Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

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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Received February 2, 2018; **Accepted** October 3, 2018; **Published** October 30, 2018

Doi: http://dx.doi.org/10.14715/cmb/2018.64.13.1

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 *E. 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 pilli *(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 *Enterococcal* 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). *E. facealis* identification was according to biochemical properties described previously (3).

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 received 300 µl of the TSB dilution as negative controls. The microtiter plates were incubated for 24 h at 37°C without shaking. All experiments were done in triplicate and mean of them considered as final absorbance. All plates were done in triplicate. After stationary aerobic incubation for 24 h at 37°C and 5% CO2, the broth was carefully drawn off and the wells were washed three times with 300 µl of sterile phosphate buffered saline (PBS) at room temperature. Then biofilms were fixed using 150 µl methanol for 20 min, flick, and air dry in an inverted position in the room temperature for about 30 min. Biofilms were stained with 150 µl of crystal violet solution(2%) for 15 min at room temperature and the wells were rinsed by placing the plate rinsed by water. Microtiter plates were inverted on a paper towel and air-dried at the warm room. In order to quantify biofilm formation, 150 µl of 33% acetic acid was added to each well to de stain the biofilms and then plates were placed at room temperature for 30 min without shaking. Thereafter, the optical density of the resolubilized crystal violet was measured at 570 nm (OD570) by using a microtiter plate reader (16, 17). *E.faecalis* (Andrewes and Horder) Schleifer and Kilpper-Balz (ATCC® 29212™) was used as positive control for biofilm formation.

PCR for the Detection of efaA

DNA extraction was performed by the boiling method (18). 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 (1.5%) and visualized under UV after staining with safe stain (19).

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 shacked after adding 200 µl chloroform, the mixture was kept on ice for 15 min and centrifuged at 12000 rpm at 4 °C for 15min. 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 and after discarding the supernatant, and adding 1 ml of 75% Ethanol, the mixture was shortly vortex to dislodge the pallet and then centrifuged at 7500 rpm for 8 min. Afterward, the supernatant was discarded and the pallet 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.

Target	Sequence (5`–3`)	Amplicon size (bp)	Reference
efaA(PCR)	F: 5`- TGGGACAGACCCTCACGAATA R: 5'- CGCCTGTTTCTAAGTTCAAGCC	101	(10)
23SrRNA	F: CCTATCGGCCTCGGCTTAG R: AGCGAAAGACAGGTGAGAATCC	100	(10)

2.5 µl Mineral oil and incubated at 42°C for 60 min and the final incubation at 70°C for 10 min. Quantitative Real time PCR was done by allele specific primers (Bioneer, Korea) (Table 1) (10). The qRT- PCR assay was performed using Rotor-Gene 6000 (Corbett Research, Sydney, Australia) and analyzed by using a software program from Real-Time Analysis Software (Roter-Gene Version 6.1, Corbett, Australia). The real-time PCR amplifications were performed in 25 µl reactions containing 2X QuantiTect SYBR Green PCR master mix (Takara, Kyoto, Japan), which includes HotStarTaq DNA Polymerase, QuantiTect SYBR Green PCR Buffer, 2.5 mmol/L MgCl2, deoxyribonucleotide triphosphate (dNTP) mix, and fluorescent dyes, RNase-free H2O (SigmaeAldrich, Germany), 0.6 Mmol/L primer (Bioneer, Korea), and 2 µl of the respective template DNA dilution. All of the experiments were performed twice. The qRT-PCR assay was optimized to the initial activation step of 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 63°C of 60 s for housekeeping gene and 64°C of 1 min for *efaA* gene and then followed by extension at 72°C for 1 min. All the results were normalized by using the housekeeping gene (23srRNA) (10).

For acidic and alkali treatments, from all isolates, two samples was selected randomly, incubated overnight at 200 rpm and 37 C. The pre-cultures were transferred to 99 ml TSB in 500 ml baffled shake flasks and incubated at 200 rpm and 37°C. At an OD600 of 1.0, corresponding to 10^8 cfu ml^{-1}, control samples were withdrawn immediately prior to the addition of 450–500 µl 6.5M HCl, which directly brought the pH down to 4.5±0.1. Samples were withdrawn 2, 5, 10 and 20 min after addition of acid and the same method for alkali shock while we used 6.5 M NaOH instead of HCl. Then complete the process as same as we described before at step 1 Quantities Real-Time PCR (20).

Statistical analysis

Expression ratio was measured by Pfaffl formula. Pfaffl formula was calculated manually(10). SPSS Version 22 (IBM SPSS Statistics, New York, USA) was used for statistical analysis. Descriptive statistics, Chi-square or Fisher's exact test were used to evaluate the data. The p-value below 0.05 was considered statistically significant (21).

Results

During this study, about 75 percent of requested patients filled consent form and participated in this study. Participants were subjected for sampling without any hazardous action. This research has been conducted in full accordance with the World Medical Association Declaration of Helsinki and was done after confirmation of the ethic committee of Tabriz University of medical sciences. All participant filled consent form and were participated voluntarily in the study. After the full explanation of the sampling process during their treatment, if patients were not agree and did not complete the consent form, sampling were canceled from these patients.

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 used 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).

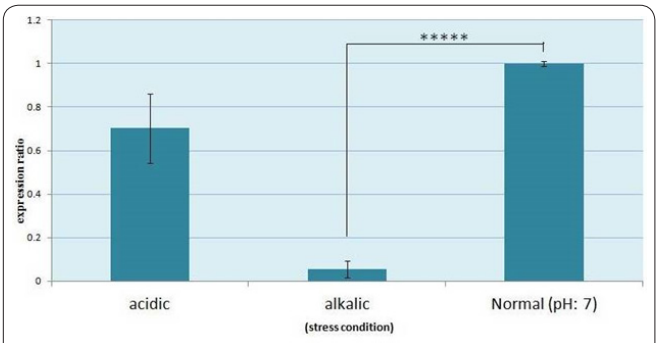

Figure 1. Changes in expression ratio of *efaA* gene in *E. faecalis* isolates after exposing with different stress condition. Change in expression after exposure to alkali stress was significant (P<0.05).

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 flahaut 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.

Funding statement

This study was supported by Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.

Acknowledgments

This study was fully supported by Immunology Research Center (Grant No. 93-129), Tabriz University of Medical Sciences. It is also a report originating from a database developed for the thesis of the first author registered in Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran. We thank all staff of Dental faculty and Drug Applied Research Center for their collaboration.

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