DOI: 10.7860/JCDR/2017/24472.9152

Original Article

Microbiology Section

Biofilm Formation by Drug Resistant Enterococci Isolates Obtained from Chronic Periodontitis Patients

SONIA BHONCHAL BHARDWAJ[1], MANJULA MEHTA[2], SHAVETA SOOD[3], JYOTI SHARMA[4]

ABSTRACT

Introduction: Enterococci are an important cause of opportunistic nosocomial infections and several multidrug resistant strains have emerged.The severity of periodontal diseases is managed by reduction in the pathogenic bacteria. There is a need to assess the prevalence and antibiotic susceptibility of enterococci colonizing the periodontal pocket and correlate its biofilm formation ability because oral biofilms provide a protective environment and are a reservoir of bacterial colonization of the gingival crevice.

Aim: To investigate possible association between antibiotic susceptibility and biofilm formation in enterococci isolates from chronic periodontitis patients.

Materials and Methods: This retrospective study was conducted at Dr. Harvansh Singh Judge Institute of Dental Sciences and Hospital, Punjab University, Chandigarh from January 2015 to October 2015. Sterile paper points were inserted in the periodontal pocket of 100 subjects and put in a transport media. Forty -six isolates were identified as enterococci. The isolates were further examined for their ability to form biofilm by microtitre plate assay and antimicrobial susceptibility testing was done by disc diffusion method for clinically relevant antibiotics.

Results: Significant relationship ($p<0.001$) was found between biofilm production with antibiotic resistance to Vancomycin, Erythromycin, Ciprofloxacin, Tiecoplanin, Amoxycillin and Gentamycin.

Conclusion: The study demonstrates a high propensity among the isolates of Enterococci to form biofilm and a significant association of biofilm with multiple drug resistance.

Keywords: Antibiotic resistance, Biofilm matrix, Enterococcus, Periodontal disease

INTRODUCTION

Enterococci are Gram positive bacteria inhabiting the gastro intestinal tract. Now, they are an important cause of nosocomial infections but were initially regarded as non virulent [1]. Enterococci are now ranked as the third most common nosocomial bacterial pathogen after coagulase negative Staphylococci, *Staphylococcus aureus* [2]. The emergence of multidrug resistant strains of enterococci to commonly used antimicrobials like tetracycline and erythromycin is a matter of concern [3].

The two most common enterococci species are *Enterococcus faecalis* and *Enterococcus faecium*. Both the species can produce biofilm, which is a population of cells surrounded by a matrix of macromolecules like polysaccharides, proteins, lipids and extracellular DNA [4]. *E. faecalis* has been recovered from periodontal pockets in 1% to 51.8% of periodontitis patients [5,6]. It has been seen that the presence of *E. faecalis* in the pockets of chronic periodontitis was significantly higher than that of a treated group [5]. The subgingival *E. faecalis* has been found resistant to routine antimicrobial agents in a high proportion [7,8]. Furthermore, recently it has been shown that enterococci are adept at acquiring transferable antimicrobial resistance and are likely to be a reservoir for diverse mobile genetic elements [9]. There is a need to assess the antibiotic susceptibility of enterococci colonizing periodontal pocket and also assess the biofilm formation ability as it may enhance the enterococcal pathogenesis in infections. The role of enterococcal biofilm and antibiotic resistance in chronic periodontitis remains unclear.

The objective of the present study was to determine the biofilm formation ability of enterococci strains in periodontitis and their antimicrobial susceptibility.

MATERIALS AND METHODS

A total of 100 subjects (52 males and 48 females) attending the periodontitis clinic of Dr Harvansh Singh Judge Institute of Dental Sciences and Hospital, Punjab University, Chandigarh were examined for presence of periodontitis. Patients with chronic periodontitis were included in the test group. The control group consisted of 30 healthy persons who did not have obvious dental disease. Subjects in age range of 18-75 years and in good general health were included in the test and control group irrespective of sex, religion. Patients who were pregnant, allergic, having diabetes mellitus, on antibiotic therapy or undergoing orthodontic therapy were excluded. The control group consisted of thirty healthy persons who did not have obvious dental disease. The study was approved by the ethics committee of Punjab University. Patients were informed of the study protocol and aim and written consent was obtained. For control, ATCC 14506 *Enterococcus faecalis* strain was used.

Sampling Procedure: The sample sites was isolated. After isolation with cotton rolls and removal of saliva and supragingival deposits, one to two sterile, absorbent paper points were introduced into each periodontal pocket for 30-60 seconds. After removal, all paper points per patient were pooled and transferred immediately to test tubes containing glucose azide broth (HiMedia Laboratories, Mumbai) and taken to the laboratory within four hours for microbiological analysis.

Bacterial Isolation and Identification: The samples were inoculated onto the blood agar (HiMedia Laboratories, Mumbai) plates and incubated in microaerobic condition. Every growth showing Gram positive cocci, positive bile esculin, positive 6.5% Nacl tests, Catalase negative was processed for further biochemical identification [10].

Antimicrobial Susceptibility Testing

The antibiotic susceptibility of the test strains to different antibiotics (amoxicillin, ciprofloxacin, erythromycin, vancomycin, gentamycin, tiecoplanin) purchased from HiMedia Laboratories, Mumbai was determined by standard disc diffusion method (Kirby Bauer sensitivity test) [11] and interpreted according to the Clinical and Laboratory Standard Institute (CLSI) guidelines [12]. The test was performed on Mueller Hinton agar (HiMedia) and results read after 24 hours of incubation at 37^0C.

Biofilm Assay

Biofilm formation was performed by microtitre plate assay [13]. Inoculums were prepared by growing in Brain Heart Infusion broth (BHI) containing 0.25% glucose and incubated at 37°C overnight. Overnight broth cultures were diluted 1:20 in fresh BHI broth supplemented with glucose. 200 ul of diluted strain was dispensed into triplicate wells in a single column of a sterile 96 well flat bottom plate (APW) and incubated at 37°C for 24 hours. The microtitre plate was gently tapped to remove the planktonic cells and wells were washed three times with 300 ul of sterile Phosphate Buffer Saline (PBS). The plates were inverted and allowed to dry for one hour at room temperature. Biofilms were then fixed with 200 ul of 0.5% aqueous crystal violet solution for 15 minutes and the wells were subsequently washed thrice with sterile PBS to remove the excess crystal violet. Microtitre plates were then inverted on a filter paper and air dried. 200 ul of 80:20 (v/v) mixtures of ethyl alcohol and acetone was added to solubilized bound crystal violet. Absorbance of the extracted crystal violet was measured at 550 nm Automatic Microplate Reader (APW). For positive control, *Staphylococcus epidermidis* and for negative control, non biofilm forming bacteria *Salmonella typhi* was used in each plate. All biofilm assays were repeated three times. The cut-off value (ODc) was established. ODc, defined as three Standard Deviations (SD) above the mean OD of the negative control. The final OD value was taken as mean OD value of test strain divided by ODc value of the triplicate assays. Any OD value above the cut-off value was indicative of biofilm production.

STATISTICAL ANALYSIS

SPSS software version 16.0 (IBM, SPSS statistics) was used for statistical analysis. t-test and Chi–square test was performed for data analysis. p-values below 0.001 were considered to be significant.

RESULTS

Forty six enterococci isolates were obtained from subgingival samples collected from 70 periodontitis patients. No enterococci isolate was obtained from control group.The mean age of all the participants in the study was 40.77 years with male participants being 52 and female participants being 48 in number. All the patients had mild to moderate periodontitis. Subjects were in the age range of 18-75 years. Based on the biochemical reactions, the species of *E. faecalis* were 39(84.78%) followed by *E. faecium* 7 (15.21%). The distribution of 46 enterococci isolates among patients in association of sex, smoking and oral hygiene is shown [Table/Fig-1].

Among the enterococci isolates tested for antimicrobial susceptibility resistance to erythromycin were 3/46(6.5%), ciprofloxacin 4/46 (8.7%), tiecoplanin 6/46 (13.0%), amoxycillin 2/46 (4.3%), gentamycin 4/46 (8.7%), vancomycin 6/46(13.3%) [Table/Fig-2].

Quantitative microtitre assay for biofilm formation was positive in 39/46 (84.78%) isolates. The remaining isolates were non biofilm producers considered as negative. *E. faecalis* strains positive for biofilm production was 71.8% and *E. faecium* was 25.6%. Statistical analysis showed significant relationship of biofilm formation with antibiotic resistance. Biofilm formation was significant in resistant isolates (p<0.001) [Table/Fig-2]. Comparison of biofilm positive and biofilm negative isolates among antibiotic resistant enterococci has been shown in [Table/Fig-3].

DISCUSSION

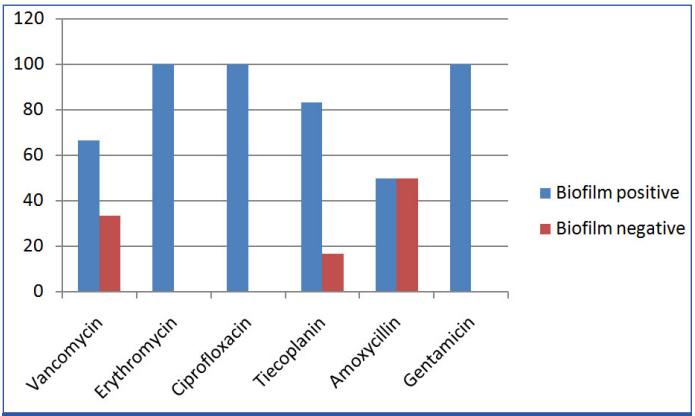
Enterococci are able to colonize the oral cavity particularly in patients with periodontitis or root canal infections associated with oral mucosal lesions and in immunocompromised patients [14]. It has been implicated that enterococci might influence periodontal antimicrobial therapy and contribute to disease progression in

Characteristics	Enterococci isolates (n=46) (%)	p value
Male Female	24/46 (52.2%) 22/46 (47.8%)	NS
Smoker Non Smoker	40/46 (87.0%) 6/46 (13.0%)	0.001
Poor oral hygiene Good oral hygeine	35/46 (76.1%) 11/46 (23.9%)	0.001

[Table/Fig-1]: Distribution of enterococci isolates in association of sex, smoking and oral hygiene among periodontitis patients. NS-non significant

Antimicrobials	No of resistant Isolates producing Biofilm/Total No Of resistant isolates.	No of sensitive isolates producing biofilm/Total No of sensitive isolates.	p value
Vancomycin	4/6 (66.7%)	33/40(82.5 %)	<0.001
Erythromycin	3/3(100.0%)	35/43(81.4%)	<0.001
Ciprofloxacin	4/4(100.0%)	34/42(81.0%)	<0.001
Tiecoplanin	5/6(83.3%)	33/40(82.5%)	<0.001
Amoxycillin	1/2(50.0%)	37/44(84.1%)	<0.001
Gentamycin	4/4(100.0%)	33/42(78.57%)	<0.001

[Table/Fig-2]: Association between biofilm production and antibiotic susceptibility pattern of enterococci isolates.



[Table/Fig-3]: Comparison of biofilm positive and biofilm negative isolates among antibiotic resistant enterococci. The y axis represents the percentage of the isolates resistant to different antibiotics and the X axis represents resistance to the antibiotic mentioned.

severe subgingival infections [15]. In this study, majority of the isolates identified were *E. faecalis* (84.78%) followed by *E. faecium* (15.21%). The study reveals that smoking and poor oral hygiene are important predisposing factors for infection with enterococci. These results agree with another study that show patients with periodontitis had more diverse combination of species as compared to healthy persons [16] and that smoking has been shown to influence oral microbiome composition [17].

Importance of biofilm formation has been described in the control of microbial infection in several areas because the biofilm can increase resistance to various physical and chemical agents especially antibiotics [18]. Biofilm formation is indirect evidence of adhesiveness and microtitre plate assay is the indirect way to measure adhesion of enterococci. In this study, enterococci isolates were resistant to multiple antibiotics. Survival advantages conferred by the biofilm community include resistance to phagocytosis and to antimicrobial agents [19].

Significant relationship was found between biofilm production with antibiotic resistance to Vancomycin, Erythromycin, Ciprofloxacin, Tiecoplanin, Amoxycillin, Gentamycin. Biofilm formation was significantly more in Erythromycin resistant enterococci isolates (100%) vs sensitive isolates (81.4%), Ciprofloxacin resistant (100.0%) vs sensitive (81.0%), Tiecoplanin resistant isolates (83%) vs sensitive

(82%) and Gentamycin resistant (100.0%) vs sensitive (80.5%) and also in the Vancomycin resistant and Amoxycillin resistant the biofilm formation was significantly more vs the sensitive isolates. The study showed that almost all enterococci strains exhibited biofilm forming ability in vitro. Biofilm exhibits more resistance to broad spectrum antibiotics [19]. This supports that biofilm adds to the virulence profile of microorganisms [20]. Other studies particularly in urinary tract infection patients have also shown a possible relationship between virulence profile and biofilm formation by enterococci [21,22]. A variety of mechanisms for the increased antimicrobial resistance of microorganism in a biofilm have been proposed including extracellular matrix in biofilm might physically restrict the diffusion of antimicrobial agents, nutrient and oxygen depletion within the biofilm cause some bacteria to enter a stationary state, in which they are less susceptible to microbial killing, a subpopulation of bacteria might differentiate into a phenotypically resistant state and some organisms in biofilm have shown to express biofilm specific antimicrobial resistance genes [23]. Recently, it has been shown that extracellular DNA (eDNA) in the biofilm matrix protects microbial cells from a variety of antimicrobial agents [4].

The present study points to the importance of biofilm susceptibility testing in clinical settings because the difference in susceptibility is remarkable under various growth conditions. The variations observed in these clinical isolates suggests that biofilm formation may be a factor when considering the virulence phenotype of periodontal strains in general. However, biofilm formation is an indirect evidence of adhesiveness and microtitre plate assay is the indirect way to measure adhesion in vitro.

LIMITATION

A limitation of this study is the lack of use of animal models as representative models of periodontitis in humans as the experimental models have the ability to mimick the pathogenesis of natural disease [24].

This study also highlights the role of enterococci in implication of periodontal disease. In a recent study of very small sample size, *E.faecalis* has been reported as a pathogen which is a critical marker of disease stage of chronic periodontitis patients [25]. Studies also suggest that periodontal infections and oral bacteria may be a risk factor for a number of prevalent systemic diseases [26]. Therefore, close attention should be given to periodontitis patients who may harbor pathogenic bacteria in the oral cavity in order to reduce the risk for development of systemic infections.

CONCLUSION

Dental Plaque is regarded as a major causative factor in dental diseases like dental caries and periodontal disease. Most of the antimicrobial resistant strains were biofilm producers as seen in our study. Hence, biofilm susceptibility testing which reflects more natural conditions in plaque related dental diseases, can lead to more appropriate evidence based therapeutic strategies.

ACKNOWLEDGEMENT

The funds for this project were sanctioned by Department of Science and Technology (DST), India. The technical help of Ms Aarti Devi is duly acknowledged.

REFERENCES

[1] Marra A, Dib-Hajj F, Lamb L, Kaezmarek F, Shang W, Beckius G, et al. Enterococcal virulence determinants may be involved in resistance to clinical therapy. Diag Microbiol Infect Dis. 2007;58:59-65.
[2] Zoletti GO, Pereira EM, Schuenk RP, Teixeira LM, SiqueiraJr JF, Santos KR. Characterization of virulence factors and clonal diversity of *Enterococcus faecalis* isolates from treated dental root canals. Res Microbiol. 2011;162:151-58.
[3] Souto R, Colombo APV. Prevalence of *Enterococcus faecalis* in subgingival biofilm and saliva of subjects with chronic periodontal infection. Arch Oral Biol. 2008;53:155-60.
[4] Jakubovics NS, Grant Burgess J. Extracellular DNA in oral microbial biofilms. Microbes and Infection. 2015;17(7):531-37.
[5] Balaei-Gajan E, Shirmohammadi A, Abashov R, Agazadeh M, Faramarzei M. Detection of *Enterococcus faecalis* in subgingival biofilm of patients with chronic refractory Periodontitis. Med Oral Pathol Oral Cir Buccal. 2010;15:e667-70.
[6] Sun J, Song X. Assessment of antimicrobial susceptibility of *Enterococcus faecalis* isolated from chronic periodontitis biofilm vs planktonic phase. J Periodontol. 2011;82:84.
[7] Sun J, Song X, Kristiansen BE, Kjaereng A, Willems RJ, Eriksen HM, et al. Occurrence, population structure and antimicrobial resistance of Enterococci in marginal and apical periodontitis. J Clin Microbiol. 2009;47:2218-25.
[8] Al-BadahSuliman AH, Ibrahim SS Abdel Nasser, Salamah AA, Ibrahim Shebl Salah S. Clonal diversity and antimicrobial resistance of *Enterococcus faecalis* isolated from endodontic infections. Electronic journal of Biotechnology. 2015;18:175-80.
[9] Song X, Sun J, Milkalsen T, Roberts AP, Sundsgford A. Characterisation of the plasmidome within *Enterococcus faecalis* isolated from marginal periodontitis patients in Norway. PLOS One. 2013;8:4.
[10] Teixeira LM, Facklam RR. Enterococcus. In Manual of Clinical Microbiology, 8th edn ed. Murray PR, Baron EJ, Jorgensen JH, Pfaller MA and Yolken RH.2003;422-433. Washington: ASM Press.
[11] Bauer AW, Kirby WM, Sherris JL, Turck M. Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol. 1966;45(4):493-96.
[12] Clinical and Laboratory standard Institute. Performance standard for antimicrobial susceptibility testing: Twentieth International Supplement M100 S20 CLSI, Wayne PA, USA, 2010.
[13] Stepanovic S, Vukovic D , Hola V, Bonaventura GD, Djukic S, Irkovic IC, et al. Quantification of biofilm in microtitre plates: overview of testing conditions and practical recommendations for assessment of biofilm production by Staphylococci. APMIS. 2007;115:891-99.
[14] Pinheiro ET, Anderson MJ, Gomes BPFA, Ducker DB. Phenotypic and genotypic identification of Enterococci isolated from canals of root filled teeth with periapical lesions. Oral Microbiol Immunol. 2007;21:137-44.
[15] Lee HJ, Kim JK, Cho JY, Lee JM, Hong SH. Quantification of subgingival bacterial pathogens at different stages of periodontal diseases. CurrMicrobiol. 2012;65:22-27.
[16] Abusleme L, Dupuy AK, Dutzman N, Silva N, Burleson JA, Strausbaugh LD, et al. The subgingival microbiome in health ad periodontitis and its relationship with community biomass and inflammation. The ISME Journal. 2013;7:1016-25.
[17] Shchipkova AY, Nagaraja HN, Kumar PS. Subgingival microbial profiles of smokers with periodontitis. J Dent Res. 2010;89:1247-53.
[18] De la Fuente-Nunez C, Reffuveille F, Fernandez L, Hancock RE. Bacterial biofilm development as a multicellular adaptation: antibiotic resistance and new therapeutic strategies. Curr Opin Microbiol. 2013;16:580-89.
[19] Mathur T, Singhal S, Khan S, Upadhyay DJ, Fatma T, Rattan A. Detection of biofilm formation among clinical isolates of Staphylococci: An evaluation of three different screening methods. Indian J Med Microbiol. 2006;24:25-29.
[20] Toledo- Aruna A, Vall J, Solano C, Arrizubeita MJ, Cucarella C, Lamata M, et al. The Enterococcal surface protein, ESP, is involved in *E. faecalis* biofilm formation. Appl and Environmental Microbiol. 2001;67:4538.
[21] Mohamed JA, Singh K, Huang W. Influence of clinical origin and by *Enterococcus faecalis*. In program Abstracts of the 43 rd Annual Interscience conference on antimicrobial agents and chemotherapy. Abstract B821. American Soceity for Microbiology. Washington. DC.2003; Pp. 42.
[22] Kafil HS, Mobarez, AM. Assessment of biofilm formation by Enterococci isolates from urinary tract infections with different virulence profiles. Journal of King Soudi University-Science. 2015;27:312-17 .
[23] Patel R. Biofilms and antimicrobial resistance. Clin Orthop Relat Res. 2005;437:41-47.
[24] De Molon RS, De Avila ED, Nogueira AVB, De Souza JAC, Campos MJA, De Andrade CR, et al. Evaluation of the host response in various models of induced periodontal disease in mice. J Periodontol. 2014;85:465-77.
[25] Yoon DL, Shukho K, Song H, Kim YG, Lee J M, Kim J. Detection of bacterial species in chronic periodontitis tissues at different stages of disease severity. J BacteriolVirol. 2015;45(4):364-71.
[26] Li X, Koltveit KM, Transtad L, Olsen I. Systemic diseases caused by oral infection. Clin Microbiol Rev. 2000;3:547-51.

PARTICULARS OF CONTRIBUTORS:
1. Assistant Professor, Department of Microbiology, Dr. Harvansh Singh Judge Institute of Dental Sciences and Hospital, Chandigarh, Chandigarh, India.
2. Associate Professor, Department of Microbiology, Dr. Harvansh Singh Judge Institute of Dental Sciences and Hospital, Chandigarh, Chandigarh, India.
3. Assistant Professor, Department of Periodontics, Dr. Harvansh Singh Judge Institute of Dental Sciences and Hospital, Chandigarh, Chandigarh, India.
4. Assistant Professor, Department of Microbiology, Dr. Harvansh Singh Judge Institute of Dental Sciences and Hospital, Chandigarh, Chandigarh, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. Sonia Bhonchal Bhardwaj,
Assistant Professor, Department of Microbiology, Dr Harvansh Singh Judge Institute of Dental Sciences and Hospital, Chandigarh-160014, India.
E-mail: sbbhardwaj2002@yahoo.com.

FINANCIAL OR OTHER COMPETING INTERESTS: None.

Date of Submission: Sep 30, 2016
Date of Peer Review: Oct 19, 2016
Date of Acceptance: Nov 10, 2016
Date of Publishing: Jan 01, 2017