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Isolation of Microbes Associated with Biofilm Formation on Removable Partial Denture (RPD) and Oral Hygiene Regimen for RPD Patients

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ABSTRACT

Biofilm microorganisms are associated with intermittent persistent oral infections and are substantially resistant to antimicrobial agent. It has been noted that more than 600 different types of bacteria are present in oral cavity which causes biofilm formation. Some are S. mutans, P. aeruginosa, S.aureus, E.coli, B.subtilis. These bacteria encounter foremost troubles such as dental caries and loss of teeth. Biofilm formed swab sample was collected of 30 patients by using swabbing technique and bacteriological examination was carried out. The isolates were recognized by standard microbiological procedure. By TM and CRA method, Biofilm detection was carried out. By Kirby-Bauer disc diffusion technique, antibiotic susceptibility was performed. TM method shows biofilm formation inside the tube and on CRA medium black colored colonies were observed by biofilm producing organism. We can conclude that S.mutans, P.aeruginosa is highly, S.aureus is moderately and E.coli is weakly responsible for biofilm formation while B. subtilis is non – biofilm producer. These organisms show susceptibility to various antibiotics.

KEY WORDS: Oral Flora, Biofilm, Antibiotic Susceptibility Test

INTRODUCTION

The Microbial multiplicity in buccal cavity is among the biggest surface as characterized in human body. By precise attention is the dental biofilm, which forms initial selective adsorption of bacteria from saliva onto tooth surface.The aggregate of microorganisms reside on the surface and in deep layers of skin, saliva, oral mucosa as well as in conjunctiva and in gastrointestinal tracts. The microflora existing in oral cavity is called oral micro flora. One of the commonly encountered problems in dentistry is loss of teeth and consequential replacement. Along with the restoration of function and aesthetic, removable prosthesis may change the oral ecology either qualitatively or quantitatively, such as increasing the total amount of oral microorganisms, (Azizah AL Mobereek. 2003 Heller et al, 2015).

Oral microbiology is the study of microorganism of the oral cavity and their interaction between oral microorganisms or with the host (Stewart et al, 2001). The environment present in mouth allows the organism to grow there. The health of our mouth mirrors the condition of our whole body. For example, if our mouth is healthy, chances of our overall health is good too. On the other hand destitute oral health may lead to many oral problems such as formation of biofilm, dental caries, oral and facial pain, problems with the heart and other major organs, digestion problems and periodontal diseases. Oral cavity is a great habitat with a stable induction and removal of microbes with nutrients. These opportunistic human pathogens colonize at several anatomically distinct surface of human body, mainly in warm and moist areas such as oral cavity. As these are opportunistic pathogens they cause various dental problems such as formation of biofilm, dental plaque dental caries and periodontal diseases (Marsh 2006). Periodontitis is frequent health difficulty caused by pathogenic biofilm forming bacteria that accelerates inflammation resulting in either reversible gingivitis or severe periodontal damage, leading to loss of healthy tooth, (Gutt et al, 2018).

Biofilm have been concerned as the main source of etiopathogenesis of dental caries and associated diseases. However biofilm can

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be removed by regular oral hygiene aids or specialized dental instruments, they have the capacity to set into dental calculus making their taking away too difficult. Consequently, these biofilms establish a great challenge to the dental practitioner in the control and suppression of biofilm associated periodontal diseases.Biofilms are totally well known concept. In the prior decades of evolutionary microbiology, Antony Von Leeuwenhoek who invented microscope was the first to observe microbial aggregates, which are now known as Biofilms, in scrapings of plaque from his own teeth. The term 'Biofilm' was coined by Bill Costerton in 1978. Wilderer & Charaklis (1989) discussed about the reasonably vague microbial community linked with a tooth surface or any other hard non-shedding material, erratically scattered in a shaped matrix or glycocalyx (Socransky 2002). Biofilm is classified into two groups on the basis of the position, mainly Supragingival- which show aggregation lying on coronal to the gingival margin and Sub gingival-which is present apical to the gingival margin (Ximenez-Fyvie 2000). Whereas, on the basis of pathogenicity it is divided into Cariogenic- which generally includes acidogenic and gram positive type of microbial flora and Periopathogenic- commonly includes basophilic and gram negative type of microbial flora.

Dental caries is the localized destruction of dental hard tissue by acidic by products from dental plaque containing acid producing bacteria. Establishment of a biofilm is a multifaceted progression that follows more than a few distinctive phases, commencing with adsorption on to the tooth surface of a habituation film derived from bacterial and host molecules finally results into tooth outbreak or tooth cleanout. This aggregation is followed by passive transport of bacteria regulated by feeble long-range forces of attraction. Irreversible attachment occurs due to strong and weak forces created by covalent and hydrogen bonds. Biofilms possess several properties like they are ubiquitous and form on almost all surfaces engrossed in ordinary aqueous environments. Biofilm confers assured properties to bacteria that are not usually found in the planktonic condition, this justifies credit of dental plaque as a biofilm, (Yu O et al, 2017).

Antibiotic resistance is one of the principal intimidations to worldwide health food security and development. By alterations of bacterial genes in response to use excess medicine or antibiotic drug, antibiotic resistivity get occurred finally resistant get developed and these bacteria possibly will infect human and resistant bacteria infection caused which are difficult to treat than sensitive bacteria (Guidelines by WHO). Day by day Antibiotic resistance is emerges rapidly at very high level in all parts of world. For that reason to avoid and manage the extend antibiotic resistance in diverse species of microbes, health professional can only distribute to local antibiotic in specific cases, by following the guideline given by WHO. Hence the scope of present study is to cure oral infection that arises due to RPD/CD those are treated with application of oral depositors (antibiotic) RPD in wearer patients.Causes of Biofilm Formation:- There are several causes of biofilm formation which are, due to improper oral hygiene, due to deposition of food particles, onto the teeth.Biofilm Associated Infectious Diseases:-Biofilms are associated with various microbial infections (by one estimate 80% of all infections). These commonly include dental caries, necrotizing fasciitis, biliary tract infection, osteomyelitis, bacterial prostatitis, native valve endocarditis, periodontal disease, otitis media, musculoskeletal infections, melioidosis, cystic fibrosis pneumonia and peri- implantitis. Significant characteristics of these infections are perseverance and chronicity (Socransky et al, 2002)

Periodontal Biofilms and treatment aids:-In dentistry, for particular disease there is appropriate treatment that facilities for every spot in each patient. Individually we must focus on treatment planning. There is a need to focus on biofilm control which is essential for the repair of oral healthiness and for avoidance of dental caries, gingivitis and periodontitis. Oral Biofilms and potential controlling aids:-One can control biofilm formation by inhibiting bacterial colonization, by changing of plaque biochemistry and Alteration of plaque natural balance by controlling bacterial growth and metabolism, Interruption of recognized plaque.

Clinical Approaches

- Involuntary Plaque controlling methods: - We should clean our tooth by dental floss, wooden tips, various perio-aids, fine brushes, Rubber tip, Oral irrigation devices, Tooth brushes- Manual as well as Electrical.
- Chemically Plaque Controlling method:-
- We can use various enzymes like Mucinase, Dehydrated pancrease, Lactoperoxidase, hypothiocyanate.
- And Antibiotics can applied whenever necessary like Penicillin, Vancomycin, Erythromycin, augmentin, (Quirynen et al, 2006).

Removable Partial Denture (RPD):A removable partial denture (RPD) is predominantly used in a partially edentulous patient who needs to have replacement teeth for functional or artistic reasons and who cannot have a bridge (a fixed partial denture) for any reason, such as a lack of required teeth to serve as support for a bridge (i.e. distal abutments) or financial limitations. This type of prosthesis is referred to as a removable partial denture because patients can remove and reinsert it when required without professional help. Conversely, a "fixed" prosthesis can and should be removed only by a dental professional.

Role of RPD in protection of teeth

It allows more rapid placement of denture and allows natural tooth position to be duplicated. It also allows teeth position to be altered and permits a test period for change in the tooth position before definitive RPD are made like its appearance, space maintenance as a vehicle for tissue treatment material. Diseases related to oral

cavity:-Dental caries, Gingivitis, Periodontitis, Thrush, Chipped tooth, Teeth grinding, Darkened teeth, Dental plaque. Oral hygiene regimen for patients who wear an RPD:-Taking measures to keep your mouth clean is essential for excellent dental health. A daily oral hygiene regimen is needed to remove the dental plaque that causes tooth decay and gum diseases. A good oral hygiene not only helps to prevent cavities, but it is necessary to battle bad breath. Practicing good oral hygiene can reduce the chances of developing complications or illness from dental diseases and could prevent the need for a costly gum diseases treatment by brushing, flossing, mouth wash, diet, professional techniques. Control of biofilm formation on RPD:-Brushin teeth and all mouth prosthesis or appliances to mechanically disrupts the biofilm. Choosing toothpaste containing antibacterial ingredients such as triclosan and rinsing ones mouth with a mouthwash containing antibacterial ingredients, such as chlorohexidine, celytpiridoinium chloride, or mixture of essential oils in alcohol works better. Soaking the prosthesis with a commercially available cleaner and if the denture line is cracked, porous or peeling, getting it repaired helps to eliminate unwanted organisms.

MATERIAL AND METHODS

This study was a laboratory based experiment which was done to isolate and identify biofilm forming bacteria and evaluates different methods of detection of biofilm formation on the removable partial denture (RPD)This experiment was carried out adopting the following materials and methods. Sample Inoculation:-The samples of 30 patients were collected from prosthodontics clinic of Akola city. Complete history and examination was performed for the study of common oral micro flora and further study of oral depositors. All patients with denture RPD/CD was subjected to bacteriological examination. Biofilm formed swab samples were collected in sterilized test tubes and the bacteria were isolated and identified in laboratory.

Media: Nutrient broth, Nutrient agar, Baired -Parker agar, EMB agar, MacConkey agar, Pseudomonas agar were used as a culture media for all the bacterial species used in this study. Biochemical studies of all isolates were carried out using media such as Trypton broth, Glucose- Phosphate broth, Citrate agar for IMViC classification and various sugars for sugar fermentation. Biofilm detection was carried out using Trypticase -Soy broth, Congo red agar medium, Brain heart infusion agar. Antibiotic susceptibility was done by using Mueller- Hinton agar.

Morphological Studies:The color of colonies were observed directly with naked eye, Gram characteristics were observed by performing Gram staining and morphological characteristics were observed under microscope.Isolation of Microorganism from Oral Cavity:-As mentioned earlier the samples of 30 patients were collected from prosthodontics clinic of Akola city. Complete history and examination was performed for the study of common oral micro flora and further study of oral depositors. All patients with denture RPD/CD was subjected to bacteriological examination. The biofilm formed swab samples were inoculated on petri plate containing different media's and incubated at 37 °c for 24 hrs. Biochemical Test:-Isolated organism was confirmed by studying their biochemical characteristics. For this IMViC classification was done and sugar fermentation was studied. Methods of Detection of Biofilm Formation:-It was complete by using various methods such as Tube method(TM), Congo red agar medium (CRA) test.

Tube Method

Christensen et al (1982) described that this is a qualitative method for biofilm detection. In 10 ml trypticase soy broth a loopful of test organism is gets inoculated with 1 % glucose solution in test tube. Then tubes were incubated at 37 °c for 24 hrs. After incubation tubes were decanted and washed with phosphate saline buffer (pH 7.3) and allow to air dry more over tubes were stained with Crystal Violates (0.1%) And excess stain was washed with deionized water. Tubes were dried in inverted position and biofilm formation was observed by observing a film lined on the wall as well as to the bottom of test tube.

Congo Red Agar Medium (CRA) Test

Freeman et al (1989) have described a simple qualitative method by using CRA. By preparing Congo red stain in a concentrated aqueous solution and allow to autoclaved at 121°c for 15 min separately from the other constituent. Then autoclaved Brain heart infusion agar was added to it with sucrose at 55°c. After proper mixing CRA plates were prepared and inoculated with test organism and incubated at 37°c for 24 hrs. aerobically. After incubation period black colonies with dry crystalline consistency were found which indicated production of biofilm. Antibiotics Susceptibility Test Was Performed By Using Kirby- Baur Disc Diffusion Method: Preparation of Plates:-Freshly prepared nutrient broth was used in which inoculation of test organism was done and incubated at 37°c for 3 hrs. Mueller Hinton agar plates were prepared and inoculated with test organisms by uniform swabbing method with the help of sterile swab and then octadisc of antibiotics is kept onto the inoculated MHA plates. The plates were inoculated at 37 °c for 24 hrs. in straight position(Baur et al 1966).

RESULTS AND DISCUSSION

The present study was conducted in the period of September 2017 to March 2018. Total 30 biofilm formed swab sample were collected from different RPD patients of different age groups from the prosthodontics clinics of Akola city. The purpose of this present investigation deals with the evaluation of biofilm forming bacteria from the removable partial denture (RPD) patients and evaluation of different methods for the detection of biofilm formation by the organisms.

Sample Characteristics:- Table 1.1 illustrates the distribution of sample according to Age, Sex, and And Type of Prosthesis. Age range of patients was 31-80 years. Mean age was 50.70 years and mode was 40 years. It was found that 76.7% females and 23.3% males were carrier of prosthesis. Almost 70% of the patients were receiving Removable Partial Denture (RPD), while, 30% of the patients were receiving Complete Denture (CD). There was age range majority of patients 41-60 was 60.1% while 31-40 years and above 80 was nearly 40%.

Microbiological Findings

Table 1.2 illustrates the distribution of predominantly cultured oral flora by the type of prosthesis. The microorganisms isolated from the samples were *Streptococcus mutans, Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli, Bacillus subtilis. The streptococcus mutans (26.7%) was the most cultivated* microorganism particularly among the partial dentures. A wide variety of the predominantly cultured bacterial strain were found among females some strain were mostly cultured from female including *Streptococcus mutans and Staphylococcus aureus*.The results of this study revealed highest load of *S.mutans followed by S. aureus, P. aeruginosa, E. coli and B. subtilis* present in the biofilm formed on the surface of RPD/ CD patient. From the above observation it was observed that from these samples we have isolated these bacteria which were responsible for the biofilm formation on Removable Partial Denture. These bacteria were confirmed by studying their cultural and morphological characteristics.

After identifying the bacteria we had checked for their biofilm forming ability. Biofilm formation was checked out by using two different methods which are Tube method(TM) and Congo red agar media (CRA). These two methods showed the qualitative estimation of bacteria responsible for the formation of biofilm onto the surface of RPD/CD patients. Table 1.4 showed that except B.subtilis all bacteria were responsible for biofilm formation on the surface of RPD/CD patients. From these the tube methods shows the dark or thick layer of biofilm formed inside the tube by the bacteria which were biofilm producer and it was stained by using 0.1% crystal violet stain, while the organisms which were non biofilm producer formed the light or thin layer inside the tubes. From these tube method we can qualitatively estimates the bacteria those were biofilm producer and non- biofilm producer.

Table. 1.1: Distribution of sample according to Age, Sex, and Type of Prosthesis

Age Group of Patients	Sex		Type		Total %
	Female No	Male No	RPD	CD	
31-40	4(13.3)	3(10)	5(16.7)	2(6.6)	7(23.3)
41-50	8(26.7)	2(6.7)	7(23.3)	3(10)	10(33.4)
51-60	8(26.7)	--	5(16.7)	3(10)	8(26.7)
61-70	3(10)	1(3.3)	3(10)	1(3.3)	4(13.3)
71-80	--	1(3.3)	1(3.33)	-	1(3.3)
TOTAL	23(76.7)	7(23.3)	21(70)	9(30)	30(100)

Further Table 1.4 illustrate that S.mutansis strongly biofilm producer as it produces thick biofilm layer inside the tube followed by S.aureus, P.aeruginosa. While E.coli is weakly biofilm producer and B.subtilisis non biofilm producer as it does not formed any layer inside the tube. It was further confirmed by another method i.e is congo red agar method which gives qualitative estimation of biofilm formation by showing the black coloured colonies onto the congo red agar medium if the bacteria was biofilm producer and it showed red color colonies if the bacteria was non biofilm producer. Table 4.1showed the bacteria that strongly responsible for biofilm formation were S. mutants > P. aeruginosa > S. aureus > E.coli. while B. subtilis was non biofilm producer.

On Mueller Hinton Agar the antibiotic susceptibility test of biofilm producing bacteria was done using the variety of antibiotic. By using Kirby-Bauer disc diffusion technique, an antibiotic susceptibility test was performed according to CLSI guidelines. Microorganisms developing in a biofilm are fundamentally more resistant to antimicrobial agents than planktonic cells. To inactivate organisms growing in a biofilm high antimicrobial concentration are necessary, as antibiotic resistance can enhance 1,000 times (Socransky & Haffajee, 2002). Higher antibiotics resistance in biofilm producing bacteria than non- biofilm producers were observed during study. Maintenance of good oral hygiene is the key to the prevention of dental diseases. The primary etiological factor for dental diseases is dental plaque. The formation of plaque on the tooth surface is characterized by the progression from a limited number of microbial species to the complex flora of mature dental plaque. This progression involves initial adherence of bacteria to the salivary pellicle and subsequent accumulation by growth and interbacterial adherence. Ultimately, that ends up in the destruction of hard enamel tissue.Biofilm producing bacteria are accountable for numerous unmanageable infections and are disreputably complicated to eliminate. Various methods for biofilm detection is there. In this study we evaluated 30 isolates by three screening methods for their capability to developed biofilms. The TM method and CRA method were used for the detection of biofilm formation. The result of this study showed that TM cannot be compulsory for general screening test to recognize biofilm forming bacteria.

In another study (Freeman et al 1989), distinguished that out of 147 isolates of S. epidermidis, by TM method ,79 (53.7 %) biofilm

Table 1.3: Distribution of predominantly cultured oral flora by age

Microorganism	Age of Patients					Total
	31-40	41-50	51-60	61-70	71-80	
Streptococcus mutans	3	4	2	-	1	10
Pseudomonas aeruginosa	-	2	2	1	-	5
Staphylococcus aureus	1	3	1	2	1	8
Escherichia coli	-	2	1	-	1	4
Bacillus subtilis	-	-	2	1	-	3
Total	4	11	8	4	3	30

formation is detected and 64 (43.5%) was detected by CRA method. They found that TM is superior for biofilm recognition than CRA. TM is used to detect biofilm formation among uropathogens (Baqai et al 2008). According to their findings, 75% of the isolates exhibited biofilm formation (Ruzicka et al 2004). The CRA method showed minute association with the other methods as well as parameters of sensitivity (11%), specificity (92%) and accuracy (41%) were very low. Three isolates were found to be false positive and 62 were false negative11 by this method, therefore CRA method for biofilm detection in their study did not suggested. CRA detected only 3.8% out of 128 isolates of S. aureus as biofilm producers as compared to TCP which detected 57.1%.

Biomechanical factors are considered for dental treatment in prior to give values like firmness and preservation. On the other hand RPD planning cannot be paying attention simply on mechanical concerns, since this will not assured an unbeaten result. The journalism undoubtedly emphasizes the necessity to judge basic ethics of RPD design and conserve the oral structures. to enhance the stability uses of bars and connectors can be done. On the other hand, the RPD design should keep away from food accumulation and biofilm formation. At what time successful biomechanical values can be recognized some bars and connectors can be detached or customized with the purpose of avoid minute retentive places close to abutment teeth.

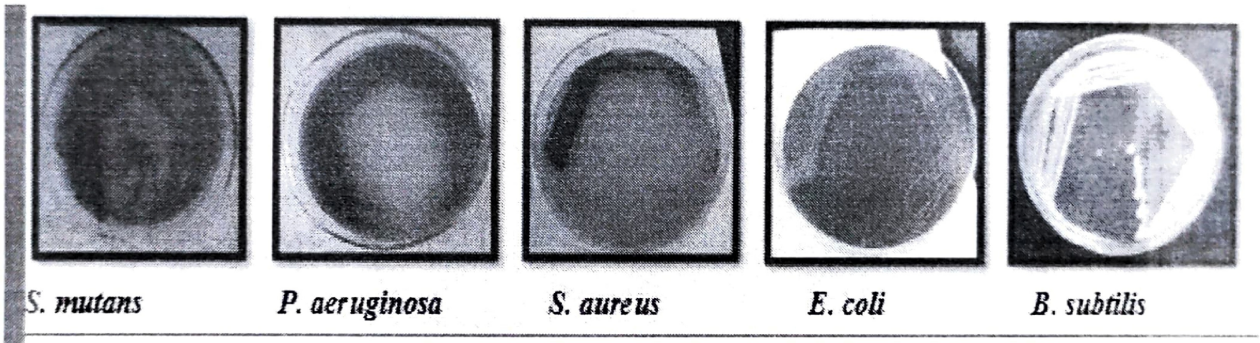

Fig. 1.1: Colonies on Selective Media

(a): Tube Method

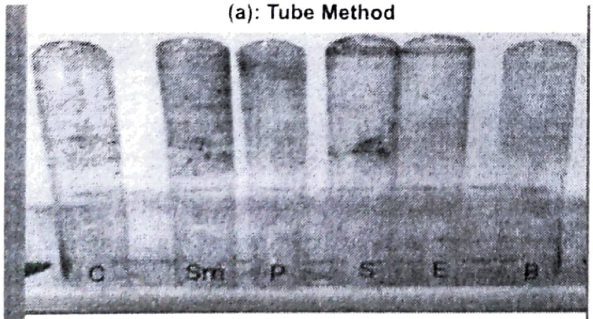

Fig. 1.2: Methods for Detection Of Biofilm Formation

Prevention must be integrated into the patient's daily routine. By taking into consideration that the tough involvement between the use of RPDs with biofilm accumulation and caries. Oral sanitation concerns must be included into the action plan. It is obvious that notice to the protective aspect of RPD treatment must contain

(b): Congo Red Agar Method

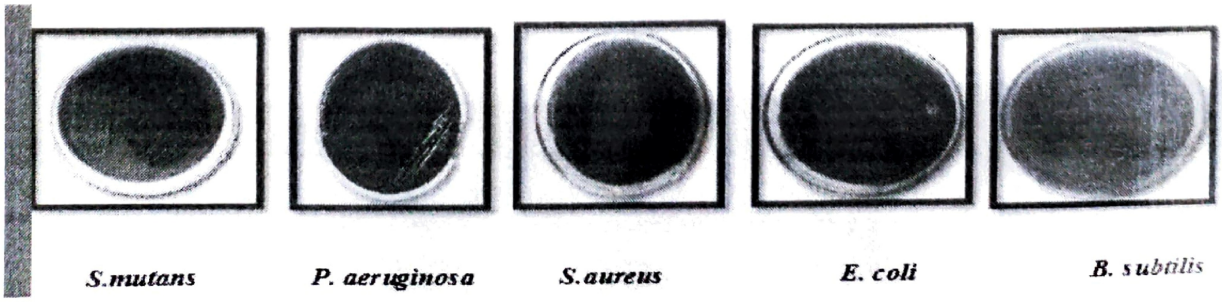

Table 1.4: Inspection of Bacteria for Biofilm Formation By Tube Method And Congo Red Agar Medium and their antibiotic susceptibility test

Organism	Biofilm producer	Tube Method	Congo Red Agar Medium	Sensitivity To Antibiotics
S. mutans	Biofilm producer	+ ve	Black colonies formed	Chloramphenicol, Ciprofloxacin, Norfloxacin Gentamycin
P.aeruginosa	Biofilm producer	+ ve	Black colonies formed	Colistin, Gentamycin
S. aureus	Biofilm producer	+ ve	Black colonies formed	Norfloxacin, Nitrofurantoin, Co- trimoxazole, Doxycycline, Vancomycine, Azithromycin
E. coli	Biofilm producer	+ ve	Black colonies formed	Nitrofurantoin, Ampicillin, Gentamycin, Cephalexin Trimethoprim, Nalidixic acid, Ciprofloxacin, Augmentin
B.subtilis	Non - Biofilm producer	--ve	Red colonies formed	Colistin, Gentamycin, Cephradine, Co-Trimoxazole, Tetracycline, Ampicillin, Ceftriaxone

more than periodic check-ups. It was confirmed in a study that S.mutans levels in saliva increased drastically 48 days after prosthesis placement, with levels above 106 CUF/mL of saliva. RPD users repeatedly have complexity removing the biofilm, even under instruction and after being instructed in the accurate use of a toothbrush and dental floss. Even if the importance of an effective oral hygiene regimen is stressed, certain RPD patients remain at risk for biofilm buildup and the progress of caries and periodontal disease. Included there are those who are ill or physically weakened. For those patients, the assistance of a second person home health aide may be needed.

The biofilm buildup and caries index are directed by the addition to in hard surfaces in the mouth subsequent placement of an RPD. As illustrious formerly, there is an enhancement in microorganism-retentive areas with the attendance of an oral prosthesis, purposely the acrylic resin base and metallic structure of the RPD. Additionaly, the high intake of fermentable carbohydrates can direct to an increased caries occurrence. By means of a diet diary, it is observed that RPD patients had greater sugar uptake in addition that improper consumption of meals can make it complicated to control biofilm accretion by standard methods(Quirynen et al 2006, pp134-69). The results of this study revealed that a fairly considerable change in oral flora does occur following the insertion of a removable prosthesis. This is very well acknowledged in the literature, and is of a particular concern since oral ecology preservation is essential to maintain oral health. This change however is not universal; rather it occurs among some of the patients. Host and properties of the mouth are all internal factors that may cause changes in the oral ecology. Those factors may include susceptibility to a particular bacterial strain, pH and nutrients available, material, type and design of the prosthesis and oral hygiene. Changes in oral ecology may affect oral health adversely. Thus it is imperative that factors such as the effect of the introduction of any appliances is investigated and

evaluated. This is to assure and maintain a healthy oral function and environment. Clinically, one should attempt to monitor such changes. Further studies are indicated to ascertain the changes and assist its clinical effects, management and prevention (Addy & Bates 1979).

CONCLUSION

Dental biofilm is a complex, organized microbial community that is the primary etiologic factor for the most frequently occurring oral diseases, dental caries, biofilm formation and periodontal diseases. Although the dental biofilm cannot be eliminated, it can be controlled with comprehensive mechanical and chemotherapeutic oral hygiene practices. Although additional research is needed, there is the possibility that these cost-effective, preventive strategies may minimize the effect of periodontal diseases on specific systemic conditions. Within the limitation of this study, it is concluded that the ecology may encounter some changes after the introduction of a removable prosthesis. These changes though minimal but may provoke some clinivcal changes such as denture stomatitis. Follow up visits, together with other factors such as elimination of surface roughness; trauma and dietary advices may assist in the elimination of some clinical complications after prosthesis insertion. RPD users can be measured at high jeopardy for enlargement of caries and related periodontal disease. Dental professionals must educate these patients and encourage them to maintain periodic recalls. RPD patients may possibly not be capable to keep appropriate oral hygiene due to higher age, physical disabilities or poor motivation. Additionaly, prophylactic measures including the submission of a chlorhexidine gel should be adopted by RPD patients in order to maintain a hale and hearty mouth.From the 30 different sample it was found that the bacteria which were qualitatively enumerates as strongly responsible for the biofilm formation on RPD were S.mutans>P.aeruginosa>S.aureus>E.coli>B.subtilis.These organisms get accumulated onto the tooth surface and formed the yellowish color layer on the surface of tooth which is known as biofilm. This is due to improper oral hygiene. From the study we can conclude that to avoid biofilm formation and diseases related to oral cavity one must maintain proper oral hygiene. By this study we can conclude that TM and CRA were the two methods which are used for the qualitative estimation of biofilm formation. Also we can conclude that there were changes in the number of microbial flora present before and after the insertion of prosthesis. We have experiential higher antibiotics resistance in biofilm forming bacteria than in non-biofilm producers.

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