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Antimicrobial resistance pattern and genetic correlation in *Enterococcus faecium* isolated from healthy volunteers

M. Asadian, J. Sadeghi, A. Rastegar Lari, Sh. Razavi, M. Hasannejad Bibalan, M. Talebi*

Department of Microbiology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran

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ABSTRACT

Enterococci are known as a cause of nosocomial infections and this aptitude is intensified by the growth of antibiotic resistance. In the present study, *Enterococcus faecium* isolates from healthy volunteers were considered to determine the antibiotic resistance profiles and genetic correlation. A total 91 normal flora isolates of enterococci were included in this study. Identification of *Enterococcus* genus and species were done by biochemical and PCR methods, respectively. Sensitivity for 10 antibiotics was determined and genetic relatedness of all isolates was assessed using Repetitive Element Palindromic PCR (REP-PCR) followed by Pulse Field Gel Electrophoresis (PFGE) on the representative patterns. None of the isolates were resistant to teicoplanin, vancomycin, quinupristin-dalfopristin, linezolid, chloramphenicol, ampicillin and high-level gentamicin. On the other hand, the resistance rate was detected in 30.7%, 23%, and 3.29% of isolates for erythromycin, tetracycline and ciprofloxacin, respectively. The results of PFGE showed 19 (61.5% of our isolates) common types (CT) and 35 (38.5%) single types (ST) amongst the isolates.

This is the first study to describe antibiotic resistance pattern and genetic relationship among normal flora enterococci in Iran. This study showed no prevalence of Vancomycin Resistant Enterococci (VRE) and high degrees of diversity among normal flora isolates by genotyping using PFGE.

1. Introduction

Enterococci are gram-positive and normal flora cocci in the gastrointestinal tract (GIT) of humans and animals [1–3]. Amongst the enterococci, *Enterococcus faecalis* and *Enterococcus faecium* are medically very important [1,2]. Although they are commensal, they can act as opportunistic pathogens and cause disease [4] such as urinary tract infections, bacteremia and endocarditis [1,2]. *Enterococcus* can survive in water, soil and food and can withstand in severe environmental conditions [3,5].

Excessive global consumption of antibiotics in general and the use of vancomycin have increased the capacity of the enterococci to acquire resistance [6]. VRE have been considered as a serious menace to human health [7].

Enterococci have a significant ability to obtain and distribute antibiotic resistance genes by different ways. This characterization has actuated them to become a major nosocomial pathogen [3]. Microbial typing results represent the amount of similarity among the isolates that benefit to performing suitable infection control procedures in medical centers [8]. Numbers of molecular epidemiology techniques have been suggested for studying enterococci clonality. However, none have received universal acceptance. Amongst the various techniques, REP-PCR has been described to have good reproducibility, typeability and a quick method for molecular typing [9]. PFGE has also suggested because of the discriminative power [9].

In this study we investigated the pattern of antibiotic resistance in *Enterococcus* strains isolated from the normal flora of healthy people and their clonality and genetic relatedness were examined.

2. Materials and methods

2.1. Bacterial isolation and identification

Stool samples were collected from twenty-four healthy

* Corresponding author. Department of Medical Microbiology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran..
E-mail addresses: Asadian.mahla@yahoo.com (M. Asadian), Javad.sadeghi1@gmail.com (J. Sadeghi), Lari@iums.ac.ir (A. Rastegar Lari), Razavi.sh@iums.ac.ir (Sh. Razavi), Meysam_hasannejad@yahoo.com (M. Hasannejad Bibalan), talebi.m@iums.ac.ir, talebi_25@yahoo.com (M. Talebi).

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volunteers in Tehran, Iran between August to October 2014. One sample was taken from each volunteer. All volunteers had normal bowel activity and subjects with a history of antibiotic and probiotic usage during the last three months prior to the study were excluded [10]. All procedures were done in accordance with the Declaration of Helsinki (1975) amended in 2013 [11]. Information including age, sex, underlying disease and history of antibiotic use were collected from each individual. A total of 24 healthy volunteers eight of them were female and 16 were male. The mean age of them was 44 years (from 12 to 72 years).

For Bacterial isolation, 0.5 g of each stool sample was dissolved in 4.5 ml of normal saline, then inoculated into m-*Enterococcus* agar medium and incubated at 45 °C for 48 h [10]. Afterwards, 24 colonies were selected randomly from each plate and sub-cultured on blood agar medium [12]. The isolates were confirmed by colony morphology, Gram staining and biochemical tests such as: catalase production, growth in 6.5% NaCl and esculin hydrolysis in the presence of bile [5]. The identification of species was performed by biochemical tests and the confirmation was done using PCR method [2,13]. All isolates were stored in BHI broth with 15% glycerol at −80 °C for subsequent experiments [5].

2.2. Antimicrobial susceptibility tests

The susceptibility of all isolates was determined to antimicrobial agents by Kirby Bauer disk diffusion assay. The common antibiotics according to CLSI guidelines include: vancomycin (30 μg), teicoplanin (30 μg), gentamicin (120 μg), quinupristin-dalfopristin (15 μg), linezolid (30 μg), tetracycline (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), ampicillin (10 μg) and ciprofloxacin (5 μg) [14]. Antibiotics discs were prepared from Mast Group (MAST House, Derby Road, Bootle, Merseyside, L20 1EA, United Kingdom) and Cypress Diagnostics (Industriepark 36, Zone B5 2235 Hulshout, Belgium). *E. faecalis* ATCC 29212 was used as the quality control strain [15].

2.3. DNA extraction and detection of resistance genes

For DNA extraction, one colony of each plate was dissolved in 200 μl distilled water and heated at 100 °C for 15 min and finally centrifuged at 14000 rpm for 20 min [12,16]. Supernatant containing DNA was stored at −20 °C until used. The resistance genes for erythromycin (*erm*(B)) and tetracycline (*tet*(M)) were tested by PCR (Eppendorf Mastercycler) in all of the isolates that showed phenotypically resistance to these antibiotics [17].

The single PCR was performed for each of the genes. The final volume of reaction was 25 μl, including: 15 μl Master Mix (Ampliqon, Denmark), 4 μl distilled water, 0.5 μl of each of the primers (F and R) and 10 μl of DNA template. PCR conditions were as follows: an initial denaturation at 93 °C for 3 min followed by 30 cycles of denaturation at 93 °C for 1 min, annealing at 62 °C for 1 min, extension at 65 °C for 4 min and a final elongation at 65 °C for 3 min. PCR products were separated on %1 agarose gel and fragments of 745 bp and 406 bp were observed for *erm*(B) and *tet*(M), respectively [17].

2.4. REP-PCR

DNA was extracted using DNA extraction kit (Qiagen, Germany). REP-PCR was performed in a total volume of 25 μl including: 1.25U *Taq* DNA polymerase, 50 ng DNA template, 1X PCR buffer, 2.5 mmol/l MgCl2, 200 μmol/l dNTP, 30 pmol BOXA2 primer. The PCR conditions were as follows: initial denaturation at 95 °C for 7 min, 35 cycles of denaturation at 90 °C for 30s, annealing at 40 °C for 1 min, extension at 60 °C for 6 min and final extension at 65 °C for 16 min [18].

2.5. Typing method (Pulse Field Gel Electrophoresis)

In this study PFGE was done according to the previous study [19]. Briefly, *Enterococcus* isolates were inoculated from blood agar to BHI broth medium. After 24 h shaking, the bacteria suspension was prepared for making plug. The plugs were thrown into lysis buffer for 24 h at 37 °C [0.25 M EDTA (pH = 7.5), 1 M Tris–HCl (pH = 7.6), 5 M NaCl, %10 Brij58,%5 Deoxycholate, %10 Sodium Lauryl Sarcosine, lysozyme 1 mg/ml (All materials are purchased from Merck, Germany), H2O], then ESP buffer [1 M EDTA (pH = 9–9.5), %10 Sarcosine, Proteinase K (5 g/l) (Merck, Germany), H2O] for 48 h at 50 °C [19]. Plugs were digested by *Sma*I restriction enzyme (Roche, Germany) and electrophoresed by CHEF-DRIII system under these conditions: The initial switch time for 5 s, the final switch time for 35 s at 6 V/cm with a run time for 22 h at 14 °C and angel 120° [13,19]. *Salmonella cholerasuis* serotype Branderup H9812 was used as a standard size marker [2,8]. The analysis of the PFGE fingerprints was performed by Gelcompare software version 6.6. Comparisons of patterns were expressed using arithmetic averages (UPGMA) clustering method by Dice band-based correlation coefficient [19].

3. Result

3.1. Bacterial isolates

A total of 576 isolates were obtained from 24 healthy volunteers and 54 patterns were resulted using REP-PCR method. For patterns with multiple isolates, 2 were chosen randomly. A total of 91 isolates were selected for further studies and all were identified as *E. faecium*.

3.2. Antimicrobial susceptibility tests

No VRE isolates were identified. All isolates were susceptible to teicoplanin, quinupristin-dalfopristin, linezolid, chloramphenicol, ampicillin and high-level gentamicin. Out of 91 isolates, 3 (3.29%) were resistant to ciprofloxacin, 21 (23%) to tetracycline and 28 (30.7%) to erythromycin.

3.3. Resistance genes detection

PCR was performed to identify resistance genes and found that a total of 21 isolates were resistant to tetracycline which harbored *tet*(M) gene. Out of the 28 erythromycin-resistant isolates, 11 (39.2%) harbored *erm*(B) gene. Fourteen MDR (resistance to at least two or more classes of antibacterial agents) (15.3%) isolates were identified. Eleven (12%) isolates revealed resistance to erythromycin and tetracycline, simultaneously. Six (54.5%) of them carrying *erm*(B) and *tet*(M) genes. Two isolates were resistant to erythromycin/ciprofloxacin, and one isolate was resistant to tetracycline/ciprofloxacin (Fig. 1).

3.4. Molecular typing

PFGE analysis showed 54 types. Thirty-five (38%) of these isolates were ST and the fifty-six (62%) isolates were belong to 19 CTs. The CT5 and CT6 contained 5 isolates each and CT3, CT9, CT13 and CT18 contained 4 isolates each (n = 26, 28%). Other isolates (n = 65, 71%) belonged to other 13 CTs. Amongst the donors, 5 persons had the same *E. faecium* patterns (J, L, O, Q, R). The other 19 persons (79.1%) had isolates with different PFGE patterns (Table 1). The isolates related to 7 volunteers (29.1%) displayed no resistance to the antibiotics. The other 17 donors (70.8%) were resistant to at least one and maximum four antimicrobial agents. Amongst the