CRISPR-cas system in the acquisition of virulence genes in the dental-root canal and hospital-acquired isolates of *Enterococcus faecalis*

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Abstract

Introduction: *Enterococcus faecalis* is one of the important causative agents of nosocomial and life-threatening infections in human. Several studies demonstrated that the presence of CRISPR-*cas* is associated with the antibiotic susceptibility and lack of virulence traits. In this study, we aimed to assess the relation between the phenotypic and genotypic virulence determinants of *E. faecalis* with the CRISPR elements in the dental-root canal and hospital-acquired isolates.

Methods and materials: Eighty-eight hospital-acquired and 73 dental-root canal isolates of *E. faecalis* were subjected for study. The phenotype tests including biofilm formation, gelatinase and hemolysis activity were performed and the genotype characteristics including *efaA*, *esp*, *cylA*, *hyl*, *gelE*, *ace*, *ebpR*, and *asa1* were performed by PCR methods. Presences of different types of CRISPR-*cas* system were determined by PCR.

Results: Biofilm formation, gelatinase and hemolysis activity were detected in 93.8%, 29.2% and 19.2% of the isolates, respectively. The most gene encoding virulence traits were *ace*, followed by *efaA* and the lowest was *cylA*. The presence of CRISPR1-*cas*, orphan CRISPR2 and CRISPR3-*cas* were determined in 13%, 55.3%, and 17.4% of isolates, respectively, which were present in proportionally more in the dental-root canal. Inverse correlation were found significantly between CRISPR-*cas* loci, *esp* and *gelE*, and direct correlations were found in *cylA*, *hyl*, *gelE* (between some CRISPR-loci), *asa1*, *ace*, biofilm formation, gelatinase and hemolysis activities

Conclusion: Findings indicates that CRISPR-*cas* might prevent the acquisition of some respective pathogenicity factors in some isolates not all, and could not be selective forces to influence pathogenic traits.

Introduction

Enterococci are ubiquitous, Gram-positive and natural inhabitants of the alimentary tracts of mammals and humans. Two important species of the *Enterococcus* genus are *Enterococcus faecalis* and *Enterococcus faecium*, which play an important role as commensals of human and animals. Both of them are also found in soil, on plants and dairy food products [4]. Like other commensals, *E. faecalis* can cause life-threatening infections in humans, such as endocarditis, meningitis, septicemia, urinary tract infections and others [6, 7]. *E. faecalis* is one of the frequent isolates of the endodontic pathogens in prevalence ranging from 30% to 90% of the cases [8, 9]. There are several potential virulence factors in *E. faecalis*, which able to acquire, accumulate and share extrachromosomal elements encoding antibiotic resistance and virulence genes. These virulence factors include secreted factors and adhesions. Cytolysin is encoded by *cyl* operon, which is carried by a plasmid or integrated into the chromosome and have both hemolysin and bacteriocin activity [11, 12]. Gelatinase is encoded by the chromosomal *gelE* gene, which is a zinc methaloprotease, and can hydrolysis gelatin, fibrinogen, collagen, casein, and insulin [13]. Another secreted factor is hyaluronidase, which is encoded by the *hyl* gene [12]. *E. faecalis* endocarditis

antigen, encoded by *efaA* gene that affects pathogenicity [15]. Cell wall-associated protein (Esp), encoded by pheromone-responsive plasmids or the chromosomal *esp* gene, involved in biofilm formation and immune evasion [15]. Aggregation substance, encoded by *asa1* gene on the sex pheromone-plasmid pAD1, are adhesions, which mediates the conjugative transfer of plasmids through the clumping of one *E. faecalis* to another and induces the formation of cell-cell contact [16]. In addition, *ebpR* has encoded an endocarditis- and biofilm-associated pilus regulator, which activates *ebpABC* operon [3]. Another adhesion factor is a collagen binding protein, encoded by *ace* gene, which mediates binding to collagen type I, collagen type IV and laminin [17].

Clustered regulated interspaced short palindromic repeat (CRISPR) loci and CRISPR-associated (Cas) protein-encoding genes are present in approximately 45% of eubacterial genomes sequenced and 90% of archaeal genomes [18]. The mechanism of action of CRISPR-*cas* system are summarized in three stages: adaptation, expression, and interference [19, 20]. In the adaptation stage, a small segment of invading mobile element is integrated into the CRISPR loci between palindromic repeats [20]. Spacers and repeats of CRISPR are transcribed and processed into small RNAs, known as crRNAs, which triggers endonucleases, encoded by the *cas* genes, to targets and cleaves invading mobile elements (DNA or RNA) from which spacers derive [20–22]. There are three types of CRISPR loci in *E. faecalis* genome, including CRISPR1-*cas*, orphan CRISPR2, and CRISPR3-*cas* [14, 23, 24]. CRISPR1-*cas* and orphan CRISPR2 was first found in the *E. faecalis* OG1RF strain: CRISPR1 is located between OG1RF homologue of EF0672 and EF0673, which has the associated *cas* genes and CRISPR2 is located between OG1RF homologue of EF2062 and EF2063, which is an orphan, consists only of spacers and palindromes and lacks of *cas* genes [23]. CRISPR3 was found in 2 genomes of strains Fly1, as a fruit fly *E. faecalis*, and T11, as a urine *E. faecalis* isolate. CRISPR3 is located between homologues of the *E. faecalis* V583 open reading frames EF1760 and EF1759 [14]. CRISPR1 possesses Nmeni subtype-specific gene *csn1* and *csn2* [14, 23], while CRISPR3 only possess *csn1*, not *csn2* [14]. Both CRISPR1 and CRISPR2 contains 7 repeats of a 37bp palindromic sequence with a no homology to any sequences 29bp spacer [23]. Nevertheless, due to small spacer sequences, it is likely that they are derived from the pheromone-responsive type plasmids, plasmids integrated within the *E. faecalis* V583 genome, and Enterococcal prophage and phage [14]. Recent studies demonstrated that the CRISPR/Cas system has applications for genome engineering and strong selective pressure for the acquisition of virulence factors and antibiotic resistance in pathogenic bacteria [14, 25–27].

In This study, we aimed to assess the relation between the phenotype and genotype virulence determinants of *E. faecalis* with the CRISPR elements in the dental-root canal and hospital-acquired isolates.

Methods And Materials

Bacterial strains

This study was approved by the regional ethics committee of Tabriz (Tabriz University of Medical Sciences, Tabriz, Iran, No. IR.TBZMED.REC.1397.188). A total of 88 isolates of *E. faecalis* were collected from teaching and treatment Emam-Reza and pediatric hospitals of Tabriz, Iran. In the same time, in order to collect 73 dental-root canal isolates of *E. faecalis*, patients in need of endodontic treatment were referred to the clinic of the faculty of dentistry of Tabriz University of medical sciences, Tabriz, Iran. Briefly, after stages of access cavity preparation by the dentist, tooth and its surroundings were washed by sterile saline solutions and were disinfected with 30% hydrogen peroxide followed by 2.5% sodium hypochlorite. Preexisting root canal filling was removed by drill and endodontic K-files without the use of chemical solvents. After the sampling of the single-multi-rooted canal of the tooth, paper points were transferred to a tube containing Enterococcal broth (Becton Dickinson microbiology systems, Cockeysville, MD) and were cultured on bile esculin azide agar (Himedia, India) and were incubated at 37 °C for 24–48h [8]. Suspected colony was identified by standard procedures of microbiology [28] and genotype detection was performed by *ddlE* primer [29] is shown in table 1. Both clinical and tooth identified isolates for further studies were stored in trypticase soy broth containing 10% glycerol at –70 °C.

Biofilm formation

Assessment of biofilm formation was analyzed by quantitative biofilm formation in 96-well flat bottom polystyrene microplates under static condition for 48h as previously described [30]. Briefly, for each isolate, fresh colony cultured on Muller-Hinton agar (Merck, Germany) containing 1% glucose were suspended in sterile saline and adjusted to 0.5 McFarland. 20 µl of the adjusted isolates were cultured in 180 µl trypticase soy broth containing 1% glucose. After incubation for 48h at 37 °C, each well was washed by 1X phosphate buffer saline (PBS; pH 7.4), fixed by methanol and stained by 200 µl 0.1% crystal violet for 30 min at room temperature. Excess crystal violet was discarded, washed by water flow. Biofilm formation was measured by absorbance of the supernatant after solubilized in 33% acetic acid at 570 nm by using a microtiter plate reader (BioTeck, Winooski, USA). The cut-off optical density (OD) for biofilm formation was considered higher than OD = 0.524 for *E. faecalis* ATCC® 29212™. Biofilm mean of the isolates was clustered by quartiles, which were obtained by Spss v17.0 (Chicago, IL, USA). Measurement of clusters were lowest at 0.524, 0.525–1.087, 1.088–1.650 and highest 1.654, which were defined none, 1+, 2+ and 3+, respectively.

Gelatinase production and hemolysis test

Hemolysis activity was assessed by blood agar plates, which prepared by brain-heart infusion agar (BHI, biomerieux, Poland, Ltd) containing 5% of group ORh⁺ human blood. Cleared or green zone around the colonies was defined as hemolysis following incubation for 24h at 37 °C [31].

Production of gelatinase was assessed by degradation of gelatin on X-ray film described by previously described [32]. The heavy inoculum of individual isolates was cultured on MHB with the X-ray

radiographic strip and was incubated for 24h at 37 °C. The cleared strip was defined as the production of gelatinase.

Genotype detection of virulence and *cas* genes

Total DNA for each isolate were extracted by the tissue buffer boiling method. Genotype analysis for each isolate were accomplished based on multiplex polymerase chain reaction (PCR) of virulence determinants encoding cytolysin activator *cylA*, *hyl*, *esp*, *gelE*, *efaA*, *asa1*, *ace*, *ebpR*, CRISPR1-*cas*, CRISPR1-*cas csn1*, CRISPR2, CRISPR3-*cas* and CRISPR3-*cas csn1*, which are shown each primer sequences and amplified size in table 1. 2 µl of total DNA was used for multiplex PCR in a 25µl reaction mixture. The mix for the detection of *esp*, *cyl*, *hyl* genes contained 12.5 µl of PCR master mix (Yekta Tajhiz Azma, Iran), 10 mM of each primer. The mix for *ebp*, *asa1* and *efaA* was the same condition. The mix for the detection of *gelE* and *ace* contained 12.5 µl of PCR master mix (Yekta Tajhiz Azma, Iran), 1.5 mM-additional MgCl₂ and 10 mM of each primer. The mix for CRISPR1-*cas csn1*, CRISPR3-*cas csn1* and CRISPR1-*cas*, CRISPR3-*cas* and CRISPR2 contained 12.5 µl of PCR master mix (Yekta Tajhiz Azma, Iran), 1mM additional MgCl₂ and 10 mM of each primer. The amplification condition was carried out with the following thermal cycling conditions: initial denaturation at 95 °C for 10 min, 34 cycles of amplification consisting of 95 °C for 30s, 30s at 58 °C for *esp*, *cylA*, *hyl*, 58 °C for *efaA*, 56 °C for *gel*, *ace*, 52 °C for *ebpR*, *asa1*, 60 °C for all *cas* genes and 72 °C for 45s, with 72 °C for 5 min for the final polymerization. PCR products were analyzed by electrophoresis in a 1% agarose gel at 100 V for 1 h in 1X TBE buffer containing DNA safe stain. PCR products size were correlated with 100 based-pair DNA ladder (Yekta Tajhiz Azma, Iran) to confirm conjunction with their expected PCR amplicon size.

Statistical analysis

Spss software version 17.0 (Chicago, IL, USA) were used for statistical analysis. *T*-test and one-tailed Fisher's exact test was performed for data analysis. Significance was set at $P \leq 0.05$.

Results

In this study, 88 hospital-acquired and 73 dental-root canal isolates of *E. faecalis* were collected. The specimen sources of hospital-acquired were included urinary tract infection (UTI) (78, 88.6%), wound (7, 7.9%) and blood (3, 3.4%). The specimens were obtained from different wards including outpatients (35, 39.8%), intensive (23, 26.1%), intensive care units (ICU) (12, 13.6%), infectious ward (13, 14.8%), emergency ward (3, 3.4%), ear-nose-throat (ENT) (1, 1.1%), urology and nephrology (1, 1.1%). 42 (47.7%) isolates were from male and 46 (52.3%) from the female. The age ranges of patients were 2 months to 86 years with a mean of 39.04 years. In dental-root canal isolates, 49 (67.1%) of the isolates were obtained from male and 24 (32.9%) from the female. The age ranges of endodontic treatment patients were 12–66 years with a mean of 32.41 years. All isolates were investigated for the biofilm formation in which the minimum, maximum and average of biofilm formation were 0.054, 2.325 and 1.611, respectively. The

most of the isolates showed strong biofilm formation (94, 58.4%) and 10 (6.2%) isolates displayed no biofilm formation. Biofilm formation of hospital-acquired isolates was significantly higher than the dental root canal isolates ($P = 0.023$). Most of the isolates were shown no gelatinase activity (70.8%), which hospital-acquired isolates were significantly shown most gelatinase activity ($P = 0.001$). In addition, the most isolates were shown no hemolysis activity (80.7%), and all hemolysis activity was found in hospital-acquired isolates (19.2%). The most presence of the virulence genes among the isolates were *ace* and *efaA* genes (88.8% and 85.1%, respectively) and the lowest presence were *cylA* and *asa1* (7.5% and 14.9%, respectively). The presence of *gelE* (contributing gelatinase activity) and *cylA* (contributing hemolysis activity) were significantly associated with phenotype gelatinase and hemolysis activity, respectively ($P < 0.001$, $P = 0.013$, respectively). In addition, the presence of *efaA*, *cylA*, and *gelE* were significantly more in hospital-acquired isolates compared to dental-root canal ($P = 0.002$, $P < 0.001$, $P = 0.008$, respectively). By comparing the presence of virulence genes among the isolates, hospital-acquired isolates had higher virulence genes than dental root canal isolates ($P = 0.007$), which all isolates had at least one virulence gene. The number of virulence genes was 1 to 7 among the hospital-acquired isolates and 1 to 6 among the dental root canal isolates. Among the hospital-acquired isolates, the presence of 5 and 4 virulence genes were highest (36.4% and 30.7%, respectively) and the presence of 4 and 3 virulence genes were highest among the isolates of the dental root canal (39.7% and 31.5%, respectively). In addition, *efaA* and *gelE* positive isolates had higher biofilm formation than negative isolates in the all isolates ($P < 0.05$). The mean of biofilm formation of isolates with different virulence genes is presented in figure 1.

The occurrence of CRISPR-*cas* is shown in table 3. Overall, the presence of CRISPR1-*cas* loci in dental-root canal isolates (4 of 73) were lower than hospital-acquired isolates (17 of 88) ($P = 0.008$), whereas the presence of CRISPR3-*cas* in dental-root canal isolates (26 of 73) were higher than hospital-acquired isolates (2 of 88) ($P < 0.001$) and orphan CRISPR2 was no differences between hospital-acquired and dental-root canal isolates. None of the isolates had both CRISPR1-*cas* and CRISPR3-*cas*, as well as, CRISPR1-*cas*, orphan CRISPR2, and CRISPR3-*cas* at the same time. The isolates were more likely to harbor orphan CRISPR2 than CRISPR1-*cas* and CRISPR3-*cas*. In addition, the presence of orphan CRISPR2 was significantly associated with CRISPR1-*cas* ($P = 0.031$), whereas was not significant with CRISPR3-*cas*. At least one CRISPR-*cas* locus was found in 106 (65.8%) of the all isolates and the results showed the isolates containing high virulence genes tended to have more frequently investigated *cas* genes. overall, the absence of CRISPR1-*cas* and either CRISPR1 or CRISPR3 were significantly associated with the presence of *esp* gene ($P = 0.005$, $P = 0.033$). In addition, the presence of either CRISPR1-*cas* or orphan CRISPR2 and either CRISPR3-*cas* or orphan CRISPR2 were significantly associated with the presence of *ace* and absence of *gelE*, respectively ($P = 0.012$, $P = 0.052$, respectively). Other significant associations were found between the absence of CRISPR1 and absence of *cylA*, *hyl*, *gelE*, *asa1* ($P < 0.05$), between the absence of CRISPR2 and absence of *gelE* ($P = 0.001$). In hospital-acquired isolates, a significant association were found between absence of CRISPR loci and absence of *gelE*, *asa1*, gelatinase and hemolysis activity ($P < 0.05$), and in dental-root canal isolates, significant association were found between absence of CRISPR3-*cas* and absence of gelatinase ($P = 0.003$), between absence of

either CRISPR1-*cas* or CRISPR2-*cas* and absence of *gelE* ($P = 0.021$), and between presence of orphan CRISPR2 and either orphan CRISPR2 or CRISPR3-*cas* and biofilm production ($P = 0.046$, $P = 0.044$, respectively) (see the table 4).

Discussion

In this study, we determined the occurrence of CRISPR loci and content of virulence factors in *E. faecalis* strains, which were isolated from different infectious sources as a pathogenic organism and dental-root canal of patients. We found that the presence of CRISPR1 and CRISPR3 loci are varied among *E. faecalis* strains, which abundance of CRISPR1 among the dental-root canal were significantly lower than hospital-acquired, whereas the reverse was significantly true for CRISPR3. These results are consistent with Burley et al. study [33], which had found the presence of CRISPR3-*cas* were significantly more in endodontic strains compared to hospital-acquired strains and the majority of strains had CRISPR3. These results are interesting but the reasons are not clear.

The results reveal that the presence of CRISPR loci were not significantly associated with less number of virulence factors. There are several virulence factors in *E. faecalis*, which play a role as antiphagocytosis, adherence, biofilm formation, exoenzyme, toxin, and quorum sensing system. Although several studies reported that there is no clear relation of origin isolation or a single gene with pathogenicity, and perhaps surface proteins of *E. faecalis* cannot be considered as virulence factors [15, 34, 35]. However, we found a correlation between the absence of CRISPR1-*cas* and presence of *esp* gene and absence of CRISPR1-*cas* and proportionally absence of *cylA* and *asa1* genes. In addition, there was a correlation between the absence of single or multi-CRISPR loci and absence of some virulence factors. The cytolysin operon, *cob* and *esp* genes reside in the same pathogenicity island, which they are located on either the chromosome or on large pheromone-responsive plasmids such as pAD1 [36, 37]. The *esp* gene is encoded a large surface protein with a variable number of highly conserved 82 amino acids repeats and contributes to promotion primary attachment, colonization and biofilm formation of *E. faecalis* [37]. Our results showed that only *efaA* gene was significantly associated with biofilm formation in hospital-acquired strains and *esp*, *hyl*, *ace*, *asa1*, *ebpR* and *cylA* genes had not associated with biofilm formation. Conflicting outcomes have been published regarding the role of the genes of biofilm formation. Duggan et al. suggested that *asa1*, *cylA*, *esp* and *gelE* have not associated with biofilm formation in oral and endodontic isolates of *E. faecalis* [38], which is compatible with our results. In addition, the results revealed that the 13.6% of hospital-acquired isolates were carried *cylA* gene, but only 35.2% of the isolates were expressed hemolysin activity (both alpha and beta hemolysis). Several studies such as Sun et al. [39], Sedgley et al. [40] and Lindenstrauß et al. [41] determined 38%, 36% and 33.3% of the chronic periodontitis, endodontitis-associated and clinical and food isolates of *E. faecalis* to be capable of producing hemolysis, respectively. These differences may be due to differences in used types of blood for determination of hemolysis activity, which we used human blood but others were used horse and sheep blood. In addition, Sun et al. [39], Sedgley et al. [40] reported the distribution of *cylA* gene was detected only in 17% and 18.18% of the isolates, respectively, which were compatible with our results. These results may be due to environmental factors such as *in vitro* and *in vivo* conditions used to test for phenotypic

characters, which could strongly influence gene expression [42] and can be causes of differences our results with others in hemolysis activity. In addition, hemolysin activity was encoded by *cyl* operon in *E. faecalis*, where *cylA* is the only reading frame required for the expression of component A, a serine protease. As well as, there is no association between CRISPR1-*cas*, biofilm-formation and hemolysis activity. Several studies reported that CRISPR loci play an inverse role in some virulence factors and acquisition of antibiotic resistance [14, 33, 41] including Palmer and Gilmore's study [43] and Burley et al.'s study [33], which reported CRISPR loci were inversely associated with antibiotic resistance and some virulence factors in *E. faecalis* strains. In addition, similar to our results, Toro et al. [44] and Touchon et al. [45] reported that there is no significant association with CRISPR-*cas* and acquisition of integrons, plasmids, antibiotic resistance and virulence genes in *Escherichia coli*. However, an analysis of 370 other Archaeal and Eubacteria genomes concluded that there is potential evidence for the propagation CRISPR-*cas* genes to occur via horizontal gene transfer [46]. These findings suggested that CRISPR loci could potentially inhibit or prevent some or part of the virulence factors and Pathogenicity Island and could not be selective forces to influence pathogenic traits of *E. faecalis*.

Conclusion

Findings of this study indicate that CRISPR-*cas* might prevent the acquisition of some respective pathogenicity factors in some isolates but not all; inverse correlations were found significantly between CRISPR-*cas* loci, *esp* and *gelE*, and direct correlations were found in *cylA*, *hyl*, *gelE* (between some CRISPR-loci), *asa1*, *ace*, biofilm formation, gelatinase and hemolysis activities. However, CRISPR-*cas* could prevent the acquisition of antibiotic resistance genes in *E. faecalis* and other bacteria. Further studies can answer the exact role of CRISPR-*cas* in the pathogenesis of Enterococcal infections.

Declarations

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

This study was approved by the regional ethics committee of Tabriz (Tabriz University of Medical Sciences, Tabriz, Iran, No. IR.TBZMED.REC.1397.188).

Informed consent

For this study, formal consent was filled by all participants before any procedure according to ethics committee approved procedure.

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Conflict of Interest

None to declare

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Tables

Due to technical limitations, Tables 1 - 4 are only available for download from the Supplementary Files section.

Figures

Figure 1

biofilm formation by *E. faecalis* isolates according to the presence of virulence factors and CRISPR loci.

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