Medical Laboratory Journal
Online ISSN: 2538-4449
Golestan University of Medical Sciences

Original Article

A Comparative Study of ERIC-PCR and BOX-PCR Methods for Evaluation of Genomic Polymorphism among Multidrug-Resistant Enterococcus faecium Clinical Isolates

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Received: 2020/11/09
Revised: 2021/08/20
Accepted: 2021/08/22

DOI: 10.29252/mlj.17.1.13

ABSTRACT

Background and objectives: *Enterococcus faecium* is a normal flora of gut microbiota. This opportunistic pathogen has attracted much attention due to its multidrug resistance and ability to survive in hostile environments. Various molecular typing methods such as pulsed-field gel electrophoresis or ribotyping have been developed for clinical and epidemiological investigation of these bacteria. However, these methods are time-consuming and labor-intensive. The present study was conducted to evaluate the discriminatory power of two common fingerprinting methods i.e. BOX-polymerase chain reaction (PCR) and enterobacterial repetitive intergenic consensus (ERIC)-PCR for *E. faecium* clinical isolates.

Methods: Fifty multidrug-resistant *E. faecium* isolates were isolated from 74 clinical specimens. The isolates were identified by specific 16S rRNA PCR. All isolates were fingerprinted using BOX-PCR and ERIC PCR. The discriminatory power and reproducibility of these two methods were also assessed.

Results: According to the dendrogram with >60% similarity, 17 different genotypes were observed using ERIC PCR. In addition, BOX-PCR produced 22 distinct patterns at a genetic distance percentage of 60%, with sizes ranging from 278 bp to 1450 bp. The discrimination index of BOX-PCR was higher than that of ERIC-PCR.

Conclusion: We concluded that a combination of ERIC-PCR and BOX-PCR may be a quicker and more reliable alternative for the discrimination of *E. faecium* clinical isolates.

Keywords: *Enterococcus Faecium*, *Comparative Study*, *Polymorphism*, *Genetic*.

INTRODUCTION

The genus *Enterococcus* is composed of more than 40 species. The most important species of this genus are *Enterococcus faecium* and *Enterococcus faecalis*, which are responsible for 90% of enterococcal infections. These Gram-positive, non-sporulating microorganisms are commensal members of the gut microbiota of humans, capable of causing a variety of infections including endocarditis, surgical wound infections, sepsis, and urinary tract infections (1).

As the main cause of nosocomial 'superinfection' in hospitalized patients, enterococci have recently attracted considerable attention. In this regard, these bacteria are among the most prevalent multidrug-resistant pathogens and the third most commonly isolated microorganism from hospital environment (2). Although *E. faecium* and *E. faecalis* are the two most clinically important species, unlike *E. faecium*, *E. faecium* is less multidrug-resistant. Conversely, a lack of bactericidal activity of β-lactam and the ability to form biofilm at higher rates is noticed in *E. faecium* compared to *E. faecalis* (87%-95% vs. 16%-29%, respectively) (3). In addition, *E. faecium* strains are tolerant to many antibiotics and acquire drug resistance either by transfer of plasmid or transposon containing genetic sequences that confer resistance to other bacteria (4). Because of the increasing clinical relevance of enterococci, especially *E. faecium*, laboratories should be able to discriminate these microorganisms from other bacterial genera within hospitalized individuals. Moreover, it is essential to distinguish different species within the *Enterococcus* genus and different strains within each species (5).

Previous studies have shown that accurate identification of different species of *Enterococcus* is important for both clinical and environmental studies, and selecting efficient fingerprinting methods is of great clinical and epidemiological importance (6). Conventionally, typing of enterococci has been accomplished by biochemical profiles, protein analysis, and antibiotic susceptibility. Nevertheless, the lack of discriminatory power of such techniques has led investigators to develop alternative molecular-based methods (6). Various nucleic acid-based techniques have been used to type and characterize the genetic variability of enterococci. Although pulsed-field gel electrophoresis (PFGE) is considered the gold standard for enterococcus typing, some studies have suggested polymerase chain reaction (PCR) as a simpler and cheaper technique that gives more accurate results within a short period (7-9). Repetitive element-based PCR has been extensively used to study the strain-specific patterns obtained from PCR amplification of repetitive DNA elements of the bacterial genome (10). Two of the repetitive elements used for molecular typing are enterobacterial repetitive intergenic consensus (ERIC) sequences and BOX elements, which are mosaic repetitive elements comprised of different combinations of three subunit sequences of DNA. These three subunit sequences, i.e., boxA, boxB, and boxC, are 59, 45, and 50 nucleotides long, respectively (8). In comparison, ERIC sequences are 126 bp long and highly conserved central inverted repeats situated in noncoding transcribed regions of the chromosome (10). This study aimed to compare the two commonly used PCR-based methods, i.e., BOX-PCR and ERIC-PCR, for determining the genetic relatedness of pathogenic clinical *E. faecium* species.

MATERIALS AND METHODS

From December 2018 to April 2019, a total of 74 clinical samples were collected from two general hospitals in Tehran, Iran. The specimens were collected from blood, wound, suction secretion, sterile body fluid, and urine samples of catheterized patients. The samples were cultured on blood agar and incubated at 37 ^{o}C for 24 hours. Then, the isolates were cultured on m-Enterococcus agar (Merck, Germany). Colonies were identified based on morphology, Gram-staining, and other differential biochemical tests. Presumptively identified bacteria were confirmed as *E. faecium* by 16S ribosomal RNA gene sequencing. Identified *E. faecium* isolates were maintained on trypticase soybean agar, and permanent stocks were conserved on tryptone soy broth with 15% glycerol at -80 ^{o}C.

All identified and collected *E. faecium* isolates were tested by the disk diffusion method following the instructions of the Clinical and Laboratory Standards Institute (CLSI). The following panel of antibiotics at the specific

concentration per disk was tested: ampicillin (10 mg), vancomycin (30 mg), linezolid (30 mg), penicillin g (10 u), quinupristin/dalfopristin (15 mg), streptomycin (10 mg), ciprofloxacin (5 mg), levofloxacin (5 mg), nitrofurantoin (300 mg), norfloxacin (10 mg), tetracycline (30 mg), and gentamicin (120 mg). Finally, the isolates were categorized as sensitive, intermediate, and resistant using the breakpoints specified by the CLSI.

ERIC-PCR

Extraction of genomic DNA for molecular analysis was performed using a QIAamp DNA extraction kit (Qiagen, Germany) according to the manufacturer's instructions. Concentration of the genomic DNA was determined by reading absorbance at 260 using a Nanodrop spectrophotometer (Eppendorf, Germany). Specific primer sequences ERIC1-R (5´-ATGTAAGCTCCTGGGGATTCAC-3´) and ERIC2-F (5´-AAGTAAGTGACTGGGGTGAGCG-3´) were used for amplification of the regions of the bacterial genome positioned between the ERIC sequences. Next, PCR amplification was carried out using a thermal cycler (Eppendorf, Germany) with a total reaction volume of 20 µl. The reaction mixture contained 4 µl of DNA template (200 ng), 1 µl of 10X reaction buffer, 2 µl of dNTP mix (10 mM), 10 pmol of each primer (ERIC 1R and ERIC 2F), 1 µl of $MgCl_2$ (25 mM), and 2 U of Taq DNA polymerase (Amplicon, USA). The PCR reaction was performed under the following conditions: initial denaturation at 95 ºC for 3 minutes, followed by 30 cycles of denaturation at 95 ºC for 1 min, annealing at 42 ºC for 40 seconds, extension at 72 ºC for 2 minutes, and final extension at 72 ºC for 10 minutes. A negative control reaction mixture without the DNA template was also included in each set of PCR reactions. The ERIC-PCR products were separated by electrophoresis on 1.5% agarose gel with ethidium bromide staining and then photographed using a UV Imaging System (Bio-Rad, USA).

BOX PCR

BOX-PCR fingerprinting was carried out using the BOXA1R primer sequence (5´-CTACGGCAAGGCGACGCT-3´) which corresponds to the bacterial repetitive BoxA subunit. Next, PCR amplification was performed in a 25 µl reaction mixture containing 50 ng of genomic DNA, 1 µl of 2 U Taq DNA polymerase, 2.5 µl of 10X Taq buffer, 1 µl of dimethyl sulfoxide (10%), 1.5 µl of $MgCl_2$, 2.0 µl of 2.5 mM dNTPS (2.5 mM), 1 µl of 10 pmol BOXA1R primers, and 0.5 µl of BSA (10 mg/ml). Cycling conditions for the BOX-PCR experiment were the same as the ERIC-PCR, except that the annealing temperature was set to 49 ºC. The amplified products were separated and visualized as stated previously. To confirm the reproducibility of the patterns, all amplifications were performed at least twice in separate assays, and only bands common to the replicate amplifications were scored and used in statistical analysis.

First, DNA fingerprints of the strains were compared for similarity by visual inspection of the band patterns. Two fingerprints were considered different if the presence or absence of at least one band differed in one of the patterns. The observed bands in the agarose gels were evaluated based on the presence (code 1) or absence (code 0) of polymorphic fragments for the ERIC products. Cluster analysis was performed using the NTSYSpc software. The Jaccard similarity coefficients were calculated, and a phylogenetic tree was constructed using the unweighted pair group method with the arithmetic mean (UPGMA) method. Next, the Simpson's diversity index was calculated, which measures the probability that two unrelated strain samples from the test population are placed into different typing groups (11). The discriminatory power (D) index was calculated using the following equation where *s* is the total number of types described, *nj* is the number of isolates belonging to *jth* type, and N is the number of isolates: $D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} n_j(n_{j-1})$. A D value of 1 is highly discriminatory, and 0 is not discriminatory at all.

RESULTS

Overall, 50 *E. faecium* strains were isolated from the clinical samples. The epidemiological characteristics of the patients are detailed in table 1. The distribution of the isolates obtained from the patients did not differ significantly according to the isolation site. In this regard, 25% of the isolates were taken from urine samples. The results of antibiotic sensitivity testing showed that all isolates were sensitive to the tested antibiotics, which were

selected according to the CLSI standards. However, two *E. faecium* isolates were resistant to vancomycin.

The ERIC1R and ERIC2R primers generated 3 to 9 amplicons with a molecular weight of 100-2000 bp (Figure 1). According to the dendrogram with >60% similarity, 17 different genotypes were observed (Figure 2). As shown in the figures below, E1, E4, E5, E9, and E12 genotypes were observed in one strain, E2, E3, E8, E10, E11, and E14 genotypes were observed in 2 isolates, while E6, E7, and E13 genotypes were observed in 7, 3, and 4 isolates, respectively. Moreover, genotype E15 genotype contained the highest number of isolates (n=19) with similarities ranging from 58% to 100%. The Simpson index of the ERIC-PCR was 0.83 (CI=95%).

Table 1- Epidemiological characteristics of the patients

Variable		Patients
Age (mean ± standard deviation)		41.2±14.1 years
Sex	Male	30 (50%)
	Female	30 (50%)
Hospital ward	Intensive care unit	24 (40%)
	Medical	20 (33.33%)
	Surgical	16 (26.66%)

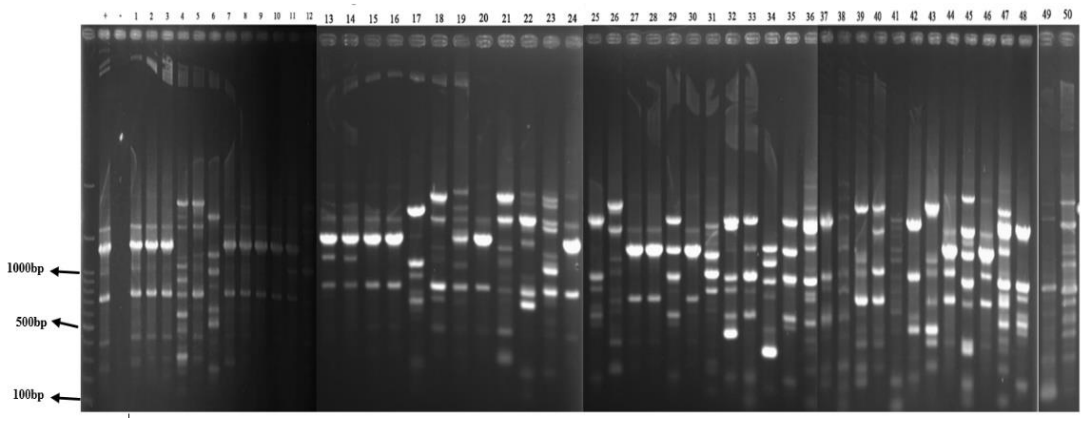

Figure 1. ERIC-PCR profile of 50 *E. faecium* isolates. The first lane denotes DNA ladder, lane + denotes positive control, and lane – denotes negative control.

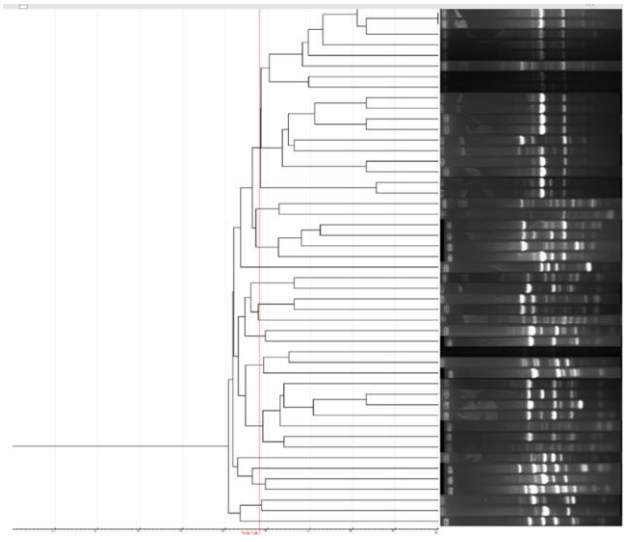

Figure 2- Dendrogram generated from ERIC-PCR fingerprinting of pathogenic *E. faecium* isolates. Similarities were calculated and the data were clustered using the UPGMA. The cut-off value of 58% similarity was used for assigning the ERIC-PCR types.

Of 50 isolates, the BOX-PCR experiment produced 22 distinct patterns at a genetic distance of 60% with sizes ranging from 278 to 1450 bp (Figure 3). The amplified banding profiles were clearly distinguishable. A dendrogram was generated with the Jaccard's similarity coefficients and UPGMA. Genotypes E17 and E18 contained the highest number of isolates (n=6) with similarity ranging from 58% to 100% (Figure 4). This was followed by four isolates in cluster E7 and E8. Moreover, 13 isolates were found to be single unique isolates in clusters E1, E2, E3, E5, E9, E11, E12, E15, E16, E20, E22, E23, and E24. The discrimination index of BOX-PCR was 0.95 (CI=95%).

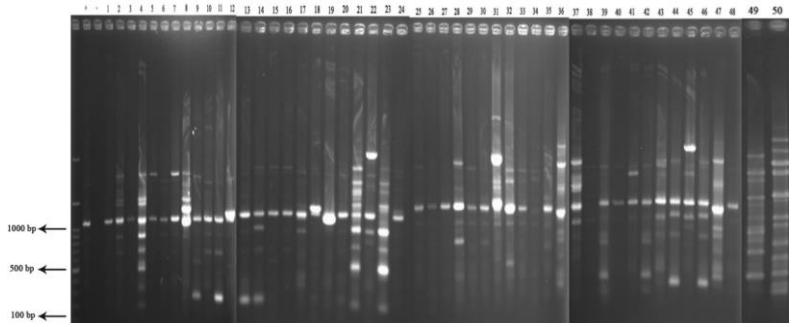

Figure 3- BOX-PCR profile of the *E. faecium* isolates. The first lane denotes DNA ladder, lane + denotes positive control, and lane – denotes negative control.

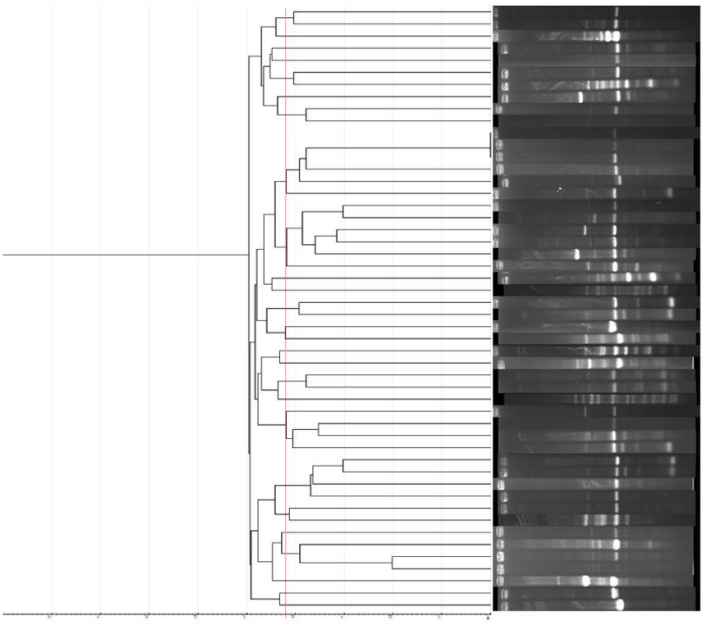

Figure 4- Dendrogram generated from BOX-PCR fingerprinting of pathogenic *E. faecium* isolates. Similarities were calculated, and the data were clustered using the UPGMA. The cut-off value of 58% similarity was considered for assigning the BOX-PCR types.

DISCUSSION

Molecular epidemiology of infections caused by enterococci species has been investigated using a variety of methods, including 16S rRNA gene sequencing, PFGE, random amplified polymorphic DNA, and (GTG)5-PCR fingerprinting. To the best of our knowledge, no study has investigated molecular typing of *E. faecium* using BOX-PCR and ERIC-PCR. The (GTG)5-PCR technique was used by Svec et al. (9) and PFGE was applied by Malathum et al. (12), Turabelidze et al. (13), and Bang et al. (14) for the same purpose. Molecular classification using BOX-PCR and ERIC-PCR has been utilized for other microorganisms. Bilung et al. reported the reproducibility and suitability of ERIC-PCR and BOX-PCR for the genetic discrimination of *Leptospira* isolates (10). Similar results were reported by Mishra et al.

in a study to compare BOX-PCR and ERIC-PCR for the determination of genetic relatedness of *Fusarium oxysporum* (8). It has been also reported that both methods were able to discriminate clinical isolates of *Proteus Mirabilis* (7) and *Pseudomonas aeruginosa* (15).

In the present study, all isolates produced bands after amplification by ERIC-PCR and BOX-PCR, suggesting the complete type ability of *E. faecium* isolates using these two typing techniques. This was corroborated by Bilung et al. (10) and Dombek et al. (16), who described the complete type ability of *Leptospira* and *Escherichia coli* isolates, respectively, using these methods. In our study, the discrimination index of the BOX-PCR method (0.95) was higher than that of the ERIC-PCR method (0.83). Controversially, Bilung et al. reported that the discrimination index for ERIC-PCR was higher than that of BOX-PCR; however, both of them gave comparable results (10). This inconsistency could be attributed to the different microorganisms investigated and their different genetic distances.

Some studies established that a higher discrimination index does not always correspond to a more accurate representation of epidemiological relatedness (10, 17). Regarding the constructed dendrograms, it can be summarized that the clinical *E. faecium* isolates in this study were highly heterogeneous and genetically diverse. A comparison was made to find similarities in clustering between BOX-PCR and ERIC-PCR. The obtained data showed that some isolates were grouped into the same cluster using both methods. Generally, of 50 isolates, three yielded the same profile in the BOX-PCR and ERIC-PCR methods. Our study showed comparable efficiency in determining the genetic relatedness of 50 *E. faecium* isolates; however, a discrimination index was obtained using BOX-PCR. Although these methods are simple and quick to perform, their resolving power is limited for discriminating genetic relatedness among *E. faecium* isolates. Despite the higher discrimination index of BOX-PCR, there are some doubts about the ability of the method to correctly project the relatedness of *Enterococcus* strains (10). About 4% of the isolates were resistant to vancomycin, but none were resistant to other tested antibiotics. The rate of vancomycin resistance was reported to be 5%, 11%, and 17% in previous studies in Iran (18), Europe (19), and Nigeria (20), respectively.

Further research with larger sample size and comprising both environmental and clinical *E. faecium* strains should be carried out to clarify our findings. Moreover, it is necessary to further evaluate these bacteria through PCR-based methods as useful molecular typing methods for the clinical investigation of *E. faecium* epidemics.

CONCLUSION

We established that the clinical isolates of *E. faecium* from Iranian patients have genetic polymorphism as detected by both ERIC-PCR and BOX-PCR. Both techniques have a high discriminatory ability, which makes them useful for clinical and epidemiological studies of *E. faecium*. We conclude that a combination of molecular methods may be more useful, rapid, and reliable for discriminating *E. faecium* isolates.

ACKNOWLEDGEMENTS

We would like to thank the staff of Pasargad Laboratory for their assistance in this project.

DECLARATIONS

FUNDING

The authors received no financial support for the research, authorship, and/or publication of this article.

Ethics approvals and consent to participate

Not applicable.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding publication of this article.

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How to Cite:
Ahmadi T, Siasi Torbati E, Amini K [A Comparative Study of ERIC-PCR and BOX-PCR Methods for Evaluation of Genomic Polymorphism among Multidrug-Resistant Enterococcus faecium Clinical Isolates]. mljgoums. 2023; 17(1): 13-19 DOI: 10.29252/mlj.17.1.13