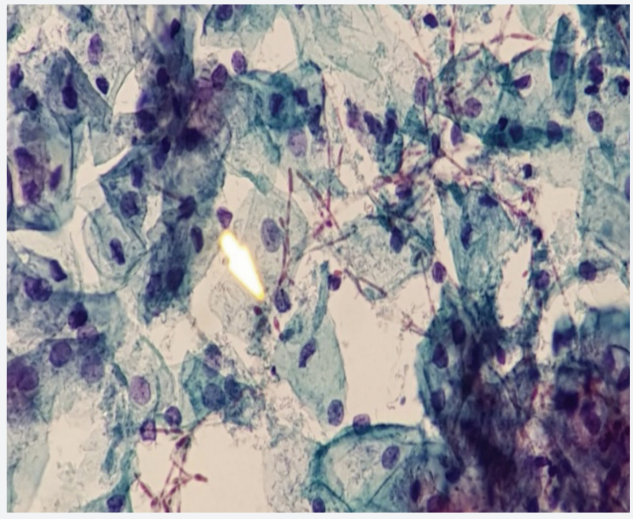

Figure 1. Yeasts, hyphae and vaginal epithelium in Papanicolau smear. X20, Pap stain

When all *Candida* species studied with CRA were evaluated, biofilm positivity was found to be 57.4% in NAC species and 38.6% in *C. albicans*. Biofilm positive NAC species were identified as 11 (15.7%) *C. glabrata*, 11 (15.7%) *C. tropicalis*, 6 (8.6%), *C. guillermondii* and 6 (8.6%) *C. krusei* (Figure 4).

Figure 2. Biofilm production by Christensen et al.[1] standard tube method with SDB positive (left) and negative (right) glass tubes -close up photo-

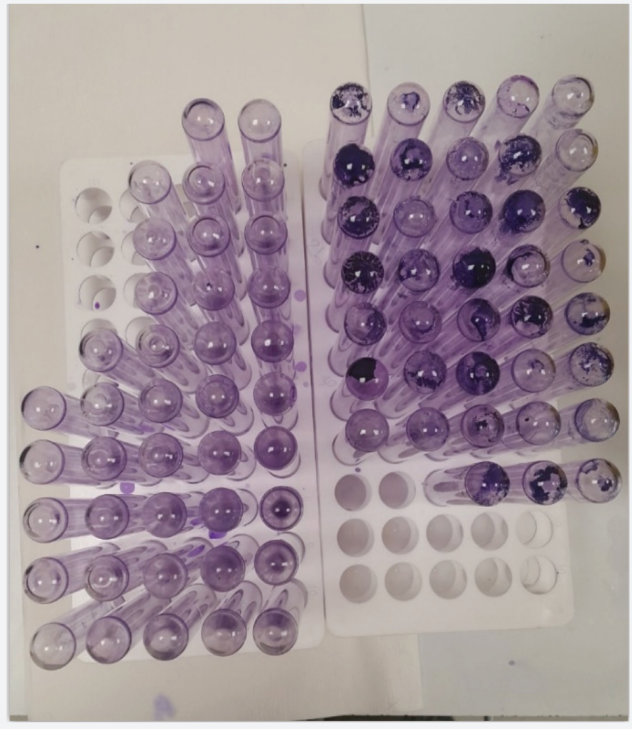

Figure 3. Biofilm production by Christensen standard tube method with Sabouraud Dextrose Broth positive (right) and negative (left) glass tubes

Table 1. Biofilm productions by standard tube method with SDB at the 24th and 48th hours

Medium	Positive	Negative	Total
SDB (24th hour)	58 (30.2%)	134 (69.8%)	192 (100%)
SDB (48th hour)	109 (56.8%)	83 (43.2%)	192 (100%)
CRA	97 (50.5%)	95 (49.5%)	192 (100%)
CRA and SDB together	88 (45.8%)	74 (38.6%)	

SDB: Sabouraud Dextrose Broth, CRA: Congo Red Agar

Table 2. Distribution of biofilm positive and negative samples by standard tube method with SDB at the 48th hour

Species	Biofilm positive	Biofilm negative	Total
C. albicans	29 (41.4%)	41 (58.6%)	70 (100%)
Non-albicans Candida	80 (65.6%)	42 (34.4%)	122 (100%)
Total	109 (56.8%)	83 (43.2%)	192 (100%)

SDB: Sabouraud Dextrose Broth

Table 3. Distribution of biofilm positive and negative samples by CRA method

Species	Biofilm positive	Biofilm negative	Total
C. albicans	27 (38.6%)	43 (61.4%)	70 (100%)
Non-albicans Candida	70 (57.4%)	52 (42.6%)	122 (100%)
Total	97 (50.5%)	95 (49.5%)	192 (100%)

CRA: Congo Red Agar

Figure 4. Biofilm positive black colonies on CRA
CRA: Congo Red Agar

DISCUSSION

The fact that some microorganisms, especially coagulase-negative staphylococci, produce biofilms under the name of slime factor was first reported by Christensen et al.[1]. The determination of slime factor production in coagulase-negative staphylococci by the CRA method has been first described by Freeman et al.[2] in 1989. Today, to detect biofilm production in coagulase-negative staphylococci, microdilution plate and standard tube methods described by Christensen or CRA method are used. The effects of biofilm production on adhesion and antibiotic resistance in bacteria and therefore its clinical importance have been shown in various studies[3,4].

Similar studies were conducted on yeasts after coagulase-negative staphylococci. Dolapçi and Tekeli[5] detected 15.14% slime factor positivity with the modified tube adherence method in a total of 350 *Candida* species (246 throat, 19 vaginal and 85 blood samples), and they also showed that the slime factor positivity was statistically significant. In the same study, they found that the slime factor positivity was significantly higher in *C. famata, C. guillermondii*, and *C. krusei*.

Li et al.[6] demonstrated the quantitative variability of adhesion and biofilm formation that allowed adhesion to polystyrene plastic surfaces in 115 *C. albicans* strain, including 47 from the oral cavity of healthy volunteers, 31 from the environment and 37 from vaginal samples of patients with candidiasis, and its relationship with genotypes. Harriott et al.[7] demonstrated *in vivo* biofilm formation and biofilm structure in the vaginal epithelium for the first-time. Calışkan et al.[8] showed that *Candida* species in the vagina could cause vaginal infections by forming biofilms and that biofilm structures that might form in intrauterine devices might cause resistance to antifungal drugs.

In the study of Yücesoy and Karaman[9], they investigated the biofilm production between *C. albicans* and NAC species with modified tube adherence and microplate methods. They showed a positivity rate of 65% with both methods. In our study, the biofilm positivity rate was found to be 45.8% with the standard tube adherence method and CRA. In the same study conducted by Yücesoy and Karaman[9], the biofilm positivity rate in different *Candida* species was between 17% and 55% in the tube adherence test. In the microplate method, it varied between 0% and 48%. In our study, SDB was found between 41.4% and 65.6% in the tube adherence test and 38.6% and 57.4% in the CRA test among *Candida* species. In the study of Yücesoy and Karaman[9] it was reported that there was no significant difference between *C. albicans* and NAC species in the tube adherence test. However, in our study, biofilm positivity was 41.4% for *Candida albicans* and 65.6% for NAC species biofilm positivity. Biofilm positivity was found to be 38.6% for *C. albicans* and 57.4% for NAC species in the CRA test. In the studies of Yücesoy and Karaman[9], it has been shown that biofilm production is a potential virulence factor for *Candida* species and is important in terms of antifungal sensitivity. In the study of Houdaii et al.[10], a positive relationship was found between biofilm formation and amphotericin B resistance.

Kumari et al.[11] detected 30.6% *Candida* species as causative agents in vulvovaginitis samples obtained from a total of 232 patients. 32.4% of them were identified as *C. albicans*, 45.07% as *C. parapsilosis*, and 22.53% as *C. glabrata*. The NAC rate of 67.6% found by Kumari et al.[11] is consistent with the rate of 63.5% in our study. While biofilm formation among *Candida* species was 70.4% in the study in question, it was found as 56.8% in the SDB method and 50.5% in the CRA method in our study.

Araurio Paulo de Medeiros et al.[12] examined their adhesion capacity to epithelial cells in 62 *C. albicans* samples isolated from the vagina and anus, biofilm formation in polystyrene microtiter plates, and proteinase activities, and they showed that virulence factors played an important role in the transition from colonization to infection in vulvovaginal candidiasis.

Kalaiarasan et al.[13], examined different virulence factors such as hemolysis, protease activity, and biofilm production, and they emphasized the importance of virulence factors, especially in NAC species. In our study, biofilm formation as a virulence factor in NAC species was found to be 63.5%.

In the study of Kıvanç and Er[14] , although there were differences between CRA and SDB microtiter plate methods for detecting biofilm production, it was stated that biofilm production was higher in *Candida* species isolated from the vagina. In the

same study, it was shown that lactic acid bacteria inhibited the production of biofilms formed by *Candida* species through competition in the adhesion area[14].

CONCLUSION

In fungal infections, the biofilm produced by the agent is directly proportional to antifungal resistance and the invasion of the infection. Therefore, determining the biofilm production of the causative fungus is important in planning the treatment. According to the values obtained from our study, the probability of biofilm positive for those with positive CRA results is 100%, while it is 87% for those with negative CRA results. CRA method with glucose is a method that can be preferred in determining biofilm production as a virulence factor in NAC species, which is increasingly common among *Candida* species, because of its simpler and shorter application compared to other methods and its objective evaluation.

Ethics

Ethics Committee Approval: The study were approved by the Okan University of Local Ethics Committee (protocol number: 13, date: 23.06.2021).

Informed Consent: Consent form was filled out by all participants.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: A.A., G.V., Concept: A.A., Design: G.V., Data Collection or Processing: A.A., G.V., Analysis or Interpretation: A.A., G.V., Literature Search: A.A., Writing: A.A., G.V.

Conflict of Interest: No conflict of interest was declared by the authors.

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