 

# Quantitative evaluation of biofilm formation in yeast nitrogen base (YNB) broth and in bovine serum (BS) of *Candida albicans* strains isolated from mucosal infections[1]

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**ABSTRACT.** *Candida albicans* is a major human fungal pathogen especially as an etiologic agent of opportunistic oral and genital infections. Moreover, *C. albicans* can be involved in the deep infections and recent evidence suggests that the majority of diseases produced by this pathogen are associated with biofilm growth. The aims of this study were to evaluate biofilm production ability of *C. albicans* strains isolated from different sources, and to evaluate the effect of serum for enhancement the growth of biofilm. The strains used in this study were obtained from three sources; 12 from feces of patients with gastrointestinal disturbances, 13 from the oral cavity of patients with oral candidiasis, and 16 from the vagina of patients with *Candida* vulvovaginitis (CVV). Polystyrene 96-well plates were used to grow biofilms and crystal violet (CV) staining method was used to evaluate the growth. There were no differences in biofilm growth expressed as CV absorbance between *C. albicans* strains from different origins neither in Yeast Nitrogen Base broth (YNB) or in bovine serum (BS) (ANOVA, $P$=0.1648, $P$=0.5106, respectively). In the BS, the biofilm production was greater than in YNB medium for all samples (ANOVA, $P$=0.0003).

**Key words:** biofilm, *Candida albicans*, crystal violet staining, candidiasis, 96-well polystyrene microplates

## Introduction

*Candida albicans* is a commensal of the human gut. But it is also a major human fungal pathogen especially as a causal agent of opportunistic oral and genital infections. Moreover *C. albicans* can be involved in the deep infection and recent evidence suggests that the majority of diseases produced by this pathogen is associated with biofilm growth [1,2]. The increase in *Candida* infections in the last decades has almost paralleled the increase and widespread use of a broad range of medical implant devices, mainly in populations with impaired immune defenses [3]. *C. albicans* can form biofilms on almost any medical device. There are also proofs that *C. albicans* can produce biofilms *in vivo* on the mucosa of vagina [4] and oral cavity [5].

The aims of this study were to evaluate biofilm production ability of *C. albicans* strains isolated from different sources, and to evaluate the effect of serum for enhancing the biofilm growth.

## Materials and methods

**Strains.** The strains used in this study were obtained from three sources; 12 from feces of patients with gastrointestinal disturbations, 13 from the oral cavity of patients with oral candidiasis, and 16 from the vagina of patients with CVV. All strains of *C. albicans* were identified based on the chlamydospores production on Rice Agar supplemented with Tween and the assimilation profiles on API 20C AUX test.

**Quantifying biofilms.** The method was adopted from Li et al. [6] and Ramage et al. [7]. Briefly, the strains were incubated 2–3 days at room temperature on Sabouraud's Glucose Agar. For each strain, a large loop of actively growing cells was transferred to a sterile Yeast Nitrogen Base (YNB) broth containing 0.9% D-glucose. After incubation at 37°C for 24 h, the cells were centrifuged and washed twice with PBS buffer (pH 7.4) by vortexing and centrifuging at 5000*g* for 5 min. The washed cells were then resuspended in YNB broth or bovine serum (BS). Optical density of cells was

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determined for each suspension and adjusted to 3 degree in McFarland scale. These cell suspensions were then used to grow biofilms. For each strain, 100 µl of the suspension was inoculated into individual wells of flat bottom 96-well polystyrene plates. Three repeats were performed for each strain. YNB broth or serum containing no *inoculum* was used as a negative control. The plates were incubated at 37°C for 90 min (adhesion period) on an orbital shaker (75 rpm). Supernatant including planktonic cells and liquid medium was then discarded and wells were gently washed twice with PBS to discard any non-adherent cells. For biofilm growth, 100 µl fresh YNB broth or BS was then added to each well. The plates were covered, wrapped with Parafilm to prevent evaporation and incubated at 37°C for 72 h on an orbital shaker (75 rpm). After biofilm formation and growth, planktonic cells were discarded through two rounds of washing with 200 µl sterile PBS buffer. Remaining cells were confirmed as biofilm cells through microscopy and these were quantified using crystal violet (CV) staining. One hundred µl of 1% CV was added to each well and incubated for 20 min at 37°C. Next, 150 ml 95% ethanol was added to dissolve the dyed biofilm cells and 100 µl of each mixture was transferred to a new 96-well microplate. The absorbance for each well was determined using a microplate reader at $A_{570}$. Similarly, wells containing only YNB broth or BS but no microbes were used as negative controls.

**Statistics.** The statistical analyzes were performed using language and environment for statistical computing R v. 2.11.1 (2010) for Linux. Additive model of two-way ANOVA was performed to determine which factor, i.e., source of strains or incubation medium, has effect on biofilm growth. *P*-values of $<0.05$ were considered statistically significant [8].

## Results

Among 41 investigated strains 35 (85.4%) grew in biofilm in YNB broth and all in bovine serum. Two strains isolated from feces (16.7%), two strains isolated from oral cavity (15.4%) and five strains from the vagina (31.2%) did not grow in biofilm in YNB medium but these differences were non significant (Fisher test, *P*=0.6368). There are also no differences in biofilm growth expressed as CV absorbance between *C. albicans* strains from different origins neither in YNB nor in BS (ANOVA test, *P*=0.1648, *P*=0.5106, respectively). In the BS the biofilm production was greater than in YNB medium for all samples (ANOVA, *P*=0.0003). The detailed results are shown in Table 1.

## Discussion

Many studies are devoted to the search of differences between *C. albicans* strains isolated from different human body sites [9–12]. They are mainly focused on the comparison of enzymatic activity of strains. There are only few studies focused on differences of biofilm production by *C. albicans* strains isolated from different sources [6,13,14].

In the view of recent reports, *Candida albicans* can form biofilm on mucosa *in vivo* [4,5]. In the present study it was attempted to find differences among strains isolated from patients with oral candidiasis, CVV and gastrointestinal disturbances, and the effect of serum on the ability for biofilm production.

Polystyrene 96-well plates were used to grow biofilms. The reason for using these devices is that method for biofilm formation in *C. albicans* has been established and standardized [7]. Therefore, obtained results can be compared to studies performed by other researchers. Moreover polystyrene plates are commercially available and relatively inexpensive. Standard method for quantification of biofilm production is measurement of mitochondrial activity with tetrazolium salt XTT. Li et al. [6] and Hasan et al. [15] found that CV and XTT biofilm quantifying methods were comparable and significantly correlated. However, the CV staining method revealed a wider range of variation of biofilm production among strains than XTT methods.

Obtained results indicate an overall lack of differences in biofilm production among samples from the three examined sources either in YNB and serum medium. Moreover, there was a high variability among strains. In the present study the mean of biofilm quantities for 41 strains of YNB and serum medium were 0.421 (±0.342 SD) and 0.968 (±0.363 SD) with a range of 0.000–1.562 and 0.135–1.974, respectively (Table 1). Mean biofilms quantified by CV staining for the 115 strains obtained by Li et al. [6] was 0.189 (±0.301 SD) with a range of 0.043–2.179. The higher absorbance values than those obtained by Li et al. [6] were related to

Table 1. *Candida albicans* biofilm formation in relation to sample origin and type of medium.

| Sample origin | *n* | Biofilm formation; mean±SD (range) | |
|---|---|---|---|
| | | Yeast Nitrogen Base | Bovine Serum |
| Feces | 12 | 0.552±0.443 (0.000–1.562) | 1.003±0.359 (0.465–1.446) |
| Oral cavity | 13 | 0.374±0.268 (0.000–0.793) | 1.001±0.454 (0.135–1.974) |
| Vagina | 16 | 0.362±0.303 (0.000–0.888) | 0.914±0.295 (0.265–1.378) |

longer period of biofilm incubation (72 h vs. 48 h) adopted from Nikawa et al. [16]. Studies of Chandra et al. [17] showed that after 72 h incubation biofilm achieved 1/3-fold higher level as measured by dry weight than after 48 h incubation. In comparison of results obtained in this study with results obtained by Li et al. [6], the mean absorbance value for the biofilm of strains isolated from the oral cavity was approximately 1/3-fold higher (i.e., 0.209 vs. 0.374); however, in the case of strains isolated from the vagina and feces more than twice (0.143 vs. 0.362 and 0.217 vs. 0.552, respectively). Hasan et al. [15] also noted that biofilm isolated both from blood and mucosa was highly variable but did not differ significantly ($P$=0.11) among *C. albican*s strains. In Li et al. [6] studies, every strain produced biofilm; however, in the present study 22% of strains did not produce biofilm in YNB medium.

There were statistically significant differences between strains incubated in serum and strains incubated in YNB broth after 72 h. Nikawa et al. [16] found that candidal biofilm formation on acrylic surfaces was enhanced by increasing concentration of serum in the pellicle. As compared to the uncoated control, significantly increased biofilm activity was observed in three of four (75%) *C. albicans* strains [16]. In the present study 35 of 41 (85.4%) strains increased biofim formation in serum (i.e., 9/12 from feces, 11/13 from the oral cavity and 15/16 from the vagina). Ramage et al. [18] noted that the level of *C. albicans* adherence was significantly elevated in comparison to that observed in untreated wells when serum was provided as a conditioning film. After 4 h there was a smaller difference between biofilm formation in the presence or absence of the serum pellicle than after 24 hours. However, *C. albicans* D1.2 showed no statistical differences between the presence or absence of the serum pellicle (99%), whereas *C. albicans* D2.2 demonstrated statistically significant differences (127%, $P$>0.001) [18]. Such results implicate that the adherence is the key stage in the biofilm formation but the low number of strains tested (i.e., 2) does not allow to generalize this conclusion. Moreover no statistical difference in adherence of blastospore after 2 h was found between „high biofilm producers" and „low biofilm producers" or among different *Candida* species [15]. This fact and obtained results suggest that stages after adherence, probably a stage of forming *hyphae*, have the effect on biofilm formation rate rather than on adherence.

The data presented above showed that *C. albicans* populations isolated from various body sites have the same ability to form biofilm. The keystage of biofilm growth is the phase after adherence and serum increasing biofilm formation. So anti-biofilm prophylaxis in patients with medical implant devices especially under immunodeficiency is essential to prevent *C. albicans* endoinfection.

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## Ocena ilościowa tworzenia biofilmu w YNB i surowicy przez szczepy *Candida albicans* izolowane z zakażeń grzybiczych błon śluzowych

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*Candida albicans* jest jednym z najczęstszych patogenów grzybiczych. który powoduje głównie oportunistyczne zakażenia jamy ustnej i narządów rodnych człowieka. Ponadto *C. albicans* może brać udział w zakażeniach głębokich, a ostatnie doniesienia wskazują, że większość z tych chorób jest związanych ze wzrostem tego patogenu w postaci biofilmu.

Celem pracy była ocena wytwarzania biofilmu przez szczepy *C. albicans* izolowane z rożnych lokalizacji oraz ocena wpływu surowicy na zwiększenie wzrostu biofilmu.

Szczepy użyte w tym badaniu pochodziły z trzech lokalizacji: 12 z kału pacjentów z zaburzeniami ze strony przewodu pokarmowego, 13 z jamy ustnej pacjentów z kandydozą jamy ustnej oraz 16 z pochwy pacjentek z kandydozą pochwy i sromu. Do badania wzrostu biofilmu użyto 96-dołkowych płytek polistyrenowych, a do ilościowej oceny wytwarzania biofilmu wykorzystano barwienie fioletem krystalicznym (CV).

Nie stwierdzono różnic we wzroście biofilmu między szczepami *C. albicans* izolowanymi z różnych źródeł, zarówno w YNB, jak i w surowicy (test ANOVA, $P$=0,1648, $P$=0,5106 odpowiednio). Stwierdzono natomiast zwiększenie wytwarzania biofilmu w surowicy dla wszystkich badanych prób (test ANOVA, $P$=0,0003).

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