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RESEARCH ARTICLE

INVITRO DETECTION OF BIOFILM PRODUCED BY MICROORGANISM ISOLATED FROM PATIENTS OF PERIODONTITIS

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Abstract

Introduction: Periodontal infections, including gingivitis and periodontitis, form a major group among the most encountered chronic diseases with infective etiologies. Microorganisms present in gingival sulcus around teeth form microbial biofilm, which is most important cause of periodontal diseases. Biofilm, a three-dimensional (3D) microbial structure with cells enclosed within a self-produced extracellular matrix that may be attached to a substratum comprises the structure of a biofilm. This study aims to detect biofilm in microorganisms isolated from periodontal pockets and establishment of relation between biofilm with tobacco chewing and comorbidies.

Material and methods:Total 100 Patients' samples were collected using bent swab from periodontal lesions. Samples were processed aerobically and identification of the isolates were done along with simultaneous demonstration of in vitro biofilm formation.

Results: Biofilm production was detected by using pre sterilized 96 well polystyrene micro titer plates. 71 samples were shown growth of microorganisms like *Streptococcus viridians* (36), *Klebsiella pneumoniae (21), E. coli (6), Klebsiella oxytoca (4), Acinetobacter baumannii (1), Pseudomonas aeruginosa (1), Staphylococcus aureus (1), and Coagulase negative staphylococcus (1).*19 isolates of *Streptococcus viridians* had formed biofilm out of 36 isolates. 16 isolates of *Klebsiella pneumoniae* had formed biofilm out of 21 isolates. 3 isolates of *Klebsiella oxytoca* had formed biofilm out of total 4 isolates. 2 isolates of *E. coli* had formed biofilm out of 6 isolates. One isolate, each of *Acinetobacter baumannii*, *CONS and Pseudomonas aeroginosa*had formed biofilm.

Discussion: Out of 43 positive oral biofilms, 21% were tobacco chewers and out of negative oral biofilm, 15% were tobacco chewers. Among positive oral biofilms, 19% had co morbidities and among negative oral biofilm, 15% had co morbidity.

Conclusion: The oral colonization by biofilm producing strains can also increase the risk of their dissemination to various human tissues and

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organs. Apart from that, biofilms cause resistance to many antimicrobial agents.

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Introduction:-

Periodontal diseases are a group of diseases that cause inflammation and destruction of the investing and supporting structures of the teeth. Periodontal diseases, including gingivitis and periodontitis are among the most common chronic diseases. The ability of bacteria to modulate gene expression allows them to finely regulate their growth rate depending on nutrient availability conditions. Around 700 microbial species present in sub gingival micro biota, among these only few species of microorganisms associated with pathogenesis of periodontal diseases. Microorganism present in gingival sulcus around teeth form microbial biofilm, which is most important cause of periodontal diseases[1]. Bacteria can also transform from an individual and free-floating (planktonic) state to a community-like condition where they improve their ability to survive in adverse environmental conditions. This self-organized arrangement, known as biofilm, consists of a three-dimensional (3D) microbial structure with cells enclosed within a self-produced extracellular matrix that may be sometimes attached to a substratum. Biofilm contributes as the main virulence factor causing biofilm-related infections. Thus, the planktonic form of bacteria gradually converts to biofilm aggregates. Bacteria presented in biofilm are more resistant to antimicrobials than planktonic bacteria and therefore infections caused by them are more difficult to treat. Hence, detection of biofilm production is of utmost importance[1,2,3]. Certain methods like microtitre plate method, tube method, congo red agar method and bioluminescent assay are employed for effective invitro biofilm detection. Other than that, these methods are also used for screening of antibacterial effect of agents against biofilm embedded microorganism[1,2,3]. Gene identification and gene expression measurement as a result of antibiofilm activity and antibacterial activity of agents can be advantageous in biofilm detection related studies[4].

Materials And Method:-

Aim:

Detect biofilm in microorganisms isolated from periodontal pockets and establishment of relation between biofilm with tobacco chewing and co morbidities.

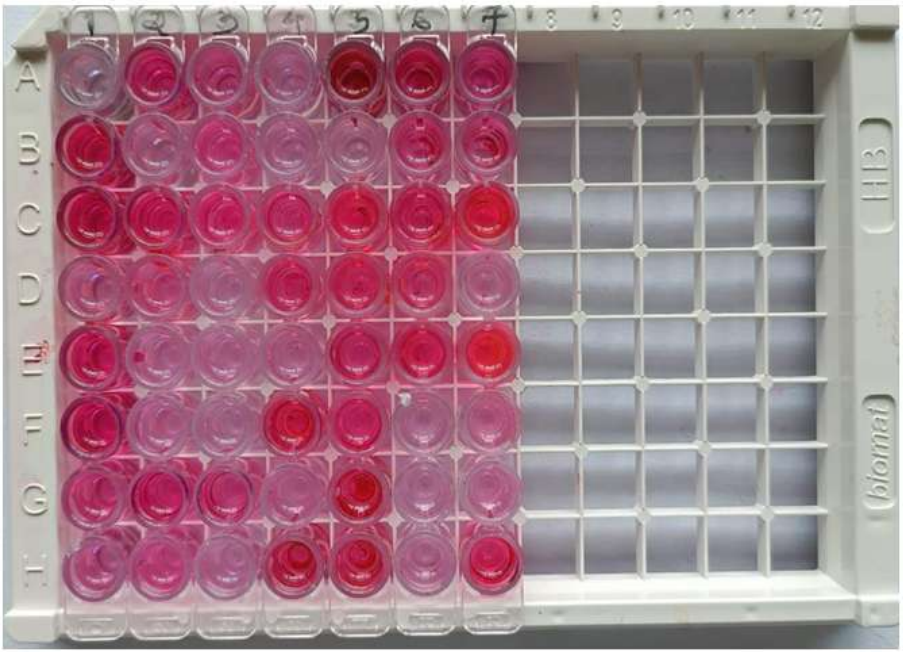
Sample Collection:

Study was started after ethical approval for the same from Human Research Ethics Committee of Government Medical College (GMCS/STU/ETHICS/Approval/19801/2022). Patients coming to dental OPD with signs and symptoms (swollen gum, bright red, dusky red gums, pus between gum and teeth, complain of pink tinged tooth brush after brushing, gums that bleeds easily etc) of periodontitis were selected for the study. Patents were informed about the study in the language they comprehended and written informed consent was taken from all the patients and/or their legal guardians. Patient's samples were collected from periodontal lesions using bent swab stick to collect proper sample from periodontal lesion pocket. Samples were transported to Microbiology laboratory in cold chain at 2-4^0 C for further processing. All the methods were carried out in accordance with relevant guidelines and regulations.

Detection of biofilm:

Samples were processed aerobically and microbiological identification was performed. All isolates were maintained by repeated subculture for further process of biofilm formation. Biofilm production was detected by micro titer plate method by using pre sterilized 96 well polystyrene micro titer plates. For each isolate and controls, a suspension from an overnight culture was prepared in sterile distilled water and it was adjusted to 1 McFarland standard. Each well of microtitre plate was filled with 180 microlitre of brain heart infusion broth with 8% glucose and 20 microlitre of prepared suspension was added along with this. After that microtitre plate was covered and incubated at 37°C for 24 hours. After incubation, the wells are washed thrice with distilled water and then stained with 1% safranin for 5 minutes [5, 6]. Then, transmittance (in percentage) of the stained wells of the plate was read at 630 nm by ELISA reader. (Fig 1)

Figure 1:- Biofilm detection in microtiter plate.



All tests were done thrice and Optical density of controls (ODc) = average optical density (OD) of negative control + three times of standard deviation (SD) of negative control. Biofilm of each isolate was quantified as; Negative (if well is clear); Weak (ODc< OD < 2 x ODc); Moderate (2 x ODc< OD = 4 x ODc), Strong (OD > 4 x ODc) [5,6].

Controls:
Positive: Staphylococcus aureus ATCC 43300
Negative: sterile BHI broth

Results:-

Total 100 samples were collected from dental OPD from patients presented with periodonitis during study period. Out of this, 71 samples were shown growth of microorganism like *Streptococcus viridians (36), Klebsiella pneumoniae (21), E. Coli (6), Klebsiella oxytoca (4), Acinetobacter baumannii (1), Pseudomonas aeruginosa (1), Staphylococcus aureus (1), Coagulase negative staphylococcus (1).*From 71 isolates, 43(61%) had formed biofilm and 28 were negative for biofilm.

19 isolates of *Streptococcus viridians* have formed biofilm out of 36 isolates. 16 isolates of *Klebsiella pneumoniae* have formed biofilm out of 21 isolates. 3 isolates of *Klebsiella oxytoca* have formed biofilm out of 4 isolates. 2 isolates of *E. coli* have formed biofilm out of 6 isolates. One isolate of each *Acinetobacter baumannii, CONS and Pseudomonas aeruginosa* has formed biofilm. Quantification of biofilm of microorganisms has been shown in Table no.1.

Table no 1:- Quantification of biofilm of isolated microorganisms.

Organism	Strong	Moderate	Weak
A. baumannii (1)	1	0	0
CONS (1)	0	0	1
E. coli (2)	0	1	1
K. oxytoca (3)	0	1	2
K. pneumoniae (16)	2	9	5
P. aeruginosa (1)	0	1	0
Strep. viridans (19)	5	7	7
Total (43)	**8**	**19**	**16**

5 isolates of *Streptococcus viridans*, 2 isolates of *Klebsiella pneumoniae* and 1 isolate of *Acinetobacter baumannii* have formed strong biofilm as described in table no-1(total 8). 9 isolates of *Klebsiella pneumoniae*, 7 isolates of *Streptococcus viridans*, 1 isolate of *E. coli*, *Klebsiella oxytoca* and *Pseudomonas aeruginosa* have formed moderate (total 19) biofilm, 7 isolates of *Streptococcus viridans*, 5 isolates of *Klebsiella pneumoniae*, 2 isolates *of Klebsiella oxytoca* and 1 isolate of each *CONS* and *E. coli* have formed weak (16) biofilm.

Table no 2:- Association between oral biofilm and tobacco chewing.

Tobacco chewing	**Biofilm**				**Total**	
	Positive		**Negative**			
	no	%	no	%	no	%
Yes	9	21%	4	14%	13	19%
No	34	79.%	24	86%	58	81%
Total	**43**	**100.0%**	**28**	**100.0%**	**71**	**100.0%**
Chi-square=0.410, p=0.522, Not significant						

In table no 2 association between oral biofilm and tobacco chewing is shown. There is no statistical significant association between biofilm production and tobacco chewing. Out of 71 samples, 13 (19%) were tobacco chewers and 58(81%) were not chewing tobacco. Out of 43 positive oral biofilms, 9 (21%) were tobacco chewers and out of 28 negative oral biofilm, 4 (14%) were tobacco chewers. Among 43 positive oral biofilm, 9 (21%) were tobacco chewers with the 95% confidence interval of proportion as (10%, 36%).

Table no 3:- Association between oral biofilm and co-morbidity.

Co-morbidity	**Biofilm**				**Total**	
	Positive		**Negative**			
	N	%	n	%	n	%
Present	8	19%	4	14%	12	17%
Absent	35	81%	24	86%	59	83%
Total	**43**	**100.0%**	**28**	**100.0%**	**71**	**100.0%**
Chi-square=0.168, p=0.682, Not significant						

Above table no 3, indicates association between oral biofilm and co-morbidity. Out of 71 samples, 12 (17%) had co-morbidity and 59 (83%) had no co-morbidity. Out of 43 positive oral biofilms, 8 (19%) had co-morbidities and out of 28 negative oral biofilm, 4 (14%) had co-morbidity. Among 43 positive oral biofilm, 9 (19%) had co-morbidities with the 95% confidence interval of proportion as (8%, 33%). There is no any statistical significant between biofilm production and co-morbid conditions of patients.

Discussion:-

In present study, the predominant isolated organism was *S. viridans* and it had formed maximum strong biofilms which is similarto the study conducted by Sonali kaustubhAmbulkar, *et al* [7]. Streptococcus species was most isolated from dental isolates and strong biofilm production was seen in most of the isolated organisms. Whereas in a study conducted by Alwan, *et al* [8]enterococcus was predominantly isolated and these too were strong biofilm producer.

In present study, most of the strong biofilms were shown by Streptococcus species, moderate biofilms mostly by Klebsiella species and maximum weak biofilm were produced by streptococcus, whereas in study by Tale Vidya,*et al* [9]maximum strong biofilm was produced by streptococcus, maximum moderate and weak biofilms were produced by enterobacter species and pseudomonas has shown moderate biofilm.

In present study, 18% were strong biofilm former, 44% were moderate biofilm former and 37% were weak biofilm formers, whereas in study by Alwan, *et al*[8], 40% were strong biofilm formers, 42% were moderate biofilm formers and 18% were weak biofilm formers. Also comparable with other study [10, 11].

In present study, the result of chi-square indicates no significant association between oral biofilm and tobacco chewing (chi-square = 0.410, p=0.522). Whereas Liu,*et al*[12] observed that different concentrations of smokeless tobacco extracts stimulate growth of microorganisms present in oral cavity. Huang,*et al* [13] found that nicotine act

as enhancer of biofilm formation and biofilm metabolic activity of *S. mutans*, this suggests that smoking can increase the risk of periodontal diseases and caries by enhancing the formation of *S. mutans* biofilms on tooth surfaces.

Some of these organisms enhance the attachment of other oral microorganisms that colonize the tooth surface, forms dental plaque and contributes to development of caries and periodontal disease. Study by Tomar, *et al* [14] and Falkler,*et al*[15]shows that growth of some species of Streptococcuswas enhanced in the presence of smokeless tobacco extracts [16].Studie of Soskolne WA,*et al* [17];Pihlstrom BL,*et al*[18] has confirmed diabetes as a highly significant risk factor for periodontitis.In present study, the result of chi-square indicates no significant association between oral biofilm and co-morbidity (chi-square = 0.168, p=0.682). Preshaw PM,*et al* [19] study shows that diabetes alters the local environment within the periodontal pockets in such a way that the growth of certain bacterial species arefavored.Individuals with poorly controlled diabetes are at risk for the other macro vascular and micro vascular complications. Since, oral diseases are highly prevalent and are associated with significant morbidity. Proper control of diabetes can be expected to reduce the risk and severity of periodontitis among other diseases.

Conclusion:-

The colonization of biofilm producing strain of oral cavity increases the risk of their dissemination to various human tissues and organs. Biofilm has over the time shown increased resistance to the available antimicrobial agents compared to isolated planktonic forms of infectious organisms. To overcome chronic and recurrent infections and to combat the increasing nature of antimicrobial resistance contributed by biofilms, it is of more important to detect and evaluate biofilms and the organisms contributing to the formation of biofilms. Present study shows that tobacco chewing and smoking are not associated with oral biofilm formation by microorganisms causing periodontitis. Epidemiological studies suggest that susceptibility to periodontitis is increased in people with diabetes. Patient with diabetes has three times higher risk of developing periodontitis. In present study, there is no correlation between oral biofilm and co morbidity.

Limitations

Determine antibiofilm activity of agents against biofilm and determine antibacterial activity of agents against biofilm embedded microorganism with the appropriate methods was not able to analyzed. Identification of genes involved in biofilm formation and measurement of gene expression as a result of antibiofilm and antibacterial activity of agents can be advantageous in biofilm studies.

Competing Interests:

The authors have no competing interests to declare.

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