

Evaluation of Real-time PCR-based DNA melting method for detection of *Enterococcus faecalis* and *Enterococcus faecium* in clinical isolates

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ABSTRACT

BACKGROUND AND OBJECTIVE: Human error and timelessness in conventional techniques to identify species *Enterococcus faecium* and *Enterococcus faecalis* are two species of the pathogenic species in humans, more accurate techniques is essential. The aim of this study is to design a method of quickly and accurately using DNA melting by Real Time PCR technique to identify and separate the two species in clinical isolates.

METHODS: In this experimental study, the bacterial isolates in the Department of Microbiology Bank of Hamadan University of Medical Sciences was used. Design of primers was done by proprietary software and selecting *DivIVA* gene for *Enterococcus faecalis* and *alanine racemase* for *Enterococcus faecium* was performed. Isolates identification was evaluated by using Real Time PCR test and melting curve temperature of DNA.

FINDINGS: Susceptibility of primers designed in *divIVA* gene (specific for *E. faecalis*) and *alanine-racemase* gene (specific for *E. faecium*) was 15CFU/ml per reaction. Specificity of designed primers by using DNA melting curve analysis was 76.6 for *E. faecalis* and 80.93 for *E. faecium* which showed considerable different in comparison with another microorganism.

CONCLUSION: Using the results obtained in this study, primers designed were sensitivity and specificity for diagnosis and differentiation of *Enterococcus faecium* and *Enterococcus faecalis* species in clinical isolates.

KEY WORDS: *Enterococcus faecalis*, *Enterococcus faecium*, Real Time PCR

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Introduction

Enterococci are gram-positive cocci that cause endocarditis and urinary infections, bloodstream infections, meningitis, infection related to babies, and intra-abdominal and pelvic infections (1).

There are at least 12 different species and the prevalence of two highly pathogenic species, *Enterococcus faecalis* (more than 70%) and *Enterococcus faecium* (more than 15%), constitute *Enterococci* isolated from human (2). *Enterococcus faecalis* is one of the most important causes of nosocomial infections worldwide and the involvement of this bacterium in the incidence of *Enterococci* has given great importance to this bacteria (3). Although *Enterococcus faecium* has smaller share in pathogenicity compared with *Enterococcus faecalis* (3, 4), it is a common cause of Enterococcal infections in patients with urinary or intravenous catheters and in patients who have been hospitalized for a long period and received various types of antibiotics (5, 6).

The potential ability of these bacteria to transfer the antibiotic resistance through mutation or receiving foreign genetic factors such as plasmids or transposons has made *Enterococcus* a serious factor in the development of resistant infections in other bacterial species (7–9). The biochemical tests for carbohydrates, culture in specific media such as Skim Milk Agar or commercial detection systems are the most common methods for identification of various *Enterococcus* species (3).

These methods are accompanied by problems in specific detection of strains in terms of time, complexity and errors (3). Therefore, efforts have been made for identification of this bacterium based on PCR methods for specific sequence of bacteria (10). In molecular methods, the bacterial species were identified through targeting specific genes of bacteria as target site and designing specific primers for the identification of these genes with appropriate sensitivity and specificity (11). One of the most sensitive methods for quick identification of this bacterium is real-time PCR-based DNA melting

method (melting curve analysis) (12). In this test, the accurate identification is based on the relationship between temperature and the range of DNA denaturation. DNA denaturation with increased temperature specifies the melting curve, which is usually sigmoid (13). *Enterococcus faecium* and *Enterococcus faecalis* species can be identified by specific genes that are active in special routes. *DivIVA* is a specific gene in *Enterococcus faecalis*, which participates in cell division and chromosome segregation (14). *Alanine racemase* also plays a significant role in biosynthesis of peptides and glycans and L- and D-alanine exchange inside *Enterococcus faecium* (15).

All these issues increased the significance of identifying this bacterium in primary nosocomial infections. Designing an accurate, fast and cost-effective method can help identify species with homologically high affinity in addition to identifying the type of bacteria. This study aims to design a fundamental method for fast and accurate identification of *Enterococcus faecium* and *Enterococcus faecalis* in clinical isolates based on DNA melting diagrams.

Methods

Designing primers: The target sites selected for *Enterococcus faecalis* and *Enterococcus faecium* primers were *divIVA* (the gene responsible for cell wall division) and alanine racemase (the gene responsible for the synthesis of the amino acid alanine), respectively. After selecting target sites for designing primers, databases related to each bacterium were prepared from NCBI website.

To assess T_m and annealing temperature of the designed primers, Oligo 6, Oligoanalyzer and Gene Runner were used (Table 1). After designing the aforementioned primers, to ensure the specificity of their function, they were blasted with human, fungal and viral samples and other microorganisms in NCBI database.

Table 1. Primers designed for the identification of *Enterococcus faecium* and *Enterococcus faecalis*

Size(bp)	Length of sequence	Target genes	Bacterium
123	F: ACGTGTCTTCCATCAACGCT	F. <i>divIVA</i>	<i>Enterococcus faecalis</i>
	R: ACTGCTGTATGTTTGTCTCCGA	R. <i>divIVA</i>	
248	F: ATCCCTCTGGGCACGCAC	F. <i>Alanine racemase</i>	<i>Enterococcus faecium</i>
	R: ACATACACGCCCAATCGTTTC	R. <i>Alanine racemase</i>	

Microorganisms used for quality assessment: In order to perform analytical tests, the clinical and standard strains of *Enterococcus faecalis* (ATCC 29212) and *Enterococcus faecium* (confirmed by sequencing) used as positive control and *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 29213), *A.baumannii* (ATCC 19606), *P. aeruginosa* (ATCC 27853) and *K. pneumonia* (ATCC 13882) used as negative control were prepared from microbial collection of Hamedan University of Medical Sciences.

Genomic DNA (gDNA) extraction: DNA extraction was done through boiling method. Based on this method, one colony of each strain was boiled in 100 µl vortexed sterile PBS solution for 15 minutes. After final centrifugation, the supernatant was used as DNA. Concentration and optical density of the purified DNA was determined using NanoDrop® ND-1000 Spectrophotometer (BioRad, USA) at wavelengths of 260 and 280 nm (15).

PCR reaction: To have a PCR reaction for each sample, 12 lambda Master Mix RED (Ampliqon, Germany) (containing Tris-HCl PH8.5, (NH₄)SO₄, 3mM MgCl₂, 0.2% Tween20, 4 mM dNTP 4, 2.0 unit Ampliqon polymeras, Insert red dye and stabilizer), 1 lambda of the extracted DNA and 1 lambda of each primer (10 pM) were poured into RNAas/DNAas free microtubes 0.2 ml (BioFil, South Korea) and distilled water was added to have a final volume of 25 lambda. Thermocycler (Eppendorf, Germany) with temperature cycle regulations as thermal shock 94 °C for 60 seconds and 25 cycles as 94 °C for 30 seconds and 72 °C for 30 seconds was used for proliferation of target genes. The annealing temperature for *Alanine racemase* and *divIVA* genes were 57 °C and 59 °C, respectively for 60 seconds. The final elongation was done at 72 °C for 7 minutes.

Electrophoresis on agarose gel 1.5%: The PCR products were separated by electrophoresis using agarose gel 1.5%; 5 µl of the final PCR product was electrophoresed in agarose gel 1.5% in buffer (0.5 X). To stand the gel, 2 µl Gel Red (Biotium, USA) was added and mixed well. Fermentas molecular marker (Thermofisher, Denmark) with sequence of 100 bp was used to determine the size of products. Finally, the obtained gel was photographed by FlashGel™ Dock.

Determining the sequence of *Alanine racemase* and *divIVA* products: After designing the target primers in this study, the PCR products were sent to the Korean company by Takapouzist Co. to ensure the accuracy of

sequences determined by the primers. The results of sequencing were used for aligning and evaluating the homology of target primers in bacterial species. Moreover, the results of sequencing the designed primers were used as the gold standard method to determine the specificity of primers.

Real-time PCR reaction: ABI TaqMan® One-Step RT-PCR (USA) was used in this study. The temperature program was performed in three stages. The first stage, which leads to denaturation of the DNA template and activation of polymerase enzyme, was conducted at 90 °C for 5 minutes. In the second stage, DNA amplification reaction continued in 30 cycles at 95°C for 15 seconds and 58 °C for 45 seconds. The final stage was conducted to draw dissociation curve or melting curve at 95 °C for 15 seconds, 55 °C for 1 minute and 95 °C for 15 seconds. Real-time PCR reactions were done in a final volume of 20 µl in 96-well plates in duplicate. The reaction mixture included 10 µl Mix Master PCR SYBR Green (TaKaRa, Japan). 1µl forward and reverse primers (10 pM concentration), 5 µl DNA template, 0.4 µl Rox color and distilled water was added to reach the final volume of 20 µl.

Determining the sensitivity of the designed primers: In order to evaluate the sensitivity of the designed primers using bacterial standard of half McFarland (1.5×10^8 CFU/ml), a bacterial dilution of 10^1 to 10^7 was prepared. Then, DNA was separately extracted from each of the prepared dilutions and real-time PCR test was conducted in three consecutive days for the prepared dilutions in triplicate and the sensitivity of the test was achieved based on the repeatability of the designed test.

Determining the specificity of the designed primers: In order to do a specificity test for the primers prepared from DNA strain, *Enterococcus faecalis* (ATCC 29212) was used as positive control and *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 29213), *A.baumannii* (ATCC 19606), *P. aeruginosa* (ATCC 27853) and *K. pneumonia* (ATCC 13882) were used as negative control.

Moreover, for final confirmation of the specificity of the test, *Enterococcus faecium* DNA and *Enterococcus faecalis* Primer were used and *Enterococcus faecalis* DNA was used for *Enterococcus faecium*. For isolating different *Enterococcus species*, melting curve analysis was used. At the end of the reaction, the standard curve was drawn based on logarithm of the concentration of DNA

of horizontal axis (cycle threshold) and vertical axis. The standard curve slope was used to calculate the efficiency of proliferative response.

Data analysis: The collected data were analyzed using SPSS Ver.19. For this purpose, descriptive statistical methods (determining the frequency and average) were used. Moreover, Chromas Ver. 2.51 was used to analyze the results of sequencing of primers designed.

Results

Results of determining the sensitivity of primers:

Finding the concentrations at 10^1 to 10^7 dilutions (Fig 1,2) specified that the primers designed for *Enterococcus faecium* can identify 15 CFU bacteria and the primers designed for *Enterococcus faecalis* can identify 15 CFU bacteria (Fig 3).

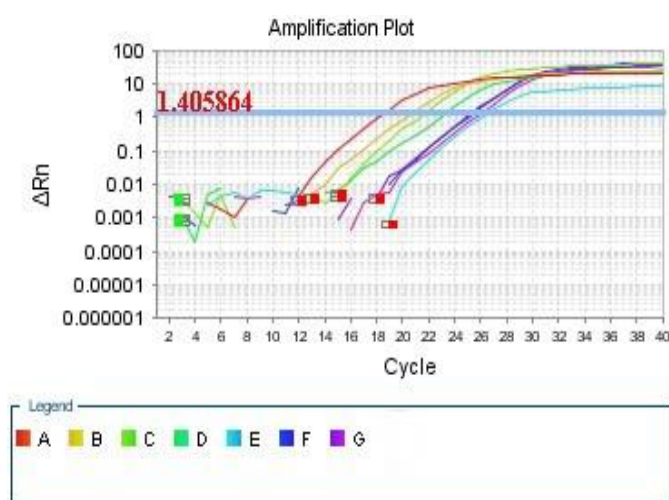


Figure 1. Threshold curve in different dilutions to determine the sensitivity of *Enterococcus faecalis* primers. The number of threshold cycles was shown on the horizontal axis, while changes in the fluorescence received (ΔRn) were shown on the vertical axis. Diagram A shows the sensitivity temperature of the designed primers at 10^7 dilution. Diagram B is related to 10^6 dilution, diagram C is related to 10^5 dilution, diagram D is related to 10^4 dilution, diagram E is related to 10^3 dilution, diagram F is related to 10^2 dilution and diagram G is related to 10^1 dilution.

Results of determining the specificity of designed primers:

The curve of *divIVA* gene amplification in *Enterococcus faecalis* showed a temperature of 76.6°C in all dilutions and the curves of *Alanine racemase* gene amplification in *Enterococcus faecium* showed a

temperature of 80.93°C . The results of primer–BLAST temperature performed in NCBI database were consistent with thermal results abstained from melting curves at the end of the reaction (Fig 4). The melting curve of *Staphylococcus aureus* and *Escherichia coli* for the two studied species were used for negative control (standard diagram). The temperature obtained at the end of the reaction and the created curves indicated failure to identify primers used in this study for other bacteria (table 2).

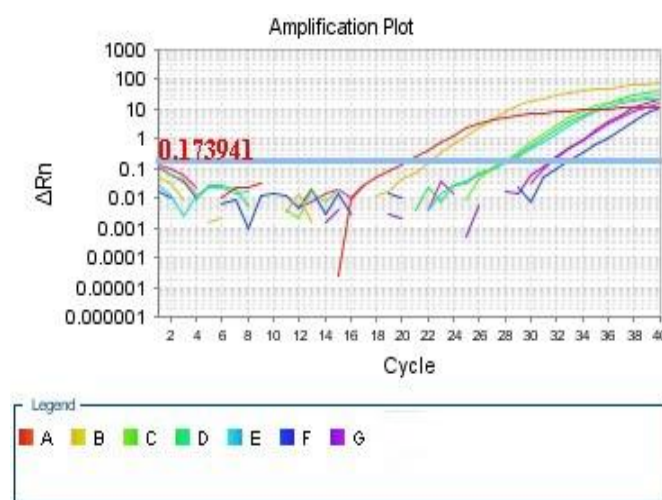


Figure 2. Threshold curve in different dilutions to determine the sensitivity of *Enterococcus faecium* primers. The number of threshold cycles was shown on the horizontal axis, while changes in the fluorescence received (ΔRn) were shown on the vertical axis. Diagram A shows the sensitivity temperature of the designed primers at 10^7 dilution. Diagram B is related to 10^6 dilution, diagram C is related to 10^5 dilution, diagram D is related to 10^4 dilution, diagram E is related to 10^3 dilution, diagram F is related to 10^2 dilution and diagram G is related to 10^1 dilution.

Results of Gel Electrophoresis: To assess the quality of the amplified genomic fragments in Real-time PCR reaction, the reaction products were uploaded in gel electrophoresis 1%. A 100 bp molecular model was used in the abovementioned reaction and 123 bp bands related to successful amplification of *divIVA* genes in *Enterococcus faecalis* and 248 bp bands related to successful amplification of *Alanine racemase* genes in *Enterococcus faecium* were obtained (Fig A3, B3).

The results of BLAST for *divIVA* gene amplification in *Enterococcus faecalis* and *Alanine racemase* gene amplification in *Enterococcus*

faecium: In a Real-time PCR test to determine the specificity of the primers, *Enterococcus faecium* and *Enterococcus faecalis* DNA were used separately along with two designed primers to find the specificity of primers for the species. In this case, *Enterococcus faecalis* primers could not identify *Enterococcus faecalis* and *Enterococcus faecalis* primer could not identify *Enterococcus faecium*. Finally, the results

were obtained as melting curves with different temperatures; when *Enterococcus faecium* DNA and *Enterococcus faecalis* primer were used, the nonspecific temperature was found to be 77.04 °C. The specificity of primers was determined based on the temperatures obtained from *Enterococcus faecium* primers (80.93 °C) and *Enterococcus faecalis* primers (76.6 °C) (Fig 4).

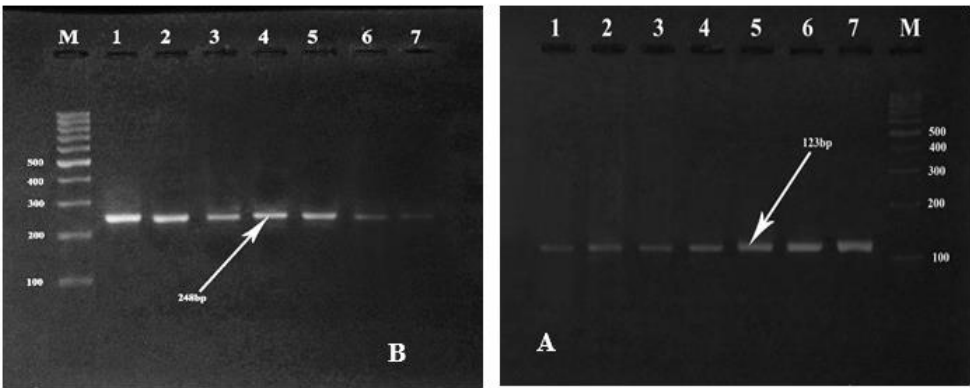


Figure 3. A: Picture of gel electrophoresis 1% related to amplification of 123 bp *divIVA* gene in *Enterococcus faecalis*. Well 1: 10⁷ dilution. Well 2: 10⁶ dilution. Well 3: 10⁵ dilution. Well 4: 10⁴ dilution. Well 5: 10³ dilution. Well 6: 10² dilution. Well 7: 10¹ dilution. Well M: 100 bp molecular marker. **B:** Picture of gel electrophoresis 1% related to amplification of 123 bp *Alanine racemase* gene in *Enterococcus faecium*. Well 1: 10⁷ dilution. Well 2: 10⁶ dilution. Well 3: 10⁵ dilution. Well 4: 10⁴ dilution. Well 5: 10³ dilution. Well 6: 10² dilution. Well 7: 10¹ dilution. Well M: 100 bp molecular marker.

Table 2. Results of determining the concentration and OD in various dilutions of DNA in *Enterococcus faecium* and *Enterococcus faecalis* bacteria using Nanodrop device

<i>Enterococcus faecium</i>				<i>Enterococcus faecalis</i>			
Positive control		Negative control		Positive control		Negative control	
<i>Enterococcus faecium</i>		<i>Staphylococcus aureus</i>		<i>Enterococcus faecalis</i>		<i>Escherichia coli</i>	
Melt	CT	Melt	CT	Melt	CT	Melt	CT
80.93	20.3	73.09	28.34	76.6	16.14	72.44	28.3

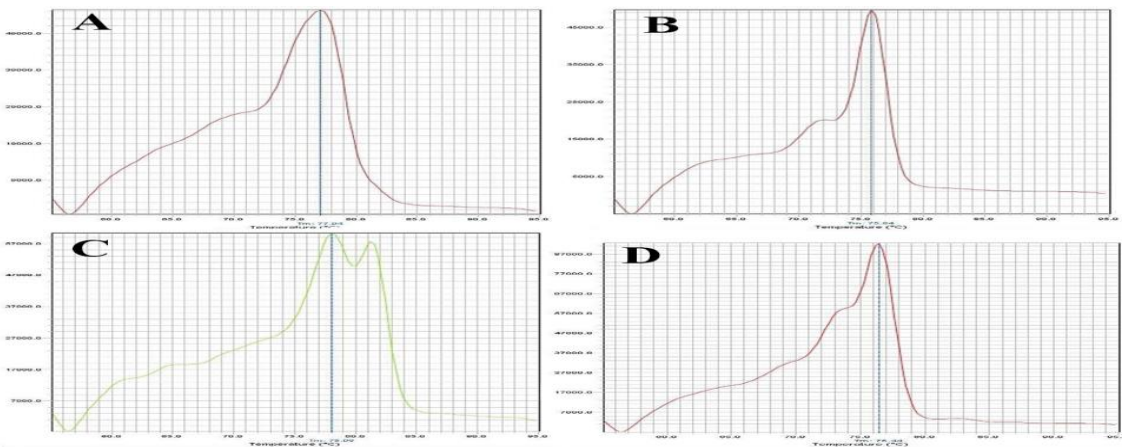


Figure 4. Melting curve of DNA obtained from Real – time PCR reaction to determine the specificity of the designed primers. A: *Escherichia coli* to provide negative control for the specificity of *Enterococcus faecalis* primer. B: *Staphylococcus aureus* to provide negative control for the specificity of *Enterococcus faecium* primer. C: *Enterococcus faecalis* and *Enterococcus faecium* primer. D: *Enterococcus faecium* and *Enterococcus faecalis* primer.

Discussion

According to the results of melting curve analysis of *Enterococcus faecium* and *Enterococcus faecalis* DNA, the best temperatures for these two bacteria were 76.6°C and 80.8°C, respectively. This temperature was consistent with the blasted primers. In similar experiments, Real-time PCR-based melting curve analysis was used as one of the most accurate and sensitive identification techniques (16). The significance of *Enterococcus* detection as an important acquired pathogen in the society and hospital (second most common nosocomial infection), particularly in intensive care units, is a new challenge.

These bacteria cause infections such as urinary tract and wound infections, bacteremia, sepsis and endocarditis (16–18). Biochemical tests such as initial detection with a variety of culture media or commercial detection systems are the common methods for detecting this bacterium. Such methods are faced with difficulties in terms of time, complexity and specific detection of strains. To overcome these problems, efforts were made to use Real-time PCR-based method for detection of the specific sequences of the bacteria (19).

In the investigations by Woksepp et al. using melting curve analysis of DNA of various bacteria, the categorization of bacteria was done with the highest sensitivity. In this study, the results of optimized MCA technique were compared with MLST technique and the results were fairly acceptable (19). Considering the results obtained from phenotypic methods in determining *Enterococcus faecium* and *Enterococcus faecalis* species, a high level error can be observed in addition to high cost (16). This becomes more pronounced when in some cases, interspecies homology in this family prevents detection using various types of sugar and may have various false negative and false positive results. In the present study, the sensitivity of the designed primers was found to be 15 CFU/ml for identification of both species of *Enterococcus faecium* and *Enterococcus faecalis*. In the studies by Cha et al. to identify vancomycin-resistant enterococci strains using melting curve analysis of DNA, it was found that we can use this

method for categorization of resistant strains in one genus or even one species due to the high sensitivity of this method (20). The diagnostic value of Real-time PCR-based melting curve analysis in cases where there are close interspecies homology can differentiate the species with close homology. This was clearly observed in the studies by Choong-Hwan Cha et al., which ultimately managed to identify *Enterococcus faecium* and *Enterococcus faecalis*. The results of this study were consistent with the present study and similar results can be observed in both studies. However, one needs to consider that in Real-time PCR-based melting curve analysis, special attention has to be paid to primer designing. In the studies by Krawczyk et al., Melting Profile was used besides the PFGE method to increase the sensitivity (11). Considering the results obtained from the sequencing of the designed primers in the present study, it can be concluded that these primers benefit from acceptable properties and sensitivity and can easily identify the purified DNA samples of various *Enterococcus* species with similar cultural and biochemical properties. A study by Martín et al. on various species of *Enterococcus* using melting curve analysis based on restriction enzymes, targeted S16 and S23 sites and the occurrence of similar homology in some cases led to false negative and false positive reports (12).

However, using more sensitive techniques such as Real-time PCR can improve false negative and false positive results (21). Based on the results of this study, the sensitivity of primers designed for identification of various species of *Enterococcus* that underwent DNA purification process, gained a fairly acceptable diagnostic value. However, we should not forget the fact that choosing an appropriate target site can highly influence the results of the study.

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