Original Research

Effect of acidic and alkali shocks on expression of efaA gene in Enterococcus faecalis, isolated from root canal infection

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Abstract: Enterococcus faecalis is an important opportunistic infectious agent involving the oral cavity and endodontics. The aim of this study was to evaluate the expression ratio of efaA gene in biofilm producer Enterococcus faecalis before and after receiving acidic and alkali shocks. One hundred E. faecalis isolates were gathered from 170 infectious root canals. After analysis of biofilm formation by the Microtiterplate method, the presence of efaA gene was examined by PCR and its expression was evaluated by Real-time PCR, one before applying any stressed to isolates and another by applying acidic and alkali shock. Chi-square method was used for statistical analysis. Eighty-two percent of samples had efaA gene. Evaluation of biofilm formation, 49% of the isolates were strong biofilm producer, 42% moderate and 10% of them had no biofilm. 59% overexpression of efaA gene was observed in biofilm producer isolates, while there were no significant changes in samples with acidic stress and decreased expression after alkali shock. Findings of the present study, indicates importance of efaA gene in biofilm formation and pathogenesis of E. faecalis. Acid had no effect of expression of this gene but alkali reduced expression of this gene in a significant level. These results indicate the importance of efaA and acidic conditions in biofilm production by E. faecalis.

Key words: Acid response; Adhesin; Biofilms; Gene expression; Endodontic infection.

Introduction

Contrary to considering enterococci as commensals of the intestinal tract, nowadays it has an undeniable role in nosocomial infections (1), with clinically the most significant species, E. faecalis, and E. faecium, by their association with infection (2, 3). Between these two, E. faecalis plays a dual role in human ecology and has frequently been associated with bacteremia and endocarditis and with infections of the abdominal cavity, pelvis, and soft tissues (4, 5). Besides these, E. faecalis has been identified to be part of the oral cavity, saliva, root canal, and found in common dental diseases, i.e., periodontitis. E. faecalis has been found occasionally in primary endodontic infections. While, in cases of failed endodontic treatments, E. faecalis has been frequently isolated (3). E. faecalis possesses certain virulence factors in order to interact with different cells and microorganisms. A group of genes may contribute to enterococcal virulence (6, 7). Several adhesions and secreted virulence factor have been described including aggregation substance (asa), extracellular surface protein (esp), E. faecalis antigen A (efaA), the adhesin of collagen from E. faecalis (ace) and endocarditis and biofilm-associated pilii (ebp) (2, 8). Enterococcal infections are difficult to treat because of intrinsic resistance to many antimicrobial agents and emergence of strains extremely resistant to high levels of vancomycin, penicillin or aminoglycosides and high toleration of environmental pH changes (9). The ability of E. faecalis to form biofilms which is considering to be one of the most important causes of high adaptation ability of this organism to difficult and challenging environmental conditions and resistance to antimicrobial agents may confer an ecological advantage in certain situations (10). Biofilm is a population of cells attached irreversibly on various biotic and abiotic surfaces, and encased in a hydrated matrix of exo polymeric substances, proteins, polysaccharides and nucleic acids (11). Bacteria in a biofilm behave differently while it can cope with a hard situation like acidic or alkali shocks from their free-floating (planktonic) counterparts (12, 13). From all virulence factors, there is an important auspice on efaA gene (14) which was proven to have a role in biofilm formation in endodontics isolates. Because of significant effects of pH changes of dental environments in the result of various food sources, this work constitutes a part of our effort to study different stress responses of E. faecalis under effects of acidic and alkali shocks on the expression of this particular gene.
Materials and Methods

Sampling
One hundred and seventy patients including failed endodontic treatments and primary endodontic infections who were referred for endodontic treatment to the Department of Endodontics of the Dental faculty of Tabriz University of medical sciences during July 2015 to Oct 2016 were enrolled in this study. Initially, each tooth was cleaned with pumice and was isolated with a rubber dam. The tooth and the surrounding field were decontaminated with a 2.5% sodium hypochlorite solution. Then sterile burs made complete access, without water spray. Samples were initially collected with a size 15 K-type file with the handle cut off. The file was introduced to a level approximately 1 mm short of the tooth apex. In case of a dry root canal, a small amount of sterile saline solution was introduced into the canal. Afterward, two sequential sterile paper points were placed to the same level and used to soak up the fluid in the canal. Each paper point was retained in the canal for 1 min. The cut file and the two paper points were then transferred with sterile forceps to BHI (brain/heart infusion broth, Merck, Germany); samples were grown at 37 °C without shaking over a night then subcultured on Enterococcus specific medium named kanamycin aesculin azide agar and grown at 37°C over a night (3). Suspected colonies of Enterococcus were checked for morphology and Gram staining characteristics and one hundred of samples were identified as E. faecalis and were stocked in TSB (Tryptic soy broth, Merck, Germany) with 20% glycerol at -70°C (15).

Biofilm formation
Sterile 96-well flat-bottom polystyrene microtiter plates (Fisher Scientific, Pittsburgh, PA) were utilized. Wells containing 180 µl TSB medium with 5% glucose were inoculated with 20 µl of enterococcal suspension (0.5 McFarland: OD600 between about 0.08 and 0.1) from the overnight culture. Wells without bacteria served as controls. Fresh 24 h bacterial cells were dissolved in 1 ml ice cold RNXplus solution (including phenol and Guanidinium) and was gently shaken after adding 200 µl chloroform, then the mixture was kept on ice for 15 min and centrifuged at 12000 rpm at 4 °C for 15 min. The aqueous phase was transferred to a new RNase-free 1.5 ml tube and added equal volume of Isopropanol and after gently mix and incubation on ice for 15min, mixture was centrifuged at 12000 rpm at 4 °C for 15 min. The supernatant was discarded and the pellet was dried at room temperature for few minutes and was dissolved in 50 µl DEPC (Diethylpyrocarbonate) (SinaClon) water. DNA quality was measured by OD260/280 ratio higher than 1.9 in Nanodrop (BioTech, USA) and Gel electrophoresis. DNAse treatment was done by adding 5U DNAse enzyme (Fermentas, Germany) to 20 ml RNA. Extracted DNA was used as a template for the amplification of efaA gene. The primer sequence and corresponding reference are in Table 1. Conventional PCR was performed in 25 µl volumes by using Red amplicon ready master mix. An initial 10 min denaturation at 94 °C was followed by 35 cycles of 1 min denaturation at 94 °C, annealing at 58 °C 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 agarose gel (1%) and visualized under UV after staining with safe stain.

Quantitative RT-PCR assay
Total RNA from 82 dental enterococci isolates were extracted using RNXplus kit (SinaClon, Tehran, Iran). Fresh 24 h bacterial cells were dissolved in 1 ml ice cold RNXplus solution (including phenol and Guanidinium) and was gently shaken after adding 200 µl chloroform, then the mixture was kept on ice for 15 min and centrifuged at 12000 rpm at 4 °C for 15 min. The aeous phase was transferred to a new RNase-free 1.5 ml tube and added equal volume of Isopropanol and after gently mix and incubation on ice for 15 min, mixture was centrifuged at 12000 rpm at 4 °C for 15 min and after discarding the supernatant, and adding 1 ml of 75% Ethanol, the mixture was shortly vortex to dislodge the pellet and then centrifuged at 7500 rpm for 8 min. Afterward, the supernatant was discarded and the pellet was dried at room temperature for few minutes and was dissolved in 50 µl DEPC (Diethylpyrocarbonate) (SinaClon) water. RNA quality was measured by OD260/280 ratio higher than 1.9 in Nanodrop (BioTech, USA) and Gel electrophoresis. DNAse treatment was done by adding 5U DNAse enzyme (Fermentas, Germany) to 20 ml RNA and followed by adding 4 µl Buffer MgCl2 and incubation at 37°C for 1 h. Reaction was stopped by adding two µl EDTA and incubation at 65°C for 10 min. cDNA synthesis was done by using STRP Kit (Sinnaclon). 5 µl of RNA was heated for 1 min and immediately transferred on ice and then vortexed again and followed by adding 17 µl Mix1 solution and 0.5 µl RT Enzyme and

Table 1. Oligonucleotide primers used in PCR and RT-PCR.

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence (5’–3’)</th>
<th>Amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>efaA(PCR)</td>
<td>F: 5’- TGGGACAGACCTCAGAATA</td>
<td>101</td>
<td>(10)</td>
</tr>
<tr>
<td></td>
<td>R: 5’- CGCCTGTTTTCTAAGTCAAGCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23SrRNA</td>
<td>F: CCTATCGGCCTCGGCTTAG</td>
<td>100</td>
<td>(10)</td>
</tr>
<tr>
<td></td>
<td>R: AGCGAAAGAGCATGGTAGAATCC</td>
<td></td>
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One hundred isolates of _E. faecalis_ were collected from infected root canals of patient's referred to the dental clinic. Approximately 68 (68%) of the isolates were from male patients and 32 (32%) were from female patients. The average age was 32 ± 10 year. All isolates were checked for the presence of _efaA_ and from all, 82 (82%) had _efaA_ gene. 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 10% (n = 10) weak or no biofilm was observed. In this study, the range of OD570 readings was from 0.09 to 2.96. No biofilm were detected in negative control wells. By comparing isolate absorbance, _efaA_ positive isolates had higher biofilm formation than negative isolates (P <0.05). All 82 _efaA_ positive isolates were examined by Real-Time PCR expression assays. Expression ratios for all 82 _E. faecalis_ show 59% overexpression for the _efaA_ gene. From all 82 _efaA_ positive isolates. Two samples were treated randomly for acidic and alkali shock with HCl and NaOH. One sample was treated as the control in normal condition. RT-PCR analysis presented acceptable amplification of the targeted gene and housekeeping gene. Analyses were done for both shock condition. No significant change was observed for _efaA_ expression during acidic shock but there was a significant decrease in _efaA_ gene expression in alkali shock treatment (Figure 1).

**Discussion**

In recent decades, investigations on oral cavity _E. faecalis_ are quite increased because of their important role at different oral and dental disease (3). Despite its proven effects on primary root infections, they are responsible for primarily failed endodontics and secondary root infections (22, 23). One of the most reasons for focusing on this bacterium is its ability to form biofilm and the resistance to different anti-agents due to this ability. On the other hand, _E. faecalis_ is known because of its high adaptation and tolerance power to pH changes of the environment as flaicult et al. the responses of _E. faecalis_ at alkali stress, they show a high adaptation mechanism for this bacteria during the shock (24). A similar work came from Erlend Bore and his colleagues on _S. aureus_ in acidic stress and they showed different changes on the expression of genes and they proved decreased growth rate on samples (20). Various factors are involved in the pathogenesis of this organism. Past
studies showed that esp gene despite its importance at pathogenesis does not affect the biofilm formation (2, 25) while there were new findings showing the importance of efaA at this process (10). The present study evaluated the role of efaA gene in biofilm formation and its expression ratio in isolates collected from primary and secondary infected root canals and changes in expression of this gene under acidic and alkali shock, E. faecalis isolates collected from the infected canals involved in this study. The presence of the efaA gene in this study shows a similar pattern to that previously reported for endodontic studies (26, 27), where efaA were found in 82% of the isolates and previous reports indicate its presence in 75 to 90% of the isolates (3, 26, 27). In this study, our efaA positive isolates were more biofilm producer and they had enhanced the ability of biofilm production in which it has shown 59% overexpression in isolates with the power of biofilm forming regarding our expression results. This fact that biofilm producer isolates had higher efaA expression can lead to better understanding of virulence and mechanisms related to biofilm in this bacterium.

The expression of efaA in E. faecalis under acidic and alkali stress was interestingly different. efaA had no significant change in expression in acidic condition. The main pathogenesis of this bacterium is under the acidic condition of the oral cavity. No change in expression can indicate this gene has the continuous expression in normal and acidic conditions. However, in alkali condition, it has reduced expression. This finding can lead us to develop new strategies to reduce biofilm production by this bacterium.

In conclusion, the findings of the present study indicates the importance of efaA gene in biofilm formation and pathogenesis of E. faecalis. This gene had higher expression in biofilm producer isolates with root dental origin. The acidic condition had no effect of expression of this gene and it had continuous expression after acidic shock, but alkali shock reduced expression of this gene in a significant level. Studies with transcriptome analysis will help us to have a comprehensive understanding regarding the pathogenesis of this bacterium. However, results of the present study showed another evidence regarding the importance of efaA and acidic conditions in biofilm production by E. faecalis.

Competing Interest
All Authors declare no conflict of interest.

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