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Virulence factors, antimicrobial resistance pattern and molecular analysis of Enterococcal strains isolated from burn patients

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ABSTRACT

The enterococci are emerging as a significant cause of hospital acquired infections. The pathogenesis of enterococci is attributed to the production of virulence factors and resistance to antibiotics. The purpose of the study was to assess the prevalence of genes encoding virulence factor, antimicrobial resistance determinant and molecular characteristic of enterococci isolated from burn patients. A total of 57 enterococci isolated from wound specimens of patients with burn injury were characterized by phenotypic and genotypic methods. The *efaA* was the most frequently detected gene (100%), followed by *ace* (89.1%), *asa1* (54.3%), *gelE* (50%), *cyl*A (30.4%), *esp* (23.9%) and *hyl* (8.7%) among *Enterococcus faecalis* isolates. The *Enterococcus faecium* strains carried *asa1* and *ace* genes. All isolates were susceptible to tigecycline and vancomycin. Inducible resistance to clindamycin was not observed and 64% of isolates had resistance to erythromycin. High-level gentamicin resistance (HLGR) was seen in 65.2% of *E. faecalis* strains. The *aac(6′)-Ie-aph(2″)-Ia* gene was found in 47.8% of *E. faecalis* isolates. Our data indicated that the *efaA*, *ace* and *asa1* were most frequent genes encoding virulence factors among Enterococci isolated from burn wound infection and the incidence of virulence factor genes was higher in *E. faecalis* rather than other isolates. The molecular analysis demonstrated high genetic diversity among *Enterococcus* populations from burn patients.

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1. Introduction

Enterococci are Gram-positive cocci and common inhabitants of the human intestine and genitourinary tract [1,2], However they are cause of nosocomial infections, particularly bacteremia, sepsis, endocarditis, urinary tract infection (UTI) and wound infections [3,4]. Skin damage, immunodeficiency situation and hospitalization makes burns patients at a high risk for acquiring nosocomial infections such as Enterococci infections [5]. A collection of different factors including resistance to antibiotics and virulence determinants are involved in the success of enterococci in the hospital setting [6]. Presence of various virulence determinants such as, gelatinase (GelE), aggregation substance (AS) proteins (Asa1), enterococcal surface protein (Esp), collagen adhesine (Ace), cytolysin (CylA) and hyaluronidase (Hyl) can enhance bacterial colonization on hospitalized patients [1,6].

Treatment of enterococcal infections could be difficult because they can survive in exposure to antimicrobial agents noticeably, they have intrinsically resistant to several antimicrobial agents including; β-Lactams, fluoroquinolones and trimethoprim-sulfamethoxazole and moreover they can acquire resistance to antibiotics such as, aminoglycosides, macrolides and glycopeptides [7–9]. Resistance to vancomycin is encoded by the *Van* gene clusters which are carried on transposon [10]. Resistance to high concentrations of aminoglycoside antibiotics is usually due to aminoglycoside-modifying enzymes (AMEs) is encoded within mobile genetic elements [11].

Enterococcal isolates has emerged as important pathogen in Iran as in other countries, which presents serious challenges for hospital infection control practitioners and clinicians treating infected patients. There are several reports on the endemicity of vancomycin resistant enterococci (VRE) in Iran [12,13], but there is limited amount of information regarding the virulence determinants in hospitalized burn patients. Therefore, the aim of the current study was to evaluate the prevalence of genes encoding virulence factors and antimicrobial resistance and molecular

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characterization of Enterococcal strains isolated from burn patients.

2. Materials and methods

2.1. Bacterial isolates

During June 2013 to June 2014, 57 Enterococcal isolates were collected from wound specimens of patients with burn injury from a burn hospital in Tehran. Only one isolate per patient was included. Identification of enterococci was performed based on a series of conventional microbiological tests [13]. The *ddlE* gene was targeted by Polymerase chain reaction (PCR) using specific primers ($ddl_{E.\ faecalis}$ F- 5′- ATCAAGTACAGTTAGTCT-3′ and R-5′- ACGATT-CAAAGCTAACTG-3′ and $ddl_{E.\ faecium}$ F-5′- TAGAGACATTGAATATGCC-3′ and R-5′-TCGAATGTGCTACAATC-3′) in order to confirm the identity of isolate as *Enterococcus faecalis* or *Enterococcus faecium* [14]. The PCR conditions consisted of a pre-denaturation step at 95 °C for 5 min, followed by 30 cycles of 60 s at 95 °C, 45 s at 45 °C for $ddl_{E.\ faecalis}$ and 47 °C for $ddl_{E.\ faecium}$ and 45 s at 72 °C. A final extension step was performed at 72 °C for 5 min.

2.2. Antimicrobial susceptibility testing

Antimicrobial sensitivity tests were done by disc diffusion method on the Mueller- Hinton Agar (Merck Co., Germany) based on Clinical and Laboratory Standards Institute (CLSI) guideline [15]. The tested antibiotics (Mast Group Ltd., Merseyside, UK.) were ciprofloxacin (5 μg), erythromycin (15 μg), clindamycin (2 μg), tigecycline (15 μg), vancomycin (30 μg) and gentamicin (120 μg). *E. faecalis* ATCC 29212 was used as reference strain for antibiotic susceptibility testing. All of isolates were evaluated for inducible clindamycin resistance by double disc synergy test (D-test) accordance with CLSI guideline [15].

High-level gentamicin resistance (HLGR) was determined by the broth microdilution method using Brain Heart Infusion broth (BHI) (Conda S.A., Madrid, Spain) and measurement of minimal inhibitory concentration (MICs) of vancomycin was performed by the standard agar dilution on the BHI agar [15].

2.3. DNA extraction and gene detection

Genomic DNA was extracted from overnight grown colonies as described previously [16]. PCR assay was performed for detection of the genes encoding virulence factor (*gelE, asa1, cylA, hyl, esp, efa, ace*) and the genes encoding to resistance vancomycin (*vanA, vanB*), aminoglycoside (*aac(6′)-Ie aph(2″)Ia*) and macrolide (*ermA, ermB, msrA, mefA*) [13,16–19].

2.4. Enterobacterial repetitive intergenic consensus (ERIC)-PCR

For molecular analysis of isolates, ERIC-PCR was done as described previously [21]. Briefly, the PCR protocol consisted of a predenaturation step at 95 °C for 5 min, followed by 30 cycles of 60 s at 95 °C, 60 s at 36 °C, and 60 s at 72 °C. A final extension step was performed at 72 °C for 10 min. PCR products were separated by electrophoresis in 1% agarose gels with 0.5X TBE (Tris/Borate/EDTA) buffer. DNA bands were observed by staining with KBC power load dye (Kawsar Biotech Co. Iran) and visualized under UV (ultraviolet) illumination. ERIC patterns were analyzed using GelCompar II. Isolates with a similarity coefficient equal or above 80% were considered as the same genotype. Only the dominant species (*E. faecalis*) were included in the analysis.

2.5. Statistical analysis

Differences in the incidence of virulence genes among HLGR and non HLGR *E. faecalis* isolates were calculated by Fisher's test for each gene. A p-value of $\leq$0.05 was considered as statistically significant.

3. Results

3.1. Antimicrobial resistance pattern

During one year study, a total of 57 *Enterococcus* strains, including *E. faecalis* 46 (80.7%), *E. faecium* 2 (3.5%) and other species 9 (15.8%) were isolated from burn wound. No isolates were found resistant to tigecycline and vancomycin (Table 1). Resistance to ciprofloxacin and clindamycin were more than 50% and 90% respectively. Inducible resistance to clindamycin was not observed and 64% of isolates were resistant to erythromycin. HLGR was seen in 65.2% of *E. faecalis* strains. The *aac(6′)-Ie-aph(2″)-Ia* gene was found in 47.8% of *E. faecalis* isolates. HLGR was found in one of the *E. faecium* strains which was negative for the *aac(6′)-Ie-aph(2″)-Ia* gene amplification. The *erm*(A), *mef*(A) and *msr*(A) genes were not detected in any of the isolates, and 25 (54.3%) of *E. faecalis* strains carried *erm*(B) gene. The *van*A, and *van*B genes were not seen in any of the enterococcal isolates.

3.2. Distribution of virulence genes

The *efaA* was the most frequently detected gene (100%) among *E. faecalis* isolates, followed by *ace* (89.1%), *asa1* (54.3%), *gelE* (50%), *cyl*A (30.4%), *esp* (23.9%) and *hyl* (8.7%). The *E. faecium* strains carried *asa1* and *ace* genes and one of them had *gelE* gene. The Prevalence of *ace* and *efaA* genes among other isolates were 22%, each and *cylA* was found in 11% of them. The *hyl* gene was significantly higher in HLGR isolates compared to non HLGR isolates (*P* 0.03).

3.3. Molecular genotyping

ERIC-PCR of genomic DNA from *E. faecalis* strains amplified 4 to 10 bands with molecular weight ranging from 100 bp to 1.5 kb. Among all strains, six isolates did not show any product in reactions and thereby were non typable. Thirty four ERIC types obtained from 40 isolates. Of the 34 ERIC types, there were 6 types each containing 2 isolates separately while, 28 isolates had unique banding pattern and were classified as a distinct genotypes (Fig. 1).

4. Discussion

Since the 1990s, Enterococci have been emerged as a significant cause of nosocomial infections [22]. Considering clinically important species of Enterococci, *E. faecalis* is the most common species cause of Enterococcal infections [1,23]. In our study, the most prevalent species was *E. faecalis* (80.7%), which is similar to result of other study in which the distribution of Enterococcal species was evaluated [13,16,24].

In the current study, the 65.2% of *Enterococcus* strains were HLGR and most of them (57.5%) carried the *aac(6′)-Ie-aph(2″)-Ia* gene. This finding is in accordance with previous studies in which have been shown that the *aac(6′)-Ie-aph(2″)-Ia, aph(3′), ant(4′)* and *ant(6)* genes encode aminoglycoside-modifying enzymes leading to high level resistance to gentamicin [16,25].

In the present study, there was no vancomycin resistant isolate. This result was different with previous studies in which the VRE strains have been isolated from burn infections [22,26]. Although our finding supports the vancomycin as an efficient choice against

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