Multi-species biofilm of *Candida albicans* and non-*Candida albicans Candida* species on acrylic substrate

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ABSTRACT

Objective: In polymicrobial biofilms bacteria extensively interact with *Candida* species, but the interaction among the different species of the *Candida* is yet to be completely evaluated. In the present study, the difference in biofilm formation ability of clinical isolates of four species of *Candida* in both single-species and multi-species combinations on the surface of dental acrylic resin strips was evaluated. Material and Methods: The species of *Candida*, isolated from multiple species oral candidiasis of the neutropenic patients, were used for the experiment. Organisms were cultured on Sabouraud dextrose broth with 8% glucose (SDB). Biofilm production on the acrylic resins strips was determined by crystal violet assay. Student's t-test and ANOVA were used to compare *in vitro* biofilm formation for the individual species of *Candida* and its different multi-species combinations. Results: In the present study, differences between the mean values of the biofilm-forming ability of individual species (*C. glabrata*>*C. krusei*>*C. tropicalis*>*C. albicans*) and in its multi-species' combinations (the highest for *C. albicans* with *C. glabrata* and the lowest for all the four species combination) were reported. Conclusions: The findings of this study showed that biofilm-forming ability was found greater for non-*Candida albicans Candida* species (NCAC) than for *C. albicans* species with intra-species variation. Presence of *C. albicans* in multi-species biofilms increased, whereas; *C. tropicalis* decreased the biofilm production with all other NCAC species.

Key words: Oral candidiasis. Biofilms. Crystal violet. Assay.

INTRODUCTION

Candida is the most frequently isolated fungal pathogen in humans causing a variety of afflictions ranging from superficial mucosal infections to systemic mycoses. Oral fungal infections develop frequently in immunocompromised patients, particularly in patients with prolonged, severe neutropenic episodes[2]. *In vivo* studies indicate that microbial contamination of denture acrylic resin occurs quite rapidly and implanted devices like denture prostheses provide refuge to candidal organisms as either single-species or multi-species biofilms[5,13]. Consequently, the immunocompromised denture wearers are more prone to the fungal infections.

One of the major factors contributing to the virulence of *Candida* is its ability in acclimatize to a variety of different habitats for growth and formation of surface-attached microbial communities known as "biofilms". Biofilms are defined as microbial communities encased in a matrix of extracellular polymeric substance (EPS), which display phenotypic features that differ from their planktonic or free-floating counterparts[7,16,19].

In vitro studies indicate that microbial contamination of denture acrylic resin occurs quite rapidly and the yeast cells adhere strongly to denture materials[4,13,18]. As bacteria and non-*Candida albicans Candida* species are often found with

Candida albicans in polymicrobial biofilms *in vivo*, it is likely that extensive interspecies interactions take place in these adherent populations[16,17,19].

A large number of *in vitro* model systems have been used to investigate characteristics of single-species and mixed-species biofilms consisting of *C. albicans* and bacteria[1,3,10,11,12,17]. Some studies have also been done on the interaction between different species of *Candida* on *in vitro* dual-species biofilm formation[20,25]. The study on the interaction between different species of *Candida* on *in vitro* multi-species biofilm formation is still scarce[27].

In the present study, the differences in biofilm-forming ability of clinical isolates of four species of *Candida* in single-species and multi-species combination on the surface of dental acrylic resin strips, commonly used for dental appliances, was evaluated.

MATERIAL AND METHODS

Organisms

The test organisms included 24 isolates of 4 different species of *Candida* i.e. *C. albicans, C. kruzi, C. glabrata and C. tropicalis* (six isolates of each), isolated from the oral lesions of multiple species (≥2 *Candida* species) Oropharyngeal Candidiasis (OPC) of the neutropenic patients (absolute neutrophil count $<1.5\times10^9$ cells/L)[28]. Pure culturing and identification was done at the Department of Microbiology, Modern Dental College and Research Centre. The identification of *Candida* species was conducted by culture characteristics on HiChrome *Candida* agar medium (HiMedia, Mumbai, India), assessing germ tube, chlamydospore formation and sugar assimilation patterns[6,22].

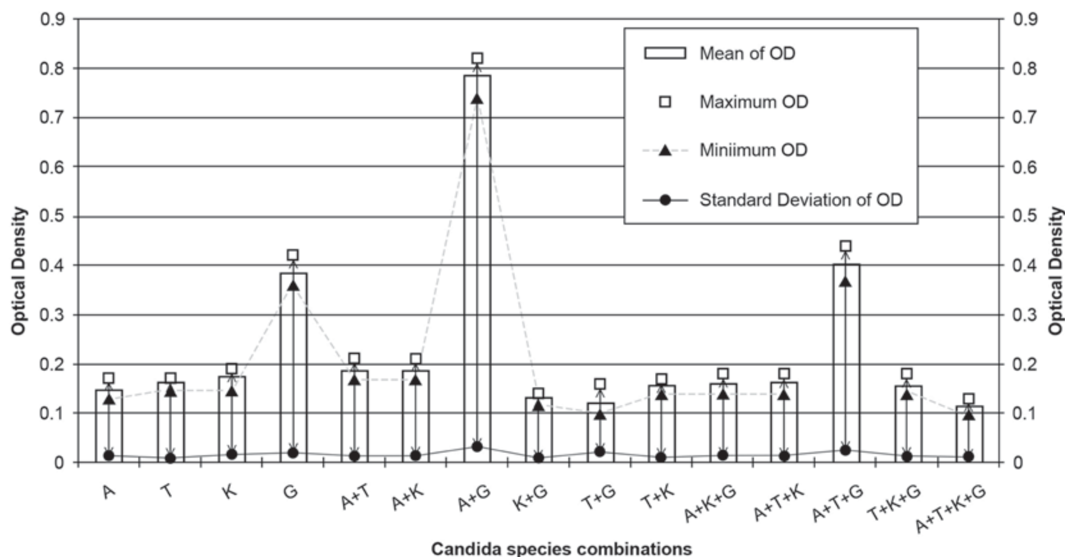
Simulated acrylic resins plates fabrication

Thirty-six square acrylic resins (polymethylmethacrylate) strips of 10x10x3 mm were fabricated. The acrylic resins strips were prepared as described by Samaranayake and MacFarlane[21] (1980) with some modifications. Wax patterns were invested in denture flasks, boiled out, packed with the denture base resins, and heat polymerized according to manufacturer instructions at a temperature 73°C for 6 h. Strips were removed from flask after bench cooling. One surface of strips was polished on buff wheel with pumice slurry. Other surface was left untouched to simulate intaglio surface. The resultant acrylic resins strips were immersed in distilled water for 1 week to leach excess monomer. Following this strips were disinfected by dipping in 70 % alcohol for 1 min, washed with sterile distilled water, dried and used for the experiment after checking their sterility.

Determination of biofilm production

Sabouraud dextrose broth (SDB) prepared from powdered Sabouraud broth (HiMedia, Mumbai, India) supplemented with 60 g of glucose *per* liter (final glucose concentration, 80 g/liter or 8%) (Qualigens, Navi Mumbai, India), was according to manufacturer's instructions. Fresh pure cultures of testing organism were prepared on SDA medium by subculturing clinical isolate. A loop full of organisms from each SDA plate was inoculated into modified Sabouraud dextrose broth (8% of glucose concentration) for 24 h at 35±2°C. The turbidity of each suspension was adjusted to the equivalent of 1×10^7 CFU/mL with SDB as determined by comparative plate counts.

Next, 1 mL of suspension of isolated species and testing combination of different species was



OD., Optical Density; A., *C. albicans*; T., *C. tropicalis*; K., *C. krusei*; G., *C. glabrata;* +, Combination of tested species

Figure 1- Optical Density of de-staining solution #

prepared by mixing equal volume of tested species, inoculated into a test tube with a screw cap (HiMedia, Mumbai, India) containing 9 mL of SDB, to make the final turbidity of each suspension to $1x10^6$ CFU/mL. Strips were placed in SDB, and then incubated at 35±2°C for 24 h without agitation. After 24 h of incubation, the culture broth in the tube was aspirated gently, and then acrylic strips were taken out for further investigation.

The acrylic resins strips, on which biofilms developed, were washed once with distilled water, and then incubated in a crystal violet (HiMedia, Mumbai, India) staining solution (0.1% in distilled water) for 15 min. These were then washed three times with distilled water. The stain was then dissolved in de-staining solution (95% ethanol) and absorbance in terms of optical density (OD) was measured at 570 nm as previously described[15]. Untreated acrylic strips were used as a control for the amount of the crystal violet stain in the de-staining solution. The absorbance values of controls were subtracted from the test values to minimize

Table 1- Paired samples t-test of *Candida* species combinations[$]

Pairs		t-test	Significance level (2-tailed)	Decision (α=0.05)
Pair 1	A - A+T	-5,452	0,003	Significant
Pair 2	A - A+K	-6,928	0,001	Significant
Pair 3	A - A+G	-65,11	0	Significant
Pair 4	A - A+K+G	-2,907	0,034	Significant
Pair 5	A - A+T+K	-2,697	0,043	Significant
Pair 6	A - A+T+G	-31,55	0	Significant
Pair 7	A - A+T+K+G	5,423	0,003	Significant
Pair 8	T - A+T	-4,443	0,007	Significant
Pair 9	T - T+G	5,477	0,003	Significant
Pair 10	T - T+K	1	0,363	Non-significant
Pair 11	T - A+T+K	0	1	Non-significant
Pair 12	T - A+T+G	-29,39	0	Significant
Pair 13	T - T+K+G	0,889	0,415	Non-significant
Pair 14	T - A+T+K+G	8,367	0	Significant
Pair 15	K - A+K	-3,503	0,017	Significant
Pair 16	K - K+G	8,73	0	Significant
Pair 17	K - T+K	4,392	0,007	Significant
Pair 18	K - A+K+G	2,169	0,082	Non-significant
Pair 19	K - A+T+K	7	0,001	Significant
Pair 20	K - T+K+G	5	0,004	Significant
Pair 21	K - A+T+K+G	8,919	0	Significant
Pair 22	G - A+G	-32,85	0	Significant
Pair 23	G - K+G	27,568	0	Significant
Pair 24	G - T+G	21,422	0	Significant
Pair 25	G - A+K+G	20,684	0	Significant
Pair 26	G - A+T+G	-1,025	0,352	Non-significant
Pair 27	G - T+K+G	37,997	0	Significant
Pair 28	G - A+T+K+G	60,374	0	Significant
Pair 29	A+T – (A,T)	5,27	0,003	Significant
Pair 30	A+K – (A,K)	5,966	0,002	Significant
Pair 31	A+G – (A,G)	50,747	0	Significant
Pair 32	K+G – (K,G)	-23,95	0	Significant
Pair 33	T+G – (T,G)	-16,84	0	Significant
Pair 34	T+K – (T,K)	-3,841	0,012	Significant
Pair 35	A+K+G – (A,K,G)	-11,87	0	Significant
Pair 36	A+T+K – (A,T,K)	0,183	0,862	Non-significant
Pair 37	A+T+G – (A,T,G)	16,021	0	Significant
Pair 38	T+K+G – (T,K,G)	-30,62	0	Significant
Pair 39	A+T+K+G – (A,T,K,G)	-25,85	0	Significant

[$]*C. albicans*; T., *C. tropicalis*; K., *C. krusei*; G., *C. glabrata;* +, Combination of tested species; (,),Average of the biofilm-forming ability of tested species independently.

background interference.

Statistical analysis

The ODs of the amount of the crystal violet in the de-staining solution, measured for different *Candida* species, were compared by the paired Student's t-test by using the SPSS Win 12.0 program (SPSS Inc, Chicago, IL, USA). Differences between the isolated species and its multi-species combinations were considered to be significant for *P* of 0.05. The null hypothesis (HO) rejected in favor of the alternative hypothesis (H1) at significance level α (0.05) if; $T>t_{n-1}$, α/2 (value of the Student table with n -1 degrees of freedom). The null hypothesis is H0: δ=0 (there is no difference in biofilm formation ability among the tested species) and the alternative hypothesis is (was) H1: δ=0 (there is a difference in biofilm formation ability among the tested species).

RESULTS

Twenty-four *Candida* species isolated from clinical samples and were used for this study. Amongst the 24 isolates, 6 each of *C. albicans*, *C. glabrata*, *C. krusei*, and *C. tropicalis* were used to test the biofilm formation ability both in single-species and in multi-species combination. All the isolates produce moderate to high degree of biofilms on the surface of acrylic material (Figure 1). The biofilm-forming ability of *C. glabrata* were reported to be the highest on acrylic substances (OD - 0.3833±0.02066), whereas; of *C. albicans* to be the lowest (OD - 0.1467±0.01366). In case of multiple species conditions, it was observed that the *C. albicans* had a positive impact on biofilms formation on acrylic substances as the highest degree of slim production occurred when *C. albicans* were inoculated with *C. glabrata* (OD- 0.7850±0.03209); whereas *C. krusei* had a negative impact in combination. Under multiple species condition when all the four species were in inoculums, the biofilms forming activity were severely hampered (OD – 0.1133±0.01033).

To test the hypothesis of no difference or no relationship between biofilm-forming ability of isolated species with that of in-combination of other species, paired t-test was performed (Table 1). For all the four species, 7 different multi-species combinations were prepared, which made a total of 39 pairs. Eighty-five percent of the pairs rejected null hypothesis in favor of alternate hypothesis. Only 15% of pairs showed no difference between the mean values of the biofilm-forming ability of single-species and in its multi-species combination.

DISCUSSION

In patients with advanced immunodeficiency, mucosal infections can lead to severe oral and esophageal candidiasis, resulting in disseminated candidiasis and sometimes early death. One of the most important virulence factors of *Candida* species is its ability to form biofilms, which has an important clinical consequence, as it confers resistance to antifungal therapy and capacity for yeast cells within the biofilms to withstand host immune defenses[3,5,6,12,19]. Changes in the oral environment effected by tooth loss or denture wearing can cause changes in oral microflora. The carriage rates of single and multiple *Candida* species were reported to be significantly higher in denture wearers[11]. In the light of above fact *Candida,* isolated from multiple species candidimia lesion, were evaluated for their biofilms formation ability on acrylic surface *in vitro*, in single-species and multi-species combination.

Candida biofilms formation has been described on polymethylmethacrylate strips which occur essentially in three overlapping phases: early (0-11 h), intermediate (12-30 h), and maturation (38-72 h) phases. The early stage is characterized by adherence and development of blastospores into distinct microcolonies. By 18 to 24 h, the *Candida* biofilm community can be seen as a bilayered structure comprising a mixture of yeasts, germ tubes, and young hyphae; this intermediate phase is distinguished by the production of extracellular polymeric substance (EPS). During maturation, the biofilms becomes a thick EPS layer in which a dense network of yeasts, pseudohyphae, and hyphae are embedded[4,18].

The biofilm-forming ability of clinical isolates of *C. albicans, C. krusei, C. tropicalis* and *C. glabrata* recovered from multiple species candidimia lesions, was evaluated by measuring absorbance of the de-staining solution containing crystal violet dye (crystal violet assay). The present showed *C. glabrata* forming thickest slim layer on acrylic resins strip followed by *C. krusei, C. tropicalis* and C*. albicans,* contrasting with the findings of previous researchers[9,24]. However Silva, et al.[23] (2009) reported that *C. glabrata* biofilms matrix was high in both protein and carbohydrates, which that probably enabled it to adsorbed more amount of crystal violet contents.

Shin, et al.[22] (2002) observed that biofilm formation was most frequent for isolates of *C. tropicalis* (80%), followed by *C. parapsilosis* (73%), *C. glabrata* (28%), and *C. albicans* (8%). This finding contrasts with the present adhesion studies, probably due to the sources of isolates. Different strains of the same *Candida* species were found to be different in their ability to form biofilms was

also reported in this study, indicating "strong" and "weak" biofilms-forming strains might exist within each *Candida* species[26]. Shin, et al.[22] (2002) also reported that biofilms-forming ability was greater for NCAC than for *albicans* species using similar protocol.

Presence of one species of microorganism on a surface can promote the adhesion of another[14]. Thein, et al.[25] (2007) reported competitive interaction in a dual-species biofilms of *C. albicans* and *C. krusei*; whereas Pereira-Cenci, et al.[20] (2008) did not report competitive interaction in a dual-species biofilms of *C. albicans* and *C. glabrata*. In the present study, the highest degree of slim production was seen with the combination of *C. albicans* and *C. glabrata*, followed by the combination of *C. albicans, C. tropicalis* and *C. glabrata*, and the lowest slim production was seen with the multi-species biofilms of all the four species of *Candida*. Presence of *C. albicans* in multi-species biofilms increased the production of slim when inoculated with all other NCAC species; whereas *C. tropicalis* retarded the production of slim under multi-species condition except with *C. albicans*. In this way, C*. albicans* might be able to successfully provide a substratum to the NCAC species on the acrylic prosthesis.

One of the most significant features of microbial biofilms is its resistance to a variety of antimicrobial agents. Studies have demonstrated drug resistance when *Candida* biofilms are even grown on surfaces like denture acrylic. The possible mechanisms of biofilm resistance to antimicrobial agents are: restricted penetration of drugs through the biofilms matrix; phenotypic switching, surface-induced expression of resistance genes and a small number of "persistent" cells[8]. Presence of two or more species in a biofilm could aggregate these factors. Synergistic effects of these factors can pose major problems to the clinicians.

CONCLUSION

Based on the results of this study, biofilm-forming ability was found greater for NCAC than for *albicans* species, isolated from multi-species oral candidiasis of the neutropenic patients. Presence of *C. albicans* in multi-species biofilms increased the slim production with all other NCAC species, whereas *C. tropicalis* impeded the production of slim under multi-species condition except for *C. albicans.*

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