Research Article

Comparison of Candidal Growth & Chlorhexidine Efficacy on Complete Denture-Biofilms of Patients with & without Denture Stomatitis - An *in vivo* Study

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Abstract

Background: Denture stomatitis is highly prevalent in Denture wearing patient, & microbial plaque accumulation on intaglio surface of removable Dentures plays a critical role in formation of Denture biofilm. The purpose of this study was to evaluate the growth of Candidal species on Denture Biofilm using new sampling technique *in vivo* & testing efficacy of Chlorhexidine as disinfectant on Denture biofilm of patients with & without Denture stomatitis. **Methods:** Patients wearing complete Dentures with partial Denture stomatitis as study group & patients without Denture stomatitis as control group were taken. Separate acrylic resin disks were inserted at Denture stomatitis lesion site and at normal mucosal sites coinciding with the Dentures of study group & control group. After 24 hours and 3 days of wear of Dentures, the disks were retrieved and compared for number of candidal colony forming unit (CFU)s per ml of samples & efficacy of 0.5% Chlorhexidine (0.5% CHX) on same samples from the lesion & normal mucosal sites respectively. **Results:** The No. of Candidal (CFU)s count on Denture biofilm increased exponentially from day-1 to day-3 with statistically highly significant order at lesion site & with statistically significant order at non-lesion site of Denture stomatitis patients. 0.5% CHX was found to be very effective & of statistically highly significant order in reducing the fungal load at lesion site of Denture stomatitis. **Conclusion:** The complete Dentures can be soaked in CHX-0.5% for specific time period to treat or to avoid Denture stomatitis in Denture wearing patients.

Keywords: Chlorhexidine; Denture stomatitis; Denture biofilm

Introduction

Oral candidiasis, also known as Denture stomatitis, is the chronic inflammation of Denture bearing area which occurs in 25-65% of Denture wearers. It is a term used to describe inflammatory changes in the oral mucosa of Denture-bearing tissues, characterized by erythema, found under complete or partial Dentures in both jaws, but more frequently in the maxilla [Figure 1].[1] These lesions are very frequent complications for the wearing of removable Dentures both in independent and frail, or dependent elderly adults.[2] The behavior of oral mucous membrane in contact with Dentures has been the subject of considerable interest. Microbial plaque accumulation on intaglio surface of removable Dentures plays a critical role in the formation of Denture biofilms. Studies have reported an association between *Candida* species & Denture stomatitis. For the first time, Cahn suggested that *Candida albicans* as the causative agent of Denture stomatitis.[3] As the association between *Candida* species & Denture stomatitis exits, targeting microbes such as *Candida* species overDenture biofilm at different stages in its development may result in better understanding of the relationship of inflammation in Candida-induced Denture stomatitis.[4] Consequently, the treatment of Denture stomatitis includes correcting Denture faults, improving Denture hygiene and prescribing antifungal agents. The *candida* cells are significantly less susceptible to antiseptics

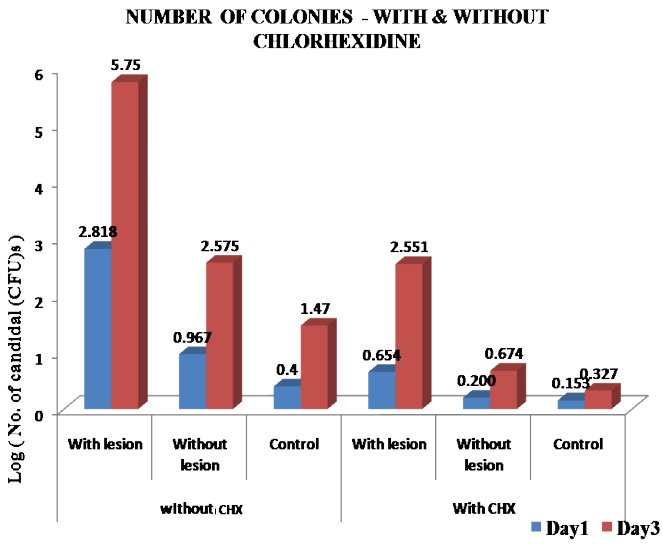

Figure 1: Partial or localized denture stomatitis lesion on palatal mucosa.

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& antifungal agents, & their mechanism of resistance is not fully understood.[5] Chlorhexidine is commonly used by dental patients as antiseptics.[6] Its efficacy on Denture biofilm needs to be tested and compared.

The purpose of this study is to evaluate the growth of *Candida* species within the Denture biofilm using new sampling technique *in vivo* and testing whether 0.5% Chlorhexidine 0.5% CHX is effective as a disinfectant on Denture biofilm in patients having Denture stomatitis & in patients not having Denture stomatitis.

Methods

Study design

The proposed study was conducted to isolate the samples of Denture biofilm formed periodically on the acrylic resin disks which were inserted on the intaglio surface of complete Dentures. The patients with Denture stomatitis & the patients not having Denture stomatitis were allowed to wear the modified complete Dentures for a specific time period of 24 hours and 3 days periodically. The samples isolated were cultured to compare for the presence of colonies of Candidal species & to quantify their differential load on the Denture biofilm samples obtained. Then the efficacy of 0.5% CHX against Candidal species cultured from various samples of Denture biofilms was evaluated. The pictorial representation of group distribution of study samples was shown in[Figure 2]. Thirty patients wearing complete Dentures for at least five years were selected with their written consent for the research work. Fifteen patients having localized or partial Denture stomatitis were considered as the experimental or study group:[Group-A]. The biofilm samples obtained from acrylic resin disks of Dentures contacting the Denture stomatitis lesion site & the normal site of the palatal mucosa of Denture stomatitis patient was termed as Group-Al samples, & Group-A2 samples respectively. Remaining fifteen patients not having Denture stomatitis were considered as Control group:[Group-B], here the biofilm samples obtained on acrylic resin disks, contacting the palatal mucosa of patients not having Denture stomatitis was termed as Group-B samples. The subjects excluded from the study were, patients with generalized Denture stomatitis, patients with a history of relevant systemic diseases like diabetes, anemia etc., which promotes Denture stomatitis & also the patients who were on immunosuppressive drugs.

Fabrication of removable acrylic resin disks inlay samples

Discs of pink modeling wax (Rolex, Ashoo sons, Delhi, B. No. MW 29) of 3x3 mm diameter and 2 mm thickness were punched from a sheet using a cork borer. Four wax discs per study group & two wax discs per control group were flasked and heat cured. The disks were de-flasked and stored in water to prevent the possibility of dimensional changes. After the routine finishing & polishing procedures commonly used in clinical prosthetic dentistry, all samples were rinsed in distilled water, ultra-sonicated twice (ULTRAsonik, Ney 28B) in distilled water for 15 min, & then rinsed once more. The disk specimens were then stored in distilled water at 37°C & water was changed every 2 days.

Method of Collection of Data

Removable acrylic resin disk inlays of 3 mm diameter in size which were custom-made were retained in the holes on intaglio surface of existing Dentures of Group-A & B patients with self-cure acrylic resins & later the Dentures were polished at that particular site. Participants were instructed to wear the Dentures with disks for the study period of total three days. Four disks were inserted to receive samples from Group-A1, and two disks were inserted to receive samples from Group-A2 [Figure 3]. After 24 hrs one disk was retrieved from the lesion site & another from normal site. After three days remaining one disk was retrieved from the lesion site & another from normal site. Only two disks were inserted to receive samples from Group-B where one disk was retrieved after a period of 24 hours & another disk after 3 days [Figure 3]. The retrieval of disk samples was done without disturbing the biofilm on intaglio surface of the disk. After the study was completed the holes in the Dentures were repaired by self-cure acrylic resin.

Inoculation

Each of the retrieved disks from Dentures of both groups was cleaned sonically in distilled water by agitation for 15 seconds to remove salivary debris. The biofilm formed on the disks was scraped thoroughly with sterile B.P. blades separately and then diluted in normal saline in two separate test tubes.

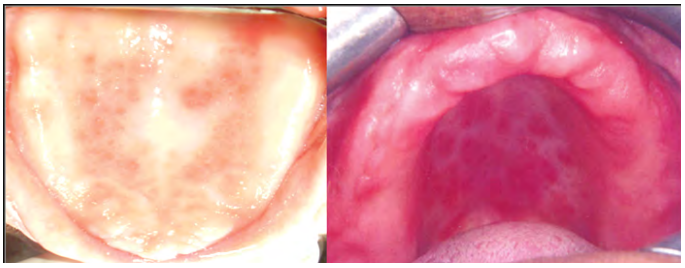

Figure 2: The pictorial representation of group distribution of study samples.

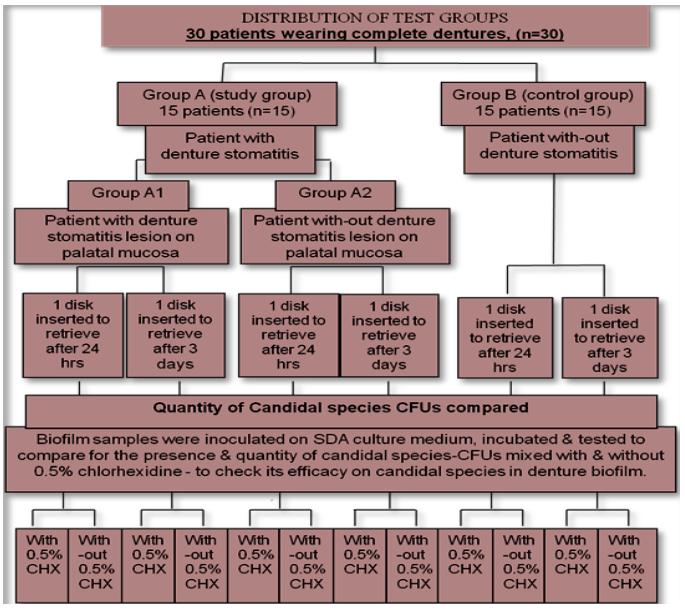

Figure 3: Site of location of disks in dentures of sample group's patient.

To test the efficacy of 0.5% CHX (Chlorhex, Universal medical supplies Co, US.) on Candidal species of biofilm, 10 ml each of biofilm samples taken from both the groups samples retrieved after 24 hours and after 3 days were inoculated with 10 ml of 0.5% CHX, incubated at 37°C for 24 hours & sub-cultured on two halves of Sabouraud Dextrose Agar (SDA) plates & incubated as before & the growth was quantified (CFU/ml) and compared separately [Figure 4].

The results obtained from the above study were collected, tabulated and statistically analyzed.

Results

Descriptive comparison of Means & Std. Deviation of No. of Candidal (CFU)s between (b/w) & within study & control groups, with & without application of 0.5% CHX on 1st & 3rd day samples was done [Table 1]. The No. of Candidal (CFU)s without CHX at lesion site on 3rd day was more than 5.7 log10 Candidal (CFU)s which was reduced to 2.5 log 10 Candidal (CFU)s with an application of 0.5% CHX. Thus fungal load was reduced to 4 log10 Candidal (CFU)s with an application of 0.5% CHX. Similarly, the comparison on 1st day with & without 0.5% CHX shows that the fungal load was reduced to 2 log10 Candidal (CFU)s. The samples from the non-lesion site when compared for means of No. of Candidal (CFU)s, with & without 0.5% CHX, the fungal load was reduced to 0.7 log 10 Candidal (CFU)s for the 1st day and was reduced to 1.9 log 10 Candidal (CFU)s for the 3rd day. The samples from group-B when compared for means of No. of Candidal (CFU)s, with & without 0.5% CHX, the fungal load was reduced to 0.25 log10 Candidal (CFU)s for the 1st day and to 1.0 log10 Candidal (CFU)s for the 3rd day.

The statistical analysis was done by taking actual values without considering the log values for the No. of Candidal (CFU)s count using a Kruskal-Wallis test (H) for group comparison[Table 2], Mann-Whitney U test (z) for inter comparison of groups[Table 3], & Wilcoxon signed rank sum test for comparison between day-1 day-3 samples[Table 4], with statistical package for social science (SPSS) version 11.5 for windows.

The comparison of Means of No. of Candidal (CFU)s b/w groups, the mean and standard deviation (SD) along with probability 'p' and 'H' values (Where 'H' indicates Kruskal-Wallis test) shows that the p-value (p-0.001) was found to be very highly significant for day-1 & day-3 samples from Group-A1, A2 & group-B without 0.5% CHX application, & for day-3 samples from Group-A1, A2 & B after application with 0.5%CHX. The 'p' value (p-0.05) was found to be not significant for day-1 samples from group-A1, A2 & B with 0.5%CHX application [Table 2]. The paired intercomparison of means of No. of Candidal (CFU)s b/w Day-1 & Day-3 samples shows that the probability p-value ($P<0.01$) was found to be highly significant for samples with & without 0.5% CHX application in Group-A1 & and the p-value ($p<0.05$) was found to be significant b/w day-1 & day-3 in Groups-A2 & B for without 0.5% CHX. But p-value ($p>0.05$) was found to be not significant b/w day-1 & day-3 in Groups- A2 & B for with 0.5% CHX [Table 4]. The paired inter-comparison of Means of No. of Candidal (CFU)s b/w samples, for with & without 0.5% CHX application from day1 & day-3 in Group-A1, was found to be highly significant. But the p-value ($p<0.05$) b/w samples, for with & without 0.5%CHX application from day1 & day-3 in Groups-A2 & B was found to be not significant [Table 3]. The overall comparative analysis of no. of Candidal (CFU)s of the samples has been depicted graphically.

Discussion

Candidal existence presents a high significance in the etiology

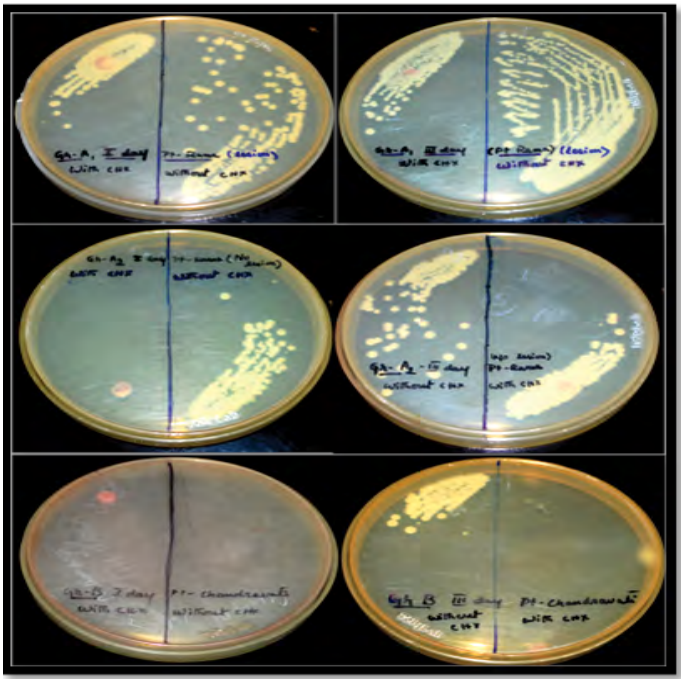

Figure 4: Comparative candidal growth from biofilm samples on SDA plates.

Table 1: Comparison of means & Std. deviation of No. of Candidal (CFU)s & efficacy of 0.5% CHX on study & control group samples.

			Descriptive				
			N	Mean	Std. Deviation	Minimum	Maximum
Without CHX	Day 1	With lesion	15	2.8177	1.90056	0.00	4.78
		Without lesion	15	0.9670	1.42911	0.00	3.30
		Control	15	0.4000	1.08815	0.00	3.70
	Day 3	With lesion	15	5.7513	3.87046	0.00	8.90
		Without lesion	15	2.5757	3.10543	0.00	8.48
		Control	15	1.4708	2.18017	0.00	4.90
With CHX	Day 1	With lesion	15	0.6537	1.42656	0.00	4.60
		Without lesion	15	0.2000	0.77460	0.00	3.00
		Control	15	0.1534	0.59412	0.00	2.30
	Day 3	With lesion	15	2.5510	2.32567	0.00	6.30
		Without lesion	15	0.6738	1.17535	0.00	2.90
		Control	15	0.3269	0.86449	0.00	2.60

Table 2: Comparison of Means of No. of Candidal (CFU)s b/w groups (Kruskal-Wallis Test).

Analysis was done by taking actual values:			Comparison between group				
			N	Mean	Std. Deviation	H	P-value
Without CHX	Day 1	With lesion	15	15333.3333	21238.46466	14.459	0.001 vhs
		Without lesion	15	333.3333	598.41059		
		Control	15	346.6667	1288.33595		
	Day 3	With lesion	13	392929230.8	356247813.8	13.593	0.001 vhs
		Without lesion	13	23933892.31	82979013.91		
		Control	14	15142.8571	26737.63450		
With CHX	Day 1	With lesion	15	2733.3333	10311.62081	1.816	
		Without lesion	15	66.6667	258.19889		
		Control	15	13.3333	51.63978		
	Day 3	With lesion	13	170846.1538	550178.10543	12.566	0.001 vhs
		Without lesion	13	146.1538	296.12887		
		Control	14	42.8571	115.78684		

a. H= Kruskal -Wallis test

Table 3: Inter -comparison of Means of No. of Candidal (CFU)s b/w samples, for with & without CHX. (Mann - Whitney U test).

	Group[a]		Group Statistics N	Mean	Std. Deviation	Z-value
With lesion	Day 1	With0ut CHX	15	15333.33	21238.46466	2.99600
		With CHX	15	2733.3333	10311.62081	p=.003 hs
	Day 3	With0ut CHX	13	3.9E+08	356247813.8	2.89400
		With CHX	13	170846.2	550178.10543	p=.004 hs
Without lesion	Day 1	With0ut CHX	15	333.3333	598.41059	1.75300
		With CHX	15	66.6667	258.19889	p=.08 ns
	Day 3	With0ut CHX	13	2.4E+07	82979013.91	1.79700
		With CHX	13	146.1538	296.12887	p=.072 ns
Control	Day 1	With0ut CHX	15	346.6667	1288.33595	.63700
		With CHX	15	13.3333	51.63978	p=.524 ns
	Day 3	With0ut CHX	14	15142.86	26737.63450	1.57100
		With CHX	14	42.8571	115.78684	p=.116 ns

a. Z= Mann - Whitney U test

Table 4: Comparison of Means of No. of Candidal (CFU)s b/w Day-1 & Day-3 samples. (Wilcoxon signed rank sum test).

			Paired Samples Test			
			Paired Differences			
			Mean	Std. Deviation	Z-value	P-value
Without CHX	With lesion	Day 1- Day 3	-3.9E+08	356238865.3	2.934	.003 hs
	Without lesion	Day 1- Day 3	-2.4E+07	82978822.26	2.366	.018 sig
	Control	Day 1- Day 3	-14771.4	25813.39630	3.023	.043 sig
With CHX	With lesion	Day 1- Day 3	-167692	539111.62490	2.666	.008 hs
	Without lesion	Day 1- Day 3	-69.2308	235.88350	.921	.357 ns
	Control	Day 1- Day 3	-28.5714	132.59871	.816	.414 ns

a. Z=Wilcoxon Signed Rank Sum Test

of Denture stomatitis. Its incidence has been reported to occur among 11-67% of the Denture wearers.[7,8] The yeasts, being a part of the Denture plaque, adhere and accumulate on the surface of the prosthesis that plays a stocking role for them.[9-

[11] The ability to form biofilm is intimately associated with the ability to cause infection and as such should be considered an important virulence determinant during Denture stomatitis.[12] The colonization and growth on prostheses by *Candida* species are of clinical importance.[13] Budtz-Jorgenson et al. in his studies on the clinical aspect of *Candida* infection in Denture wearers hypothesized that the Candida-induced Denture stomatitis usually does not reflect on any deep-seated systemic abnormality, but the Dentures are primary predisposing factors. Therefore preventive measures must be taken against colonization of *Candida* on palatal mucosa and Dentures.[14] Various drugs have been employed to treat Candida-induced Denture stomatitis & CHX is commonly used by dental patients as antiseptics.[6] In the present study an attempt has been made to evaluate the growth of *candida* species in the Denture biofilms using new sampling technique *in vivo*,[4] & to test and compare the efficacy of 0.5% CHX as a disinfectant on Denture biofilm in patients with & without Denture stomatitis. Olsen. I did a study on five patients of Denture stomatitis who were treated with a combination of Amphotericin-B lozenges and Denture soaking in 0.2% chlorhexidine to assess their long-term disinfection ability,[7] but Budtz-Jorgenson et al.[14] report Chlorhexidine gluconate (0.2-2%), as a disinfectant for *candida* infections in Denture wearers. However according to Therand et al.[6] Chlorhexidine at 0.5% was the only fungicidal agent effective on pure cultures, yeast mixtures, and biofilm. So 0.5% CHX was tested for its efficacy on candidal biofilm & was observed for the reduction of fungal load on Denture biofilm before & after 0.5% CHX application over the lesion and non-lesion site of Denture stomatitis patient as well as on normal Denture wearing patients for 1st day & 3rd day. According to Chandra et al. only chlorhexidine (0.5%) reduces a fungal load in biofilm conditions by more than 4 log10 yeast cells per ml & thus fungicidal.[5] In accord with these results, the results of the present study show that the mean No. of Candidal (CFU) s without CHX at lesion site on 3rd day was more than 5.7 log10 Candidal (CFU)s and the fungal load reduces to 2.5 log10 Candidal (CFU)s with an application of 0.5% CHX [Table 1]. Catalan et al.[15] in a study on Denture plaque on palatal mucosa in patients with & without Denture stomatitis revealed that the microbiologic analysis of Denture plaque samples in patients with healthy mucosa showed the presence of gram positive cocci in all & no presence of yeast even in single patient studied. But the samples from Denture stomatitis patient showed the presence of gram positive cocci in all & presence of yeast in all except one patient studied.

In this study, the targeted micro-organisms were only Candidal species. In the study group, 1st day samples showed a presence of yeast in all samples except two patients & 3rd day samples showed a presence of yeast in all samples of biofilm retrieved from Denture stomatitis patient. However two patients who didn't show any presence of yeast on the 1st day did not return on 3rd day & in two patients results were not tabulated because of contamination of disks probably due to technical problems, and in both conditions, these samples were excluded from the study. Though the sample size had been reduced the results were not affected much, as the sample size taken were relatively large with prior considerations of the study subjects & technical factors. In control group, the 1st day samples showed a presence of yeast in only two samples & 3rd day samples showed a presence of yeast in only four sample of biofilm retrieved from non-Denture stomatitis patient. However in control group also one patient who didn't show any presence of yeast on the 1st day did not return on 3rd day & in two patients results were not tabulated because of contamination of disks probably due to technical problems, and in both conditions, these samples were excluded from the study. The results obtained in this study were relatively similar to the results obtained from Catalan et al. [15] study, except the presence of yeast in two & four biofilm samples of non-Denture stomatitis patients respectively from 1st & 3rd day samples. The presence of yeast in nonDenture stomatitis patients may be due to commensals or may be due to dormant Denture stomatitis without actual lesions. According to Chandra et al.[5] the biofilm associated with candidal cells compared with planktonic form cells are resistant to the antifungal agent used to treat Denture stomatitis & only Chlorhexidine (0.5%) reduces a fungal load in biofilm conditions by more than 4 log 10 yeast cells per ml & thus fungicidal. In the present study the biofilm samples though extracted from sample patients were in their original form, but during subjecting them for growth on SDA-plates & further during efficacy testing with 0.5% CHX, the original form of biofilm could not be maintained & were converted into planktonic form & is considered as one of the limitations of this study not to subject the biofilm for study in its original form instead of planktonic form under the present conditions.

Further investigations should be made to overcome the before mentioned problems associated with a microbiologic study to determine the candidal count in Denture biofilm. However exclusive electron microscopic analysis gives better information regarding biofilm forms but rarely gives quantitative information of *Candida* in a biofilm condition.

Conclusion

Within the limitations of this study, the following conclusions can be drawn:

- The No. of Candidal (CFU)s count in Denture-biofilm increases exponentially, with the statistically highly significant order from day-1 to day-3 at lesion site of Denture stomatitis. The No. of Candidal (CFU)s count in Denture-biofilm increases exponentially with a statistically significant order from day-1 to day -3 at non-lesion site of patients with and without Denture stomatitis.
- 0.5% CHX was very effective & of statistically highly significant order in reducing the fungal load at lesion site of Denture stomatitis. 0.5% CHX was also effective in reducing the fungal load at non-lesion site of patients with & without Denture stomatitis, but statistically not significant.

As the No. of Candidal (CFU)s count in Denture-biofilm decreases significantly, with 0.5% CHX at Denture stomatitis lesion & non-lesion site, & also in nonDenture stomatitis patient, the Dentures can be soaked in 0.5% CHX for a specific time period to treat or to avoid Denture stomatitis in patients wearing Dentures.

Competing Interests

The authors declare that they have no competing interests.

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