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Original Research

Virulence genes in biofilm producer *Enterococcus faecalis* isolates from root canal infections

M. Akbari Aghdam[1], M. H. Soroush Barhaghi[1*], M. Aghazadeh[1], F. Jafari[2], M. Beomide Hagh[1], M. Haghdoost[3], M. Y. Memar[4,5], M. Ahangarzadeh Rezaee[3], H. Samadi Kafil[1*]

[1] Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[2] Department of Endodontics, Dental School, Tabriz Branch, Islamic Azad University, Tabriz, Iran
[3] Infectious and Tropical Medicine Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[4] Student Research Committee, Tabriz University of Medical Sciences, Tabriz, Iran
[5] Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

Correspondence to: mhsoroushb@yahoo.com, kafilhs@tbzmed.ac.ir

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Abstract: *Enterococcus faecalis* is occurring in opportunistic infections involving the oral cavity. This study aimed to evaluate the presence of *E. faecalis* virulence genes in dental root canal isolates recovered from advanced chronic periodontitis patients. One hundred *E. faecalis* isolated from dental root canal during July 2015 to Oct 2016. After analysis of biofilm formation by the semi-quantitative determination in 96-well flat bottom polystyrene plates, the presence of *asa*, *esp*, *efaA*, *ace, ebpR, gel and hyl* gene were studied by PCR. Gelatinase and hemolytic activity were detected by phenotypic methods. Ninety-one percent of isolates had *ebpR* gene, 85% *ace*, 82% *efaA*, 81% *gel,* 56% *esp*, 33% *asa1*, 2% *hyl* and 0% *cyl* gene. Evaluation of biofilm formation by microtiter plate method presented 49% of the isolates as strong biofilm producer, 42% displayed moderate biofilm formation, and 10 % weak or no biofilm was observed. *asa1*, *efaA*, *esp*, and *ebpR* positive isolates had significantly higher biofilm formation than negative isolates, while no significant differences were found when comparing *ace*-positive and -negative isolates. Present study showed that the *ace* genes do not seem to be necessary nor sufficient for the production of biofilm in *Enterococcus faecalis* but the presence of *asa1*, *efaA*, *esp*, and *ebpR* correlates with increased biofilm formation of dental root canal isolates.

Key words: *Enterococcus faecalis*; dental root canals; Biofilm; Virulence genes.

Introduction

Apart from nosocomial infections, *Enterococcus faecalis*, although not normally considered to be part of the healthy oral flora (1), has been recovered from root canal, saliva, mucosal surface, and gingiva and also found in common dental diseases, i.e., periodontitis, periimplantitis and caries (2, 3).

E. faecalis possesses certain virulence factors to interaction with host cells and other microorganisms, as well as alter host responses (4). Several adhesions and secreted virulence factor have been described including aggregation substance (*asa*), extracellular surface protein (*esp*), *E. faecalis* antigen, A (*efa*A), adhesin of collagen from *E. faecalis* (*ace*) and endocarditis and biofilm-associated pilli (*ebp*) (5) . Enterococcal infections can be difficult to treatment due to intrinsic resistance to some antimicrobial agents and emergence of strains resistance to high levels of aminoglycosides, penicillin or vancomycin (6). The mobile genetic elements act as reservoir for acquirement and dissemination of drug resistance factors among *E. faecalis* strains. Diverse plasmids have been described in *E. faecalis* which some of these encode important resistance gene including vancomycin, aminoglycosides, erythromycin and multi drug resistance (7).

The ability of *E. faecalis* to form biofilms may confer an ecological advantage in certain situations. For example, clinical strains of *E. faecalis* isolated from infective endocarditis patients were significantly associated with greater biofilm formation than non-endocarditis clinical isolates (8). This may be attributable in part to specific virulence traits such as gelatinase production and presence of the adherence determinant *esp*; this combination was shown to be associated with the formation of thicker biofilms (9, 10). These virulence traits and others have also been identified in clinical isolates of *E. faecalis* from root canals and the oral cavity (11, 12).

Some studies claimed correlation among the presence of certain factors and biofilm formation (13) but others suggest that these genes do not seem to be necessary for the production of biofilm in enterococci (14). More recently, biofilm-associated infections of artificial medical devices have been attributed to enterococci (15). These are difficult to treat due to increased antibiotic resistance and also, the slow metabolic rate of microorganisms in biofilms as well as the extracellular matrix can impede the effectiveness of many antimicrobials (16, 17). For example, the inhibition of *E. faecalis* biofilms require very high concentrations of antibiotics such as ampicillin, vancomycin, and linezolid (18).

In view of the limited data on this potential oral pathogen, the present study examined the occurrence of *E.*

faecalis in dental root canal samples recovered from advanced chronic periodontitis (CP) patients in the Tabriz, Iran, frequency of biofilm producer and co-occurrence of biofilms and certain virulence factor genes.

Materials and Methods

Bacterial isolates

One hundred *E. faecalis* isolates from dental root canals were collected from dental clinics affiliated to Tabriz University of Medical Sciences Tabriz, Iran during July 2015 to Oct 2016. All isolates were obtained from patients in need of endodontic treatment referred to the clinics. After supragingival root canal removal by scaling, each tooth was cleansed with pumice and isolated with a rubber dam. The tooth and the surrounding field were cleansed with 3% hydrogen peroxide and disinfected with a 2.5% sodium hypochlorite solution. After isolation and disinfection of the operative field, coronal restorations were removed when present. For this purpose, sterile high-speed carbide burs were used until the root filling was exposed. If a post was present, removal was attempted through ultrasonic vibration; if unsuccessful, post removal was performed with a sterile high-speed carbide bur. After completion of the endodontic access, the tooth, clamp, and adjacent rubber dam were once again disinfected with 2.5% NaOCl. For sterility control, two paper points were scrubbed on the disinfected tooth crown and transferred to a tube containing Enterococcosel broth (Becton Dickinson Microbiology Systems, Cockeysville, MD), a selective medium with bile-esculin and sodium azide, and incubated for 72 hours at 35°C.

Culture Procedures

After incubation for 72 hours at 35°C, samples that showed growth in Enterococcosel broth were plated onto trypticase soy agar plates containing 5% sheep blood, and incubated at 37°C for 72 h. Suspected colonies of *Enterococcus* spp. were checked for morphology and standard methods of microbiology (19).

Biofilm formation assay

Biofilm formation were analyzed by the semi-quantitative determination of biofilm formation in 96-well flat bottom polystyrene plates, under static conditions for 48 h as previously described (10, 20). For biofilm development, inoculums of around 10^7 CFU/mL were prepared by adjusting culture grown bacterial suspensions in trypticase soy broth (TSB) (Himedia, India) from overnight cultures to an optical density ($OD_{600\ nm}$) of 0.1 and adding 100 µL of every inoculum to wells. Taking after 48 hours incubation, plates were tenderly washed one time with 1X phosphate buffered saline (PBS; pH 7.4) and stained with 100 µL of 0.1% Crystal Violet (CV) for 30 min at room temperature. Excess crystal violet was expelled by washing, and biofilm was evaluated by measuring absorbance of the supernatant at 570 nm utilizing a microtiter plate reader (BioTeck, Winooski, USA) taking after the solubilization of CV in 95% ethanol. The cut-off optical density (OD) for Biofilm formation by isolates was defined as the optical density higher than OD570= 0.524 (absorbance of biofilm produced by *E. faecalis* ATCC 29212).

Hemolytic-assay

Hemolytic activity of isolates were detected on blood agar plates prepared with Blood Base Agar (Hi-Media, India) containing 5% defibrinated sheep blood, by examination of hemolysis zone around colonies following incubation for 24 h at 37°C(21).

Gelatinase activity

We assessed gelatinase activity by inoculating *E. faecalis* isolate onto tubes containing 5 ml Mueller-Hinton broth with the radiographic strip. After 24 to 48h incubation at 370 C, clearing of strip and tarnishing of media was considered as positive test.

Detection of *E. faecalis* Virulence Genes

DNA extraction was performed by the tissue buffer boiling method (22). Extracted DNA served as a template for the amplification of virulence genes specific for *E. faecalis* includes *asa* , *esp* , *efaA* , *ace* and *ebp*). All primer sequences and corresponding references are listed in Table 1 (5). Conventional PCR was performed in 25 µl volumes reactions that contained 20–200 ng DNA, 0.5 µM of 1 µl of each specific primers for each gene, 1.5 mM $MgCl_2$, and 200 µM of each dNTP, 1× PCR buffer and 2 U DNA Taq-polymerase (Cinnage, Tehran, Iran) (23). An initial denaturation at 94 °C for

Table 1. Oligonucleotide primers which were used in the present study.

Target(s)	Sequence (5`–3`)	Amplicon size (bp)	Reference
asa1	F: 5`-GCACGCTATTACGAACTATATGA R: 5'- TAAGAAAGAACATCACCACGA	375	(10)
ace	F: 5`-GGAGAGTCAAATCAAGTACGTTGGTT R: 5`- TGTTGACCACTTCCTTGTCGAT	101	(5)
efaA	F: 5`- TGGGACAGACCCTCACGAATA R: 5'- CGCCTGTTTCTAAGTTCAAGCC	101	(5, 28)
ebpR	F: 5`- AAAAATGATTCGGCTCCAGAA R: 5`- TGCCAGATTCGCTCTCAAAG	101	(39)
esp	F: 5`- GGAACGCCTTGGTATGCTAAC R: 5`- GCCACTTTATCAGCCTGAACC	95	(40)
hyl	F:ACAGAAGAGCTGCAGGAAATG R:GACTGACGTCCAAGTTTCCAA	276bp	(5)
gel	F: TATGACAATGCTTTTTGGGAT R:AGATGCACCCGAAATAATATA	213bp	(5)
cyl	F: ACTCGGGGATTGATAGGC R:GCTGCTAAAGCTGCGCTT	688bp	(5)

10 min was followed by 35 cycles of 1 min denaturation at 94 °C, annealing at 58 °C (for *esp*, *efaA* and *ace*)/52 °C (for *ebpR* and *asa1*) for 1 min and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. PCR products were analyzed in 1.5% agarose gels and visualized under UV after staining with 0.5 $\mu g \times ml^{-1}$ DNA safe stain.

Statistical analysis

All statistics have been analyzed by means of SPSS software version 22.0 (SPSS Inc, Chicago, IL, USA). Comparative data was calculated utilizing the Fisher's exact and two-tailed $\chi 2$ test, once appropriate. A p-value of ≤ 0.05 was thought to be significant.

Results

Among the study period, non-duplicated 100 isolates of *E. faecalis* were collected from dental root canals of patient's referred to dental clinics. Based on the patient's gender, 68 (68%) of the isolates were from male patients and 32 (32%) were from female patients. The average age was 32 ± 10 yr. All isolates were investigated for the presence of virulence genes and 56 (56%) had *esp* gene, 33 (33%) had *asa1* gene, 85 (85%) had *ace* gene, 82 (82%) had *efaA* gene and 91 (91%) had *ebpR* gene. Evaluation of biofilm formation by microtiter plate method presented 49% (n = 49) of the isolates as strong biofilm producer, moreover these isolates had stronger initial attachment capacity to microtiter wells. About 42% (n = 42) displayed moderate biofilm formation, furthermore indicated initial binding activity; while, in 9 % (n = 9) weak or no biofilm was observed. In the present study, the range of OD570 readings was from 0.09 to 2/96. No biofilm was detected in negative control wells. By comparing isolate absorbance, *asa1* positive isolates had significantly higher biofilm formation than *asa1* negative isolates as well as *efaA*, *esp*, and *ebpR* positive isolates had higher biofilm formation than negative isolates (P <0/05). No significant differences were found when comparing *ace*-positive and - negative isolates (P >0.05). The frequency of virulence genes among biofilm producer and non-producer isolates were presented in Table 2.

Gelatinase activity was detected in 77% of isolates by phenotypic method. However, the PCR results were shown the presence of the equivalent genes in 81% of isolates.

The number of isolates positive for *hyl* gene by PCR was more than the number of isolates showing positive result by phenotypic detection.

Discussion

The interest in the microbial composition and frequency of various microorganisms in endodontic infection has been focused in recent decades on the anaerobic bacteria due to their predomination in untreated teeth with necrotic pulps (3). *E. faecalis*, on the other hand, are very seldom isolated in those infections and little attention has therefore been paid to them in the development of periapical destruction. In the last decade, several studies have focused on the relationship between periodontal diseases and oral bacteria and systemic diseases (24). Likewise, bacterial pathogens that cause medically important diseases may colonize the oral cavity (25). The current investigation examined the virulence factors and biofilm formation *E. faecalis* isolates from dental root canals samples from patients with chronic periodontitis and periodontal health.

The presence of *E. faecalis* in periodontitis lesions may have important therapeutic implications. Established pathogens in the dental biofilm may be more difficult to eradicate due to their higher resistance to antimicrobials agents and immunological defense mechanisms, increasing the probability of reinfection and treatment failure (24). In fact, *E. faecalis* isolated from root canal infection was capable of forming a very thick biofilm on gutta-percha points. Finally, enterococci originating from periodontal sites may seed into the bloodstream and act as a source for life-threatening septicemias in immuno-compromised patients (26). Of interest, *E. faecalis* has been frequently encountered in the subgingival microbiota of severely immunosuppressed HIV individuals; i.e. with low levels of TCD4 lymphocytes (<200 cells/mm3).

The most important adhesion factors tested in this study was Asa, Esp, EfaA, Ace and Ebp. Other important secreted pathogenic factors of *E. faecalis* with a value in pathogenesis are CylA (cytolysin), GelE (gelatinase) and Hyl (hyaluronidase) (27, 28). Several studies investigated the role of these virulence factors in biofilm formation by *E. faecalis* (10, 28). esp and gelE were the main factors investigated in strains from different origins(10, 29). Prevalence of *E. faecalis* and its virulence factors differ from one study to another.

Of interest was whether greater biofilm production capacity was associated with virulence traits identified in the same strains (12). Our investigated isolates had diverse presence of virulence factors from lack to high amount of virulence genes. Overall, except to the *ace*, , relationship was found between the ability of *E. faecalis* isolates to form biofilms and presence of the virulence determinants *efaA, esp, and ebpR* (P >0.05). Conflicting

Table 2. Frequency of virulence genes among biofilm producer and non-producer isolates (%).

Virulence gene	No of biofilm producer (%)	No of Non-biofilm producer (%)	Total*
*efa*A	74(90.24)	8(9.75)	82
ace	77(90.58)	8(9.41)	85
ebp	84(92.3)	7(7.69)	91
asa	28(84.84)	5(15.15)	33
esp	49(87.5)	7(12.5)	56
gel	73(90.12)	8(9.87)	81

* These numbers indicates number of isolates harboring subjected gene from all collected isolates.

results have been reported regarding the role of the *esp* gene in biofilm formation. Some authors have suggested that *esp* promotes biofilm formation; however, additional determinants may contribute to biofilm formation in *E. faecalis* (30, 31). However, finding of our study and others suggest that the *esp* gene does not seem to be necessary nor sufficient for the production of biofilm in *E. faecalis* (10, 32-34). These findings add more contrary to the role of *esp* on biofilm formation by *E. faecalis*

Similar to our study, Zoletti G et al. reported high frequency of *efa*A and *ace* genes among oral cavity isolated *E. faecalis*(35).

The presence of the gelE gene without of gelatinase activity in Enterococcus has been related with operations in laboratory, silent genes, and low level expression or down regulation of the gelE gene. Similar to other studies, our results indicated that strong biofilm formation correlated with gelE genes presence ($p < 0.05$).

Zoletti G et al. reported the phenotypic tests can detect gelatin hydrolase among 50% of all *gel*E positive isolates (35).

Hancock and Perego were reported it stimulates the aggregation of the planktonic cells in microcolonies to procedure the primary attachment site and following expansion into a three-dimensional construction. The gelE gene might directly or indirectly degrade tissue proteins of host cells such as collagen protein, which may further lead tissue injury (36, 37).

Differences in detection of *E. faecalis* observed in the literature may result from differences in the study population, oral or systemic status of the patient, type and number of clinical samples, and methods of detection. Studies have shown that the periodontal microbiota may vary markedly in frequency and proportions in populations with distinct ethnic background (24, 38). Geographical differences in bacteria detection have also been observed in endodontic infections.

In summary, the oral cavity has been shown to be a reservoir for *E. faecalis*. More importantly, our findings provide additional evidence for the association of this microorganism with the presence of periodontal infection. Some of the virulence determinants detected in the present study may help to describe the development of *E. faecalis* biofilm in the oral cavity that may influence infection progression and outcome.

Considering that eradication of this species from subgingival biofilm in deep periodontal pockets may be limited, close attention should be given to these patients in order to reduce the risk for development of systemic diseases caused by *E. faecalis* in other areas of the body.

Ethics Committee Approval:

Ethics committee approval was received for this study from Tabriz University of Medical Sciences Ethics Committee for Experimental studies (permit no:1394.4.11).

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