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Rapid Identification of *Enterococcus Faecalis* Isolates by Polymerase Chain Reaction

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ABSTRACT

BACKGROUND AND OBJECTIVE: Rapid detection of *Enterococcus faecalis* as a frequent cause of nosocomial infections in immunocompromised patients is an important issue. Herein, the current study developed a PCR assay based on the *ef0737* gene to detect *E. faecalis* isolates.

METHODS: In this cross-sectional study, 150 clinical isolates including *E. faecalis*, *E. faecium* and *Staphylococcus aureus* were collected. A set of pair primers was designed using the sequence of *ef0737*. All isolates were examined for the presence of *ef0737* gene by PCR assay. The sensitivity of PCR assay was evaluated according to 50 clinical isolates of *E. faecalis*. The specificity of PCR primers was also determined using non-*E. faecalis* species including 50 *E. faecium* and 50 *S. aureus* isolates.

FINDINGS: In this study, from 150 clinical isolates that were collected; all the 50 *E. faecalis* isolates showed positive results for the *ef0737* gene which showed 100% sensitivity. No amplification were observed in other isolates include *E. faecium* and *S. aureus*.

CONCLUSION: PCR assay is a more efficient and sensitive tool for detection and characterization of *E. faecalis* especially in patients with the critical condition. Identification of the preserved *ef0737* gene in clinical samples may be able to determine infections caused by *E. faecalis*.

KEY WORDS: *Enterococcus faecalis, ef073, PCR.*

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Introduction

Enterococcus faecalis as the most important species of Enterococcus genus is a part of the human gut microbiota and frequent cause of serious infections in hospitalized patients, including surgical wound infections, urinary tract infections, bacteremia and endocarditis (1,2). According to epidemiological reports, enterococci have now become one of the major nosocomial pathogens, with a mortality rate of more than 60% among patients (3) Therefore, rapid and correct identification of *E. faecalis* in the clinical specimens is essential for initiation of treatment and reducing the spread of multidrug-resistant *E. faecalis* (4). Traditional methods for detection of *E. faecalis* are based on phenotypic and culture characteristics (5). Phenotypic methods demonstrated to have several limitations and errors with respect to laboratory microbiological diagnosis. several studies showed misidentification of *E. faecalis* with other gram-positive cocci. So all of this evidence can represent a drawback for the treatment of patients and infection control in hospitals (6, 7).

In recent years, molecular methods have been introduced to increase the diagnostic sensitivity of detection. PCR-based detection test is a widely applied and accepted method for identifying bacterial infection (8). This method can serve as an alternative to conventional methods to time-saving and decrease of misidentification (9). In various studies, several genes were used for molecular detection of *E. faecalis* in clinical specimens. Some of these genes such as *rrs* and *ddls* are diagnosis markers for *E. faecalis* that used in blood samples (10). Some other virulence genes such as *gelE*, *esp*, *hly* and *efa* were examined, although their presence in all isolates of *E. faecalis* was not confirmed (11-13). In this study, we applied the PCR assay based on *ef0737* gene. This gene which encodes the EF0737 protein (14) that has not been examined so far. Our bioinformatic studies have shown that preserved *ef0737* gene exists in all strains of *E. faecalis* with a significant sequence. Therefore, it seems a new genetic marker that is preserved and exists in all isolates of *E. faecalis* can be useful for the clinical detection of such bacterial species. the aim of this study was to investigate the efficacy of *ef0737* gene in the molecular identification of *E. faecalis* isolates.

Methods

Bacterial Isolates: This study was approved by the Ethics Committee of the Tehran University of Medical Sciences with ethics code: IR.TUMS.SPH.REC.1396.2067. In this cross-sectional study, a total of 150 clinical isolates (approved by phenotypic methods) referring to pathobiology laboratories of Tehran University of Medical Sciences were collected from different clinical sources such as blood, urine and wound infection during April to August 2016. These isolates were including 50 *E. faecalis*, 50 *E. faecium*, and 50 *Staphylococcus aureus.* Since no study has been done on this gene and its presence in *S. aureus* and *E. faecium*, the ratio of the *ef0737* gene in each bacterium was considered 0/5 and the number of isolates were determined with 95% confidence and a maximum error of 15%. Isolated numbers for each bacterium was obtained from the relative formula.

DNA extraction: Genomic DNA of isolates was extracted using a DNA extraction kit (Bioneer, Seoul, South Korea,) according to the manufacturer's instructions and was used as template for PCR amplification.

Optimization of Polymerase Chain Reaction: The specific primers to amplify the *ef0737* genes were analyzed and designed according to *ef0737* gene information in the GenBank database. A 26-bp forward primer (5′-ATGTCTAAATTTTTAAAAGTAATCGG-3′) and 25-bp reverse primer (5′-CTGCTCATCTCTATTTATTTTTTTA-3′) were used in PCR to obtain a 1587-bp product.

A standard strain *E. faecalis* ATCC 29212 and reaction without DNA were used as a positive and negative control, respectively. The positive resulting product was sent to Takapouzist Company for sequencing. The PCR method was conducted on the total volumes of 25 µL using 12 µL of mixed mastermix (Sinaclon, Iran), 2 µL of DNA, 1 µL of each primer (20pmol/µl), and 9 µL of double distilled water (D.D.W). DNA amplifications were performed with the following temperature profiles: 1 cycle of 95°C for 5 min; 30 cycles of 95°C for 60 s, 63°C for 45 s, 72°C for 90 s; and finally 1 cycle of 72°C for 10 minutes. The PCR products were analyzed on 1% agarose gel electrophoresis (90 v, 2 hrs).

Sensitivity and Detection Limit of PCR assay: A 10-fold serial dilution (range: 10^{-1} to 10^{-9} of DNA) of the DNA was prepared in deionized distilled water (D.D.W), and PCR assays were carried out using different DNA concentrations. The sensitivity of PCR assay was also evaluated by 50 clinical isolates of *E. faecalis.*

Specificity of PCR Primers: The specificity of the PCR assays was evaluated using non-*E. faecalis* species including *E. faecium* and *S. aureus.*

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Results

Optimization of PCR: In all, 150 isolates of *E. faecalis*, *E. faecium*, and *S. aureus* were used in the current study and subjected to PCR amplification for the 1587 bp-*ef0737* gene. Our results showed that this gene was detected in all *E. faecalis* isolates and none of *S. aureus* and *E. faecium* isolates was harbor this gene (Fig1).

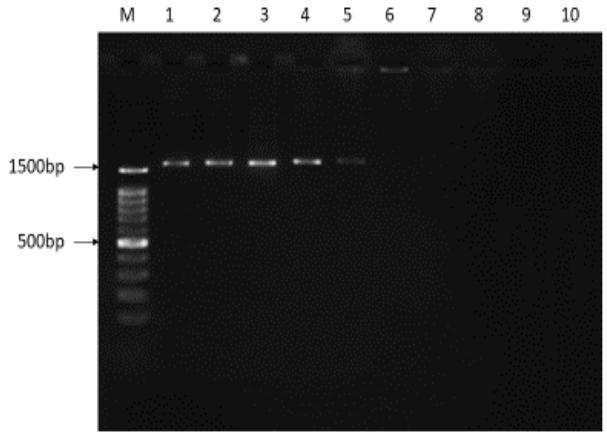

Figure 1. Electrophoresis of PCR product with an approximately 1587-bp band for evaluating the presence of *ef0737*gene on 1%agarose gel. (M) Marker 100bp (Gene On, Germany), 1: positive control, 2-5: isolates of *E. faecalis*, 6-7: *E. faecium* isolates, 8-9: *S. aureus* isolates, 10: negative control

Sensitivity and Detection Limit of PCR assay: In order to determine the lowest detection limit of PCR (using *ef0737* primers), PCR performed with 10-fold serial dilutions of purified DNA. The results of amplification showed that the minimum copy number of DNA detectable by PCR assays was 10 copies/reaction, respectively. PCR assay for 50 clinical isolates of *E. faecalis* was positive, thus the sensitivity was 100% using the 2x2 table of sensitivity.

Specificity of PCR: The specificity of the primer set was examined using DNA samples of non-*E. faecalis* isolates and negative results indicating specific primers. Only the *E. faecalis* strains were recognized by the primer set with 100% specificity.

Sequencing Result: Gene sequencing results compare with an original sequence in the GenBank database. Our sequencing results were exactly in line with the reference sequence and no mutations were observed in the sequence. According to gene sequencing, Basic Local Alignment Search Tool (BLAST) and multiple alignments, results showed that *ef0737* gene was completely similar in different strains of *E. faecalis* and could not be found in other bacteria.

Discussion

In the present study, we have developed an *E. faecalis*-specific PCR-based assay targeting the *ef0737*gene. Based on testing with DNA from 150 clinical isolates (50 *E. faecalis*, 50 *E. faecium*, 50 *S. aureus*), this PCR assay was shown to be specific and sensitive for clinical isolates of *E. faecalis* tested. In our previous studies, the importance of enterococcal infections in our region was shown (15, 16).

Although traditional techniques for the detection of pathogens and antimicrobial resistance evaluation clearly are winning a place in routine diagnostics, in some cases there is an urgent need to find rapid tests to improve disease diagnosis and treatment decisions (17, 18). On the other hands, an issue concerns the loss of cultivability of *E. faecalis* that may enter into the viable but non-culturable (VBNC) state under adverse conditions, where they are alive or metabolically active and able to express virulence genes, but will not form colonies on culture media (19).

Several studies have pointed to the importance of molecular diagnosis and limitations of phenotypic diagnosis. For instance; in the previous study by Velasco D, *et al.* it was shown that there was no correlation between phenotypic techniques and PCR-based genotypic methods for detection of Enterococcus species. Their results showed 15% *E. faecium* strains misidentified by phenotypic methods and accurate detection was done by PCR assay based on *ddl* gene (20). In the current study, we have shown that *ef0737* gene is present in all clinical isolates of *E. faecalis* with no exception. we amplified *ef0737* gene as a conserved gene for specific detection of *E. faecalis*, which was not detected in similar species. There are studies based on identification of genetic elements of *E. faecalis* such as *esp*, *hyl*, *asa1*, *van*, *ddl* (21) and *gelE*, *esp*, *cylA*, *hyl*, *ace* (22), although the prevalence of these genes was the main goal of the studies but most of them were not found in all isolates.

In our previous study based on EF0737 protein that encodes by *ef0737* gene, we demonstrate EF0737 protein interact with sera from patients with *E. faecalis* infection and *ef0737* gene express during infection (23), it showed this gene may appropriate marker in detection of *E. faecalis* infection which requires complementary studies on this gene in the clinical specimens. Rapid

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identification of *E. faecalis* by PCR amplification of *ef0737* gene in clinical samples is possible and can be effective in the diagnosis of infections caused by *E. faecalis.* Delayed diagnosis of infections caused by *E. faecalis* can result in more serious disease.

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