

Molecular Detection of Enterococcal Surface Protein (*esp*) Gene in *Enterococcus faecalis* Isolated from Dental Calculus of Patients in Sari, Iran

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Abstract

Background: Enterococci are important gram-positive bacteria causing dental calculus in human beings; however, the role of these bacteria in oral cavity is unclear. The aim of this study was to investigate the presence of Enterococcal Surface Protein (*esp*) gene in *Enterococcus faecalis* isolated from dental calculus in the city of Sari, Iran.

Materials and Methods: In the present study, 207 dental calculus samples were collected from patients. The isolates were identified by growth on Bile Esculin agar, Gram stain, Catalase test, Growth at 6.5% NaCl, PYR and arabinose fermentation test. Antimicrobial susceptibility pattern of the isolates was determined by disk agar diffusion method. The presence of *esp* gene was assessed by polymerase chain reaction (PCR).

Results: Among the 56 (27%) enterococci isolated from dental calculus, 43 (76.7%) were determined as *E. faecalis*. The resistance rate to ampicillin, vancomycin, tetracycline, ciprofloxacin and erythromycin in *E. faecalis* isolates was estimated as 13.9%, 4.6%, 11.6%, 6.9% and 13.9%, respectively. The *esp* gene was detected in 18.6% of *E. faecalis* isolates. Among the isolates containing *esp* gene, 33.3%, 50%, 40%, 33.3% and 33.3% of them were resistant to ampicillin, vancomycin, tetracycline, ciprofloxacin and erythromycin, respectively.

Conclusion: *E. faecalis* is an important organism causing dental calculus but the presence of *esp* gene had no correlation with the resistance to tested antimicrobial agents.

Keywords: *Enterococcus faecalis*; Dental Calculus; *esp* gene; PCR

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Introduction

Enterococci are gram-positive facultative anaerobic cocci that normally inhabit the gastrointestinal tract of humans and animals (1, 2). Moreover, these bacteria are found in other parts of the body such as oral cavity and vagina as well as in water, soil, food, plants and insects (2, 3). *Enterococcus faecalis* and *Enterococcus faecium* are the most common species causing human infections and frequently have been associated with nosocomial infections throughout the world (4). *E. faecalis* is not a normal flora of the mouth but has been observed in diseases such as dental caries, periodontitis and tooth root infections (1). Several virulence factors can cause the

accumulation of these bacteria and initiation of dental infections (4, 5). Enterococcal surface protein (ESP) is one of these virulence factors (2). The *esp* gene encodes ESP with iterative structure causing bacterial adhesion and biofilm formation. ESP is a high molecular weight superficial protein containing 1873 amino acids, which has N-terminals, central core, and C-terminal regions. The C-terminal domain contains a membrane hydrophobic region. Recently, it has been assumed that the N-terminal of ESP participates in interaction with the host and the central region of this protein has an important role in accumulation of the bacteria and hides the mentioned protein from the

host immune system (6, 7). Biofilm is a layer consisting of a mass of bacteria adhering to each other. This layer is composed of a polymer-extracellular matrix that is made by the bacteria (8). For example, Enterococcus form the biofilm in dental root canal which was filled previously (1). Biofilm causes bacterial protection against environmental changes, host immune response and antimicrobial agents, thus prevents the treatment of the infections caused by the biofilm producer bacteria (6, 9). About 80% of the bacterial infections are associated with biofilm production (10). *In vivo* and *in vitro* experiments demonstrated that minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) of biofilm-forming bacteria are about 1-1000 times higher than that of Planktonic cells, resulting in limitation in treatment of infections caused by these strains (8). Biofilm causes the bacterial colonization on medical instruments and dental surfaces. This leads to accumulation of other bacteria that produce acid due to the metabolism and initiation of dental calculus (8, 10-12). The aim of this study was to determine the prevalence of *esp* gene in *E. faecalis* isolated from dental calculus in patients referred to a dental clinic.

Materials and Methods

Sample collection

This study was conducted on 207 samples collected from non-repeated patients referred to the Mostafavian dental clinic in Sari, Iran. This project was explained to the patients and sampling was carried out with the consent of patients. The samples were taken by sterile swabs from the patients' dental calculus. Then, swabs were placed immediately in Brain hurt infusion (BHI) nutrient broth and transferred to the laboratory.

Table 1. The primer sequences used in this study.

Gene	Primer	Primer Sequence (5'→3')	Amplicon size(bp)	Reference
<i>esp</i>	esp 11 - esp 12	F: TTG CTA ATG CTA GTC CAC GAC C R: GCG TCA ACA CTT GCA TTG CCG AA	933	(16)

The amount of each primer, DNA and master mix (Amplicon, Denmark) used in this test was equal to 10 pmol, 300 ng/μl and 8 μl respectively, and the final volume of reaction was 15 μl.

The PCR process was carried out by an Eppendorf AG thermal cycler (Germany) as follows: initial denaturation at 94 °C for 1 min, followed by 30 cycles of denaturation at 94 °C for 45 sec, annealing at 60 °C for 60 sec, extension at 72 °C for 60 sec with a final extension at 72 °C for 5 min.

Distilled water and *enterococcus faecalis* ATCC 29212 were chosen as *esp* negative and positive control, respectively.

Microbiological and antimicrobial susceptibility testing

The samples were cultured on blood agar containing 5% sheep blood under sterile conditions, and incubated for 24 hours at 37°C. Morphological comparison with grown colonies of *Enterococcus faecalis* ATCC 29212 (Pasteur Institute of Iran) as a standard strain was used to confirm the suspected colonies of enterococci. The pure cultures of suspected colonies were sub-cultured on Bile Esculin agar, and incubated for 48 hours at 37°C. Moreover, Gram stain, catalase test, growth at 6.5% NaCl and PYR test were performed for early identification of enterococci (13). In this study, arabinose fermentation test was employed to differentiate *E. faecalis* and *E. faecium* (14).

The susceptibility pattern of the isolates against ampicillin, vancomycin, tetracycline, ciprofloxacin and erythromycin was determined by disk agar diffusion method according to the clinical and laboratory standards institute (CLSI) guidelines (15). *E. faecalis* ATCC 29212 was chosen as control strain in the antimicrobial susceptibility testing.

DNA extraction

Genomic DNA was extracted using a DNA extraction kit (Thermo Scientific, Waltham, Massachusetts, United States) according to the manufacturer's instructions.

Detection of the *esp* gene

PCR was used to detect the presence of *esp* gene in *E. faecalis* isolated from dental calculus. The sequences of primers used in the present study are shown in Table 1.

Electrophoresis

The PCR products along with a 100 bp DNA Ladder and DNA of *esp* positive control were electrophoresed on 2% agarose gel (Figure 1). The results were observed with gel documentation device (Vilber lourmat, France), after staining with safe stain (Aryatous, Iran).

Statistical analysis

Data were analyzed using Statistical Package for Social Science (SPSS, version 22) software. The binomial test was used for analysis of the data and *P*-value < 0.05 was statistically significant.

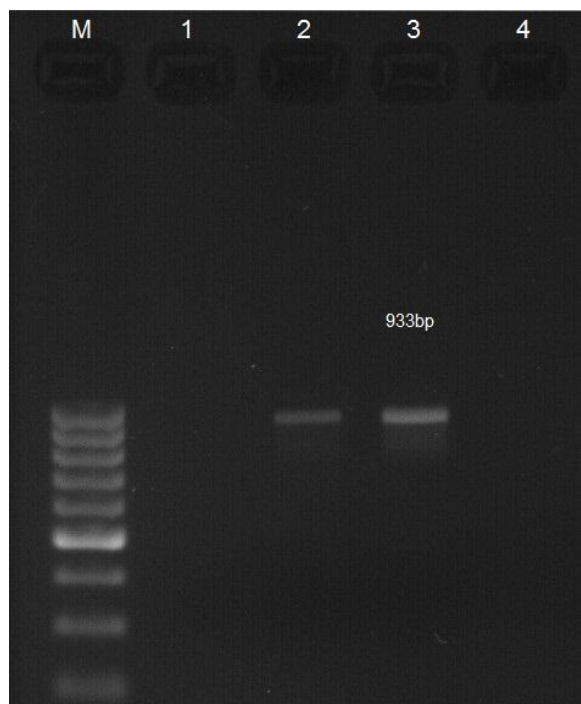


Figure 1. M: DNA marker (100bp); Line 1: negative sample; Line 2: positive sample; Line 3: positive control; Line 4: negative control.

Results

According to the standard microbiologic tests (13), 56 (27%) of dental calculus isolates were identified as Entrococci. Among these isolates, 43 (76.7%) of

them were confirmed as *enterococcus faecalis* and others were identified as *enterococcus faecium*. In this study, 13.9%, 4.6%, 11.6%, 6.9%, and 13.9% of the isolates were resistance phenotypes against ampicillin, vancomycine, tetracycline, ciprofloxacin and erythromycin, respectively. The molecular assay showed that 8 (18.6%) *enterococcus faecalis* isolates were *esp* positive. Interestingly, none of these patients used toothbrush and one patient had denture. There was no significant correlation between the presence of *esp* gene and the sex of the patients ($P > 0.05$). Also, the presence of this gene had no significant correlation with the resistance rate against ampicillin, vancomycin, tetracycline, ciprofloxacin and erythromycin (Table 2).

The association between the isolation of *enterococcus faecalis* and some problems which are related to the patients is shown in Table 3.

Discussion

The present study showed that *E. faecalis* was more prevalent than *E. faecium* in the samples collected from dental calculus. There was no significant relationship between the variables of questionnaire in patients and *E. faecalis* prevalence rate. However, there was an important correlation between antibiotic usage and the prevalence of *E. faecalis* (P -value < 0.01).

Table 2. Antimicrobial susceptibility results and its correlation with the presence of *esp* gene in *enterococcus faecalis* isolates.

Antibiotics		Susceptibility pattern of the all isolates No. (%)	No. (%) of positive isolates	No. (%) of negative isolates	P-value
Ampicillin	R	6 (13.9%)	2 (33.3%)	4 (66.6%)	$P > 0.05$
	I	-	-	-	
	S	37 (86.04%)	6 (16.2%)	31 (83.7%)	
Vancomycin	R	2 (4.6%)	1 (50%)	1 (50%)	$P > 0.05$
	I	13 (30.2%)	2 (15.3%)	11 (84.6%)	
	S	28 (65.1%)	5 (17.8%)	23 (82.1%)	
Tetracycline	R	5 (11.6%)	2 (40%)	3 (60%)	$P > 0.05$
	I	3 (6.9%)	1 (33.3%)	2 (66.6%)	
	S	35 (81.3%)	5 (14.2%)	30 (85.7%)	
Ciprofloxacin	R	3 (6.9%)	1 (33.3%)	2 (66.6%)	$P > 0.05$
	I	8 (18.6%)	2 (25%)	6 (75%)	
	S	32 (74.4%)	5 (15.6%)	27 (84.3%)	
Erythromycin	R	6 (13.9%)	2 (33.3%)	4 (66.6%)	$P > 0.05$
	I	1 (2.3%)	-	1 (100%)	
	S	36 (83.7%)	6 (16.6%)	30 (83.3%)	

Abbreviations: R, resistant; I, intermediate resistant; S, susceptible.

Antimicrobial susceptibility testing showed that *E. faecalis* isolated from dental calculus had low levels of resistance to ampicillin (13.9%), vancomycin

(4.6%), tetracycline (11.6%), ciprofloxacin (6.9%), and erythromycin (13.9%).

Table 3. Association of enterococcus faecalis isolation with patients' underlying problems.

<i>E. faecalis</i>	Dental infection	Gastrointestinal diseases	Endoscopy	Taking antibiotics	Filled teeth	Extracted teeth
Positive (43 isolates)	41 (95.3%)	8 (18.6%)	4 (9.3%)	11 (25.6%)	19 (44.2%)	37 (86%)
negative (164 isolates)	160 (97.6%)	32 (19.5%)	29 (17.8%)	75 (45.7%)	83 (50.6%)	138 (84.1%)

The prevalence of *esp* gene in our isolates was lower than other studies (1, 2, 4, 17-19), and that may be due to geographical differences or various clinical samples which were used in their studies. Moreover, the resistance rate to ampicillin, vancomycin, tetracycline, ciprofloxacin and erythromycin in our study had no significant correlation with the presence of *esp* gene. However, some studies have reported a high prevalence of this gene (17, 18). According to a study conducted from 2008 to 2010 in Iran (17), the prevalence of *E. faecalis* isolated from urinary tract infections was 73.4% while 47.1% of the isolates contained the *esp* gene. This difference in the prevalence of *esp* gene may be due to different samples used in the two studies. The above-mentioned study (17) also showed that 64%, 97%, and 100% of their vancomycin-, ampicillin- and ciprofloxacin-resistant isolates contained the *esp* gene, while we did not find any correlation between them. However, the resistance rate to ampicillin in our study was higher than that of their isolates. These data show that the presence of *esp* gene may have a significant correlation with the resistance to these antibiotics in their study. However, the production of biofilm due to the presence of *esp* gene can facilitate the acquisition of some antibiotic resistance genes (17). Another study from Iran (18) has reported that 16.5%, 16.3%, 87.8%, 43.9% and 65.3% of their *E. faecalis* isolates were resistant to ampicillin, vancomycin, tetracycline, ciprofloxacin and erythromycin, respectively. However, 74.6% of their isolates contained the *esp* gene. This was probably due to different clinical samples used in their study. This difference in the prevalence of *esp* gene was in concordance with other studies conducted in Bulgaria and Brazil, which used various clinical samples (2, 18, 19). The high prevalence of *E. faecalis* encoding this gene in different areas may be dependent on the high prevalence of this organism in animals and human carriers, which can be as reservoirs of these bacteria.

The prevalence rate of *esp* gene in *E. faecalis* isolated from clinical and saliva/plaque samples collected in Germany (1), was 60% and 86.5%, respectively.

Moreover, the prevalence rate of this gene in endodontic samples in the mentioned study was higher than that in our study (38.1% vs. 18.6%).

E. faecalis is associated with various dental diseases and can lead to oral biofilm formation (1). The pathologic role of several virulence factors identified in *E. faecalis* is still under discussion and the function of these factors is unknown in the clinical isolates (1). A study carried out in Chile (20) investigated different clinical samples showed that the prevalence of *esp* gene in their *E. faecalis* isolated from urinary infection and bacteremia was 42% and 52%, respectively, however this gene was not found in endodontic isolates. The results of these studies on *E. faecalis* indicate that the prevalence rate of this gene is different in various clinical samples. Current knowledge on the features of virulence factors in the pathogenesis of infections caused by this bacterium is still limited. The production of biofilm in this bacterium is dependent on multiple genes such as, *epa*, *atn*, *fsr*, *srtA*, *srtC*, *ebpA*, *ebpB*, and *ebpC*, suggesting that more comprehensive studies should be conducted on this subject.

Conclusion

The presence of *esp* gene is not the only cause of the acquisition of resistance genes due to biofilm formation. Antibiotic resistance is associated with several mechanisms of which the most important mechanism is to obtain the resistance genes from other bacteria present in the biofilm. Considering that none of the patients who were positive for *esp* in *E. faecalis* isolate used toothbrush, it is reasonable to expect that oral hygiene plays a major role in preventing biofilm formation.

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