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Genotypic and Phenotypic Detection of Certain Virulent Determinates of Entrococcus Faecalis Associated With Dental Caries- an in vitro study

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Abstract:
Enterococci have transformed over the past century from being an intestinal commensal organism of little clinical importance to becoming the second most widespread nosocomial pathogen and is related with considerable mortality and morbidity. Considering its multitude of resistance to different infections with high grade severity, we have attempted this study. A sum of 20 E. faecalis isolates from patients with dental caries were subjected to antibiotic sensitivity test followed by the detection of hemolysin and gelatinase enzyme activities and determination of ace and efaA genes encoding forcollagen binding protein and endocarditis respectively by PCR analysis.Of the 20 dental caries isolates of E. faecalis, 14/20 (70%) and 8/20 (40%) of them were found to be positive for hemolysin and gelatinase activities respectively. Among the 20 E. faecalisstrains, 4 (20%) of them found to have efaA gene encoding for endocarditis, whereas none of the isolates showed positive for ace gene encoding for collagen binding protein. However, 12/20 (60%) of them were harboring both ace and efaA genes together. Our study showed that most of our strains produced hemolysin followed by gealtinase and they found to be sensitive to most of the commonly used antibiotics. Many were equally have the ability to exhibit both collagen binding protein and endocaridtis.

Key words: *E. faecalis, antibiotic sensitivity pattern, hemolysin,gelatinase, ace, efaA, PCR.*

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I. Introduction

A large group of aerobic and anaerobic bacterial populations are being colonized in the human dental cavity. *Enterococcus faecalis* is being regarded as an important nosocomial pathogen that could cause several infections, of which many of them known to be isolated from root canal failed cases [1]. Several studies have reported that *E. faecalis* is commonly encountered from oral infections such as gingivitis, periodontitis, pulpitis etc [2,3,4]. Virulence factors of *E. faecalis*such as adherence, aggregation formation, enterolysin/cytolysin and pheromone production play a cardinal role in determining its pathogenicity [5].

Enterococci have transformed over the past century from being an intestinal commensal organism of little clinical importance to becoming the second most widespread nosocomial pathogen and is related with considerable mortality and morbidity [6,7].

Determining itsvirulence factors in such notorious isolates would be useful in predicting its role in dental caries [8]. Considering its multitude of resistance to different infections with high grade severity, we have attempted this study.

II. Materials And Methods

A sum of 20 *E. faecalis* isolates from patients with dental caries have been included in this study. The duration of the study was between May 2017 and September 2017. During this period patients were diagnosed with multiple dental caries have been subjected to this study. Of all the isolates obtained, only *E. faecalis*were involved in this study.

Specimen collection:

Saliva samples were collected from known multiple caries individuals of fifty four. This study was conducted in Saveetha Dental College and Hospitals, Chennai, Tamilnadu, after getting a proper Institutional Ethical Committee clearance. All specimens were transferred immediately to the Dental Research Cell located at our Institution. As soon as the specimens were transferred to the lab, they were poured into sterile tubes and centrifuged at 15,000rpm for 10min. The resultant supernatant was discarded and the pellet was suspended with 1mlof sterile normal saline using vortex machine [9].

Isolation of *E. faecalis:*

A loopful of pellet suspension were seeded onto Enterococcus confirmation medium and Bile Esculine agar (HiMedia, Mumbai). Formation of deep blue coloured pinpointed colonies and deep black colouredpigemented colonies on Enterococcus confirmation agar and Bile Esculine agar respectively. These suspected enterococcal isolates were further confirmation by standard biochemical tests. These confirmed *E. faecalis* isolates were stock cultured into Brain Heart Infusion semisolid glycerol stock and preserved at -20ºC for further use [10].

Antibiotic sensitivity test by disc diffusion method:

This was determined by standard Kirby-Bauer disc diffusion method for *E. faecalis*isolates as per CLSI guidelines [11].Antibiotics were procured from Himedia, Mumbai. A standard culture of *Staphylococcus aureus* 25923 was also included as quality control. The lists of antibiotics used are ampicillin (10µ), vancomycin (30µ), teicoplanin (30µ), erythromycin (15µ), ciprofloxacin (5µ), amikacin (200µ), gentamycin (10µ), tetracycline (30µ) and linezolid (30µ). The zone of interpretations were measured and referred with the CLSI interpretation chart after 24 hours of incubation at 37 ºC.

Detection of hemolysin:

The *E. faecalis*isolates were grown overnight in brain heart infusion broth at 37ºC with aeration. Samples were taken and 0.1ml of two folds dilution of bacterial suspensions in PBS (pH 7.2) were mixed with 0.05ml of 3 percent suspension of sheep RBC in PBS and incubated at 37ºfor 1 hour. The haemolytictitre was defined as the highest dilution in which no visible RBC button was observed at the bottom.[12]

Detection of gelatinase:

Gelatin medium was prepared by adding 3% of gelatin to LuriaBertani agar, HiMedia. The medium was autoclaved then molten agar was poured on the sterile petridishes and cooled. Overnight grown cultures of *E. faecalis*were spot inoculated on gelatin medium. The inoculated plates were incubated at 37ºC for 24 hours. On the next day, the cultures were flooded with saturated solutions of ammonium sulphate to precipitate the unhydrolysed gelatin. The development of opacity in the medium and zone of clearing around the growth was considered as positive for gelatinase production.[13]

Detection of ace and efaA genes in *E. faecalis*by PCR:

*E. faecalis*isolates were detected for the presence of ace and efaA genes that codes for collagen binding protein and endocarditis respectively by PCR analysis. Detection of the gene was carried out using primer as depicted in table 1. Bacterial DNA was extracted byboiling lysis method. 1 µL of DNA extract was used as template for PCR reaction. The reaction mixture contained 2mM of Mgcl20.2mM dNTP mix and 0.5µM of ace and efaA genes with IU ofTaq polymerase (New England Biolabs) in a 1x PCR buffered reaction. A positive control of *E. faecalis*for these genes was also included in this study.

PCR amplification was carried out usingthermal cycler machine (Eppendorf) with the following cycling condition. Initial denaturation at 94ºC for 1 min and 30 cycles for 40s, 51ºC for 1min and 74ºC for 1min, followed by a final extensionof 10 min at 74 ºC. PCR products were resolved in 2% agarose gel. A 100bp ladder was including in all the gel analysis.[10]

Table 1: Showing the primer gene sequences of ace and efaA genes

Primer	Primer sequence	Product size
ace	5′-TTGGAAACGGTTAAAACGA-3′ 5 ′-GAACCTTCCCATCAAAAAC-3′	501 bp
efaA	5′-TCGCATCAAACTGACAAAC-3′ 5′-GCAGGTACTCTATAAGTGC-3′	324bp

III. Results

Antibiotic susceptibility testing:

Wehave found increased percentage of isolates were shown to be resistant to all the antibiotics used in this study. For ampicillin, amikacin, erythromycin, gentamicin, our isolates were found to resistant between 80-90%. Better sensitivity was observed in linezolid, teicoplanin and vancomycin antibiotics. The detailed results of antibiotic sensitivity patter of *E. faecalis*were given in table 2.

Table 2: Results of antibiotic sensitivity patter of *E. faecalis.*

Antibiotics	Sensitivity (%)	Intermediate (%)	Resistance (%)
Ampicillin	1(5%)	2(10%)	17(85%)
Vancomycin	20(100%)	0(0%)	(0%)
Teicoplanin	12(60%)	3(15%)	5(25%)
Erythromycin	2(10%)	0(0%)	18(90%)
Ciprofloxacin	6(30%)	0(0%)	14(70%)
Amikacin	1(5%)	1(5%)	18(90%)
Gentamycin	2(10%)	2(10%)	16(80%)
Tetracycline	4(20%)	4(20%)	12(60%)
Linezolid	18(90%)	1(5%)	1(5%)

Results of hemolysin and gelatinase activities and PCR for ace and efaA genes:

Of the 20 dental caries isolates of *E. faecalis*, 14/20 (70%) and 8/20 (40%) of them were found to be positive for hemolysin and gelatinase activities respectively. Among the 20 *E. faecalis*strains, 4 (20%) of them found to have efaA gene encoding for endocarditis, whereas none of the isolates showed positive for ace gene encoding for collagen binding protein. However, 12/20 (60%) of them were harboring both ace and efaA genes together.

The detailed results were shown in table 3.

Table 3: showing the results of virulence determinants of *E. faecalis*

Hemolysin (n=20)	Gelatinase (n=20)	ace + efaA (n=20)	efaA (n=20)
14 (70%)	8 (40%)	12 (60%)	4 (20%)

Fig 1: Presence of RBS button at the bottom of wells indicates the hemolysin produced by *E. faecalis*

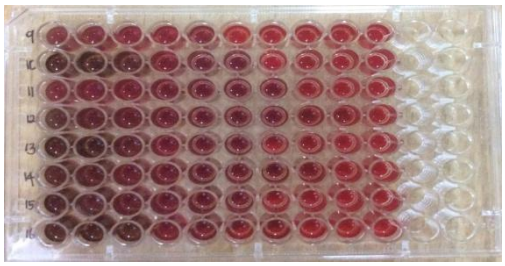


Fig 2: Presence of clear zone around the colonies indicates the gelatinase produced by *E. faecalis.*

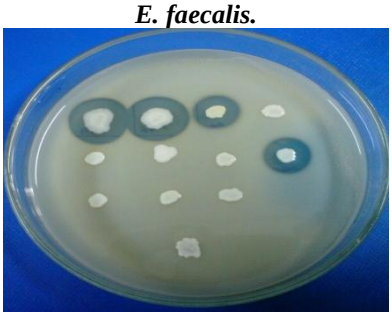
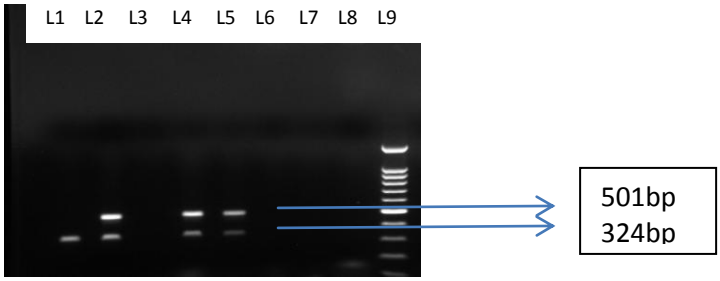


Fig 3: Representative gel picture showing the presence of efaA and ace genes by PCR.



Note: L9: 100bp ladder; L4 &L5: 501bp (ace) and 324 bp (efaA)

IV. Discussion

Due to the increased resistance to different antibiotics, enterococcal infections are gaining much importance in clinical settings regarded as nosocomial pathogen. Of all the species of Enterococcus, *E. faecalis*is known to cause 90% of the enterococcal infections in humans, and it was often isolated from obtured root canals [14-18].

This study represents that, the production of hemolysin and gelatinase enzymes as important virulent markers were not commonly expressed by *E. faecalis* associated with dental caries. However, in our study we have observed 70% and 40% of the isolates were producing hemolysin and gelatinase enzymes respectively. Similar kind of work conducted by Salah 2008 from Jordan have observed 25% of *E. faecalis*expressed hemolysin and 37.5% gelatinase activity by invitro methods [10]. Studies performed by Sedgley [9,19] found the inappropriate results for hemolysin and gelatinase activities. This proves that there is no correlation between cause of dental caries and such virulence attributes by *E. faecalis.*

Work done by Salahin 2008 [10] found that there were no enterococcal strains from healthy populations, while the isolates from diseased patients harbor the following virulence determinants such as ace gene (100%) efaA gene (100%) and cylA gene (25%) by PCR. In contrast, our study showed 20% of positivity for efaA gene and 60% of them carring both ace and efa genes. None of our isolates carried ace gene independently. This shows the association between the formation of dental caries and efaA gene.

V. Conclusion

As the pathogenicity of *E. faecalis* is not well defined that made its consideration to attempt this study.Our study showed that most of our strains produced hemolysin followed by gealtinase and they found to be sensitive to most of the commonly used antibiotics. Many were equally have the ability to exhibit both collagen binding protein and endocaridtis. This warrants a detailed study on virulence factors associated with the formation of dental caries by *E. faecalis.*

References

[1]. Bhalla A, Pultz NJ, Gries DM, Ray AJ, Eckstein EC, Aron DC, et al. Acquisition of nosocomial pathogens on hands after contact with environmental surfaces near hospitalized patients. Infect Control HospEpidemiol 2004;25(2):164-7.
[2]. Sedgley C, Buck G, Appelbe O. Prevalence of Enterococcus faecalis at multiple oral sites in endodontic patients using culture and PCR. J Endod 2006, 32:104-109.
[3]. Johnson EM, Flannagan SE, SedgleyCM.Coaggregation interactions between oral and endodontic Enterococcus and bacterial species isolated from persistent apical periodentitis. J Endod 2006, 32:946-950.
[4]. Pinheiro ET, Gomes BP, Ferraz CC, Sousa EL, Teixeira FB, SouzaFilho FJ. Microorganisms from canals of root-filled teeth with periapical lesions. IntEndod J 2003, 36:1-11.
[5]. Kayaoglu G, Orstavik D. Virulence factors of Enterococcus faecalis: Relationship to endodontic disease. Int Am Asso for Dent Res 2004, 15:308-320.
[6]. GiridharaUpadhyaya PM, Ravikumar KL, Umapathy BL. Review of virulence factor of enterococcus: An Emerging nosocomial pathogen. Indian J Med Microbiol 2009;27:301-5.
[7]. Schouten MA, Hoogkamp-Korstanje JA, Meis JF, Voss A. Prevalence of vancomycin resistant enterococci in Europe. Eur J ClinMicrobiol Infect Dis 2000;19:816-22.
[8]. Creti R, Imperi M, Bertuccini L, Fabretti F, Orefici G, Di Rosa R, et al. Survey for virulence determinants among Enterococcus faecalis isolated from different sources. J Med Microbiol 2004;53(1):13-20.
[9]. Sedgley CM, Lennan SL, Clewell DB. Prevalence, phenotype and genotype of oral enterococci. Oral MicrobiolImmunol 2004, 19:95-101.
[10]. Salah R, Das-Odeh N, Hammad OA, shehabi AA. Prevalence of putative virulence factors and antimicrobial susceptibility of enterococcus faecalis isolates from patients with dental diseases. BMC Oral Health 2008;8(17):1-7.
[11]. Clinical Laboratory Standards Institution: Performance standards for antimicrobial susceptibility testing. In NCCLS approved standard M2-A8. Wayne, PA USA: CLSI,2014.
[12]. M Mishra; YS Thakar; AA Pathak.Haemagglutination, hemolysin production and serum resistance of Proteus and relted species isolated from clinical sources. Indian J Med Microbiol 2001;19(2):5-11.

[13]. N Cevahir; M Demir; I Kaleli; M Gurbuz; S Tikvesli.Evaluation of biofilm production, gelatinase activity, and mannose-resistant hemagglutination in Acinetobacterbaumannii strains.J MicrobiolImmunol Infect 2008;41(6):513-518.
[14]. Hancock HH rd, Sigurdsson A, Trope M, Moiseiwitsch J. Bacteria isolated after unsuccessful endodontic treatment in a North American population. Oral Surg Oral Med Oral Pathol Oral RadiolEndod 2001;91(5):579-86.
[15]. Sjogren U, Figdor D, Persson S, Sundqvist G. Influence of infection at the time of root filling on the outcome of endodontic treatment of teeth with apical periodontitis. IntEndod J 1997;30(5):297-306.
[16]. Peciuliene V, Balciuniene I, Eriksen HM, Haapasalo M. Isolation of Enterococcus faecalis in previously root-filled canals in a Lithuanian population. J Endod 2000;26(10):593-5. 22.
[17]. Dahlén G, Samuelsson W, Molander A, Reit C. Identification and antimicrobial susceptibility of enterococci isolated from the root canal. Oral MicrobiolImmunol 2000;15(5):309-12.
[18]. Frank KL, Vergidis P, Brinkman CL, Greenwood Quaintance KE, Barnes AM, et al. Evaluation of the Enterococcus faecalisbiofilmassociated virulence factors AhrC and Eep in rat foreign body osteomyelitis and in vitro biofilm-Associated antimicrobial resistance.PLoS One 2015;10(6):e0130187.
[19]. Sedgley C, Nagel A, Dahlen G, Reit C, Molander A: Real-time quantative polymerase chain reaction and culture analyses of Enterococcus faecalis in root canals. J Endod 2003, 32(3):173-177.

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