

A comparative *in-vitro* microbiological study to evaluate the penetration by *Candida albicans* of different heat cure acrylic resins after denture brush abrasion

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CONTEXT: Failure to maintain adequate hygiene and surface roughness of the denture have been shown to be associated with a high level of oral *Candida* colonization. **AIMS:** To evaluate and compare the short term adhesion and long term penetration of *Candida albicans* into different types of heat cure acrylic materials, with and without denture brush abrasion and to compare the adherence and penetration among these materials. **MATERIAL AND METHODS:** Three different heat cure denture base materials viz., DPI high impact, DPI conventional and DPI tooth colored were used in the study. Thirty-one test samples were fabricated for each. Nineteen test samples were subjected to brushing. All of them were incubated and examined for adherence using fluorescent microscopy at time intervals of one hour and six weeks, and for penetration after six weeks of incubation. **STATISTICAL ANALYSIS USED:** Analysis of variance (ANOVA) was used to compare the mean *Candida albicans* cells of the various denture base materials at one hour adherence, six weeks adherence and six weeks penetration. **RESULTS:** There was less surface adherence and penetration in high impact denture material as compared to conventional and tooth colored ones. **CONCLUSIONS:** High impact acrylic resin shows less adherence and penetration of Candidial cells compared to the conventional and tooth colored acrylic resins. Brushing alone does not completely eradicate all the Candidial cells present. Samples brushed initially show more Candidial cells as compared to unbrushed ones.

Key words: *Candida albicans*, denture base resins, denture brush, fluorescent microscopy surface adherence, penetration

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INTRODUCTION

Failure to maintain adequate hygiene of the prosthesis has been shown to be associated with a high level of oral *Candida* colonization, with surface roughness of prosthesis providing niches in which the microorganisms are protected from shear forces and oral hygiene measures. This surface roughness can also be attributed to the use of denture brush, which results in a ploughing type of surface of the denture.^[1]

Hence, there is a need for a study to evaluate and compare the short term adhesion and long term penetration of *Candida albicans* into different types of heat cure acrylic materials, with and without denture brush abrasion and to compare the adherence and penetration among these materials.

MATERIALS AND METHODS

An *in-vitro* study was conducted in the Department of Prosthodontics, M.S. Ramaiah Dental College and Department of Microbiology, M.S. Ramaiah Medical College to evaluate the adherence and penetration of

Candida albicans to various denture base materials. The materials used in this study were high impact polymethyl methacrylate, conventional polymethyl methacrylate and tooth colored polymethyl methacrylate resin.

Known laboratory strains of *Candida albicans* were used for this study.

Preparation of Specimen

To produce test surfaces of comparable smoothness, the different heat processed polymethyl methacrylate resins viz., high impact (DPI), conventional (DPI) and tooth colored (DPI) were processed against a glass plate to obtain 31 test samples of dimension 2 cm x 2 cm x 3 mm. The glass-smooth surface of 19 test pieces was subjected to mechanical brushing employing 20,000 brushstrokes in soapy water to simulate approximately 2 years of denture wear. The remaining 12 test pieces were used as control and were not subjected to brushing.

The test pieces subjected to brushing were incubated in *Candida albicans*, Sabouraud's broth culture medium at 37 degrees C:

- 5 test pieces for 1 hour for surface adherence assay

- b) 5 test pieces for 6 weeks for surface adherence assay
- c) 2 test pieces for 6 weeks for penetration assay
- d) 5 test pieces for 6 weeks with the test samples brushed every 24 hours using soapy water and denture brush.
- e) 2 test pieces for 6 weeks with the test samples brushed every 24 hours using soapy water and denture brush.

After the incubation period of 1 hour the respective test pieces were viewed under a fluorescent microscope to observe the adherence of *Candida albicans* to the surface.

To check the depth of penetration after 6 weeks of incubation, the test pieces were sectioned and the cut surface viewed under a fluorescent microscope.

The 12 test pieces kept as control were also incubated similarly and tested likewise.

Culture preparation

Laboratory isolates of *Candida albicans* were used for this study. Before each adhesion experiment 10 ml of Sabouraud's broth was incubated at 37°C for 18 hours with *Candida albicans*, after which the broth was plated onto Sabouraud's agar for purity checks and to obtain a standardized cell suspension by comparing the optical density with 0.5 McFarland solution for subsequent experiments.

Inoculation

A) Adherence after 1 hour:

Five test pieces of each heat cure acrylic material used were sterilized by ethylene oxide gas sterilization. Each test piece was then placed in a sterile plastic 25 ml bottle in a vertical position. Two ml of sterile artificial saliva and 2 ml of the standardized cell suspension were added to each bottle and the apparatus was incubated statically (without agitation) at 37°C for 1 hour.

After this, the test pieces were removed from the suspension, dipped gently 3 times in 20 ml sterile PBS (phosphate buffered saline) to remove loosely attached cells, and left to dry lying horizontally.

B) Adherence and Penetration assay after 6 weeks: without daily brushing:

Seven sterile test pieces of each heat cure acrylic material were placed into 25 ml glass bottles containing autoclaved 10 ml artificial saliva. Each of the bottles was inoculated with 0.1 ml of the standardized cell suspension and incubated at 37°C for 6 weeks. Test substratum units were transferred to fresh, sterile artificial saliva on a weekly basis. After 6 weeks incubation, test pieces were removed from the bottles and placed in glutaraldehyde, diluted to 4% with the

addition of PBS for at least 2 hours to fix the cells. Substrata plus attached cells were then dehydrated in ethanol (30, 50, 70, 90, and 100%) for 10 min for each concentration.

C) Adherence and Penetration after 6 weeks: with daily brushing:

Seven sterile test pieces of each heat cure acrylic material were placed into 25 ml glass bottles containing autoclaved 10 ml artificial saliva. Each of the bottles was inoculated with 0.1 ml of the standardized cell suspension and incubated at 37°C for 6 weeks. Test pieces were subjected to manual brushing every 24 hours employing 20 strokes in 15 seconds in one direction with soapy water and denture brush (Senolin denture brush used and prescribed at M.S. Ramaiah Dental College). Test substratum units were transferred to fresh, sterile artificial saliva on a weekly basis. After 6 weeks incubation, test pieces were removed from the bottles and placed in 4% glutaraldehyde for at least 2 hours to fix the cells. Substrata plus attached cells were then dehydrated in ethanol (30, 50, 70, 90, 100%) for 10 min for each concentration.

Fluorescent study

A) For 1 hour adherence assay:

After drying, the attached cells remaining on the test pieces were fixed by immersion in 100% methanol for 1 min, and then stained by 2 minutes immersion in 0.03% acridine orange in distilled water, followed by washing in distilled water and allowed to dry.

Test samples were viewed using fluorescence microscopy at x1000 magnification under oil immersion. The number of adherent cells in 20 random fields was counted for each test piece and the mean number of adherent cells per field was calculated.

B) For 6 weeks adherence assay:

Sections were immersed in 0.03% acridine orange for 1 minute, rinsed in distilled water, then allowed to dry. Test samples were viewed using fluorescence microscopy at x1000 magnification under oil immersion. The number of adherent cells in 20 random fields was counted for each test piece and the mean number of adherent cells per field was calculated.

C) For 6 weeks penetration assay:

Dried test pieces were held horizontally with a clamp and sectioned. Four sections were cut across each piece to provide 5 replicate samples, with a diamond disk of width 0.2 mm. Sections were immersed in 0.03% acridine orange for 1 minute, rinsed in distilled water, then allowed to dry lying horizontally with the cut section kept uppermost. Replicate sections were then attached to a microscope slide for examination.

Sections were viewed via fluorescence microscopy, x1000 magnification, and oil immersion. The objective was focused on one end of the section, and moved down so that the entire length of the sample was examined. This procedure was repeated 3 times: (level one) once with the objective focused on the outer layer of the sample (upper surface of the material); (level two) once in the middle of the material; and (level three) once adjacent to the lowermost layer of the material (the one processed against dental stone). The penetration of yeast into the heat cure acrylic material was measured by counting the number of candidial cells visible within each microscopic field. The number of candidial cells counted was then totaled for each level and the mean calculated for each level.

RESULTS

The study showed that there was a significant difference in the number of cells which had adhered to the three test materials ($P < 0.05$). There was also a significant increase in the degree of adherence in conventional, high impact and tooth colored specimens which had been brushed as compared to the unbrushed ones ($P < 0.05$) [Tables 1, 2]. It was observed that high impact acrylic resin showed least adherence in comparison to conventional and tooth colored acrylic resins [Tables 1, 2]. This was statistically analyzed and was found to be significant ($P < 0.05$).

Among the three acrylic resins, conventional acrylic resin and tooth colored acrylic resin showed more penetration than high impact acrylic resin [Table 3].

Table 1: Descriptives one hour surface adherence

	N	Mean	Std. Deviation	95% Confidence interval for mean				
				Std. Error	Lower bound	Upper Bound	Minimum	Maximum
Conventional Brushed	5	2.8100	0.2408	0.1077	2.5110	3.1090	2.50	3.15
Conventional Unbrushed	5	1.1000	0.1173	5.244E-02	0.9544	1.2456	1.00	1.30
High Impact Brushed	5	1.6200	0.2280	0.1020	1.3369	1.9031	1.35	1.85
High Impact Unbrushed	5	0.5700	0.3421	0.1533	0.1453	0.9947	0.15	1.10
Tooth Colored Brushed	5	2.7800	0.4522	0.2022	2.2185	3.3415	2.25	3.45
Tooth Colored Unbrushed	5	1.2900	0.4393	0.1965	0.7445	1.8355	0.80	1.95
Total	30	1.6950	0.9021	0.1647	1.3581	2.0319	0.15	3.45

ANOVA one hour surface adherence

	Sum of squares	df	Mean square	F	Sig.
Between Groups	21.049	5	4.210	39.575	0.000
Within Groups	2.553	24	0.106		
Total	23.602	29			

Table 2: Descriptives 6 weeks surface adherence

	N	Mean	Std. Deviation	95% Confidence interval for mean				
				Std. Error	Lower bound	Upper Bound	Minimum	Maximum
Conv Daily Unbrushed	5	1.1000	8.660E-02	3.873E-02	0.9925	1.2075	1.05	1.25
Conv Daily Brushed	5	0.9100	0.1387	6.205E-02	0.7377	1.0823	0.75	1.05
Conv Unbrushed		0.4100	0.1194	5.339E-02	0.2618	0.5582	0.25	0.55
High Impact Daily Unbrushed	5	0.8100	7.071E-02	3.162E-02	0.7122	0.8878	0.70	0.85
High Impact Daily Brushed	5	0.6700	0.1095	4.899E-02	0.5340	0.8060	0.50	0.80
High Impact Unbrushed		0.3000	5.000E-02	2.236E-02	0.2379	0.3621	0.25	0.35
Tooth Colored Daily Unbrushed	5	1.2400	0.1475	6.595E-02	1.0569	1.4231	1.00	1.35
Tooth Colored Daily Brushed	5	1.1500	0.2475	0.1107	0.8427	1.4573	0.85	1.45
Tooth Colored Unbrushed	5	0.3800	4.472E-02	2.000E-02	0.3245	0.4355	0.30	0.40
Total	45	0.7730	0.3573	5.327E-02	0.6660	0.8807	0.25	1.45

ANOVA 6 weeks surface adherence

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5.036	8	0.630	38.938	0.000
Within Groups	0.582	36	1.617E-02		
Total	5.618	44			

Table 3: Descriptives 6 weeks penetration

	N	Mean	Std. Deviation	95% Confidence interval for mean				
				Std. Error	Lower bound	Upper Bound	Minimum	Maximum
Conv Daily Unbrushed	10	1.1550	6.852E-02	2.167E-02	1.1060	1.2040	1.05	1.25
Conv Daily Brushed	10	.9300	.1418	4.485E-02	.8286	1.0314	.70	1.10
Conv Unbrushed	10	.2800	.1653	5.228E-02	.1617	.3983	.00	.45
High Impact Daily Unbrushed	10	.8550	.3304	.1045	.6187	1.0913	.15	1.25
High Impact Daily Brushed	10	.3600	9.661E-02	3.055E-02	.2909	.4291	.25	.55
High Impact Unbrushed	10	.2250	7.546E-02	2.386E-02	.1710	.2790	.10	.35
Tooth Colored Daily Unbrushed	10	1.2450	9.560E-02	3.023E-02	1.1766	1.3134	1.10	1.40
Tooth Colored Daily Brushed	10	.8150	.3659	.1157	.5532	1.0768	.35	1.40
Tooth Colored Unbrushed	10	.2500	.1700	5.375E-02	.1284	.3716	.00	.50
Total	90	.6794	.4272	4.503E-02	.5900	.7689	.00	1.40

ANOVA 6 weeks penetration

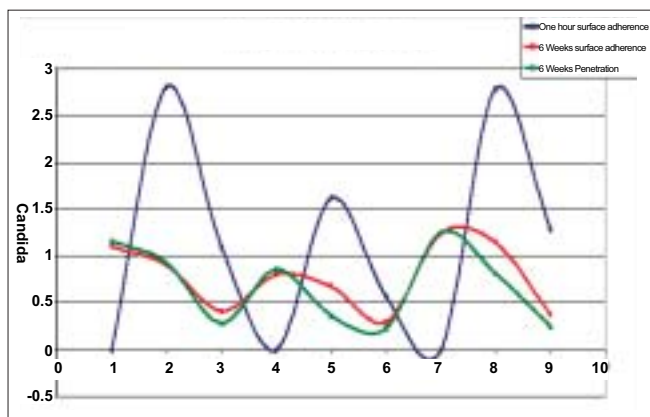
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	13.105	8	1.638	42.336	.000
Within Groups	3.134	81	3.869E-02		
Total	16.239	89			

This was found to be statistically significant ($P < 0.05$). Moreover, the surface adherence of candidial cells between these resins also showed similar results.

The samples which were brushed daily also showed more surface adherence as well as penetration of *Candida albicans* into the denture base when compared with the unbrushed test samples ($P < 0.05$) [Tables 1 and 2, Graph 1].

The conventional acrylic resin pieces which had been subjected to daily brushing showed more surface adherence than high impact acrylic resin, but not statistically significant on a routine usage. However, the difference in the penetration observed between the two was statistically significant with high impact acrylic resin showing less penetration.

High impact acrylic resin also showed statistically significant less surface adherence and penetration than tooth colored acrylic resin. On the other hand not much difference was seen between the conventional acrylic resin and the tooth colored one.



Graph 1: Mean no. of *Candida* at different time intervals

Statistical analysis

We used analysis of variance (ANOVA) to compare the mean *Candida albicans* cells of conventional brushed, conventional daily unbrushed, conventional unbrushed, high impact brushed, high impact daily unbrushed, high impact unbrushed, tooth colored brushed, tooth colored daily unbrushed and tooth colored unbrushed groups at one hour adherence, six weeks adherence and six weeks penetration.

NULL HYPOTHESIS H_0 : There is no significant difference between the groups i.e. $\mu_1 = \mu_2 = \mu_3 = \mu_4 = \mu_5 = \mu_6 = \mu_7 = \mu_8 = \mu_9$

ALTERNATE HYPOTHESIS H_1 : At least one pair of the groups differs.

LEVEL OF SIGNIFICANCE: $\alpha = 0.05$

DECISION CRITERION: The decision criterion is to reject the null hypothesis if $P < 0.05$ and accept the alternate hypothesis. Otherwise we accept H_0 .

TEST STATISTIC: We used the F-test to compare the different means.

INFERENCE: The following tables give us the significance value i.e. the “P-value” for various computations. The ANOVA table gives us a p-value which is < 0.05 leading us to reject the null hypothesis H_0 and conclude that there is a significant difference among the groups. Post-hoc comparison among the groups is done using Tukey’s HSD. From this comparison it is evident that the mean difference is significant between the groups.

DISCUSSION

This study was carried out to evaluate the surface adherence and penetration of *Candida albicans* to three commonly used denture base materials. The three denture base materials used for this study were

DPI high impact heat cure acrylic, DPI conventional heat cure acrylic and DPI tooth colored heat cure acrylic resin. Acrylics and metals have been used in the fabrication of denture bases, however acrylics are more economical and easy to manipulate and have been more widely used. A comparative study of the surface growth and penetration into high impact, conventional and tooth colored acrylic resins by *Candida albicans* has not been evaluated. Moreover, it has been reported that other factors such as surface free energy, hydrostatic forces, hydrophilic or hydrophobic nature of the material, pervious nature of the material and water sorption alter the nature of adherence of the yeast to the denture base material.^[2-5] Therefore three types of polymethyl methacrylates were used inferring some difference in the above factors. Hence, it was decided to use these three commonly available materials for the study.

The methodology chosen was based on a study by Bulad *et al.*,^[6] with the following modifications. The surface topography of the specimen was standardized by processing the acrylic resin against glass. The surface of some of the test samples was altered by brushing the surface with a denture brush. The specimens were incubated in a standardized cell suspension of *Candida albicans* and observed under incident beam fluorescent microscope and 20 fields were counted for each specimen for the presence of *Candida*.

The specimens were observed for adherent cells after one hour and six weeks as well as for cells that penetrated into the denture material after an incubation period of six weeks.

One hour surface adherence assay [Figures 1 and 2]

High impact acrylic resin (mean value of candidial cells - 1.6200) which had been subjected to brushing showed the least adherence to candidial cells when

compared to the conventional (mean value of candidial cells- 2.8100) and tooth colored (mean value of candidial cells - 2.7800) brushed samples [Table 1], [Figure 1]. This when statistically evaluated was found to be significant ($P < 0.05$). Among one type of material the brushed samples showed more adherence of *Candida* cells as compared to the samples that had not been brushed (the surfaces being processed against glass and kept unchanged). This too was found to be significant when evaluated statistically.

These can be attributed to the increased surface roughness of the conventional and tooth colored acrylic resins when compared to the high impact denture material as well as to the ability of the brushing action to abrade the surface of the test samples.^[1]

Six Weeks Surface Adherence and Penetration Assay

There was a significant relation ($P < 0.05$) between the daily brushed and the unbrushed samples.

Polymethyl methacrylate when examined for adherence showed that both the conventional and tooth colored polymethyl methacrylate specimens exhibited more adherence and penetration when compared to the high impact polymethacrylate specimens. This can be related to the relatively smoother surface of the high impact polymethacrylate specimens than the conventional and tooth colored polymethyl methacrylate specimens which show a rougher surface.^[1] The decreased adherence to high impact specimens may also be attributed to the surface hydrophobicity and surface energy of the material. Conventional and tooth colored acrylic resins show a greater contact angle with distilled water as compared to the high impact one (based on a pilot study conducted under Prof. Ashok Raichur at the Department of Metallurgy, Indian Institute of Science, Bangalore). Thus, they have a more hydrophobic surface than high impact acrylic resin, implying that they possess greater surface energy values. Hence, their surface



Figure 1: Candidial cells visible during surface adherence assay

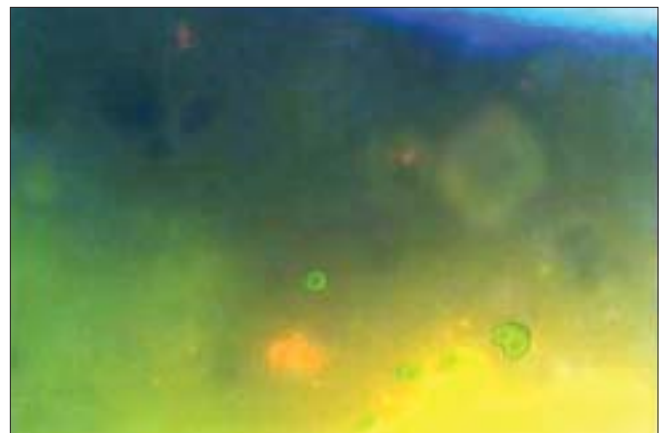


Figure 2: Candidial cells visible during penetration assay

energies are closer to that of *C. albicans*, because of which the difference in the free surface energy between these acrylic resins and *C. albicans* would be less, ultimately leading to more adherence of *C. albicans* to these surfaces and consequently more penetration into them. It has also been said that surface roughness of conventional acrylic resin is higher compared to high impact acrylic resin. This phenomenon has also been observed after brushing of their surfaces which leaves the surface of conventional (non-high impact) acrylic resin bases grooved and ploughed as compared to a relatively smoother surface of high impact acrylic resin bases.^[1] This grooved surface facilitates the growth of *C. albicans* as the troughs shield the microorganism from the cleansing action of the brush, thereby allowing it more time to firmly adhere to the surface and penetrate into material [Figure 2]. We also observe more penetration of *C. albicans* into conventional and tooth colored acrylic resin as compared to high impact. This may be explained by the difference in the density of the materials. Even after denture brush abrasion the high impact acrylic resin showed a smoother surface than the other two, implying that its particles were more closely packed together and were denser than the conventional (non-high impact) acrylic resin.^[1]

It has been reported that acrylic resins exhibit water sorption. The water sorption may help the candidial cells to adhere or to even penetrate the surface of the acrylic resin. However, this is postulated and yet to be proved.^[2]

Furthermore, it was also observed that brushing alone did not effectively remove all the *Candida* present on the test pieces. But it was observed that the brushed samples showed lesser adherence and penetration of *Candida* than the samples where daily brushing had been stopped [Tables 1-3]. This finding is consistent with the findings of a study done by Dills *et al.*,^[7] on the antimicrobial capability of an abrasive paste and chemical denture cleansers. They found that brushing alone with the abrasive paste was not capable of removing all the micro organisms present on the dentures and that soaking alone in a chemical denture cleanser was resulting in a significantly lower microbial count than brushing. Hence, it can be said that for proper oral hygiene the use of a denture cleanser in conjunction with daily brushing of the dentures should be advocated.

Further, the results obtained in the six weeks surface adherence assay complemented the ones seen in the six weeks penetration assay as those surfaces showing greater surface adherence of *C. albicans* were also showing greater amount of *C. albicans* penetration. However, it was observed that the penetration was limited to the upper 1/3rd of the surface only. A few hyphal forms were also observed, but these were also limited to the upper 1/3rd of the acrylic resin pieces. These findings suggest that the high impact denture bases are far better than the conventional and tooth colored acrylic denture bases in terms of the incidence of denture stomatitis due to candidial infection. Further they are seen to be more impact resistant and possess better strength than the conventional and tooth colored ones. However, their usage might be affected by their slightly higher cost than the other two. Besides, the patient's oral hygiene and attitude and approach towards treatment should be given prime importance irrespective of the denture base material used.

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