Prevalence of Virulence Factors and Vancomycin-resistant Genes among *Enterococcus faecalis* and *E. faecium* Isolated from Clinical Specimens

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### Abstract

**Background:** The aim of this study was to determine the occurrence of virulence determinants and vancomycin-resistant genes among *Enterococcus faecalis* and *E. faecium* obtained from various clinical sources.

**Methods:** The study was performed on the 280 enterococcal isolates from clinical specimens in Hamadan hospitals, western Iran in 2012-14. Antibiotic susceptibility testing was performed using disk diffusion and Minimal Inhibitory Concentration (MIC) methods. The presence of vancomycin-resistant genes and virulence genes was investigated using PCR.

**Results:** Totally 280 enterococcal isolates were identified as follows: *E. faecalis* (62.5%), *E. faecium* (24%) and *Enterococcus* spp (13.5%). The results of antibiotic susceptibility testing showed that resistance rates to vancomycin and teicoplanin in *E. faecalis* and *E. faecium* isolates were 5% and 73%, respectively. Of Sixty vancomycin-resistant *Enterococci* strains, fifty-one isolates were identified as *E. faecium* (VREfm) and nine as *E. faecalis* (VREFs). Prevalence of *esp*, *hyl*, and *asa* genes were determined as 82%, 71.6%, and 100%, respectively in *E. faecium* strains; and 78%, 56/6%, and 97%, respectively in *E. faecalis* strains.

**Conclusion:** The increased frequency of VREF, as seen with rapid rise in the number of vanA isolates should be considered in infection control practices.

**Keywords:** *E. faecalis*, *E. faecium*, Vancomycin-resistant enterococci, Minimum inhibitory concentration

### Introduction

Vancomycin-resistant *Enterococci* (VRE) strains were reported first in United Kingdom and France in 1986, and after that in the United States (1). At present, these organisms are significantly isolated in hospitals around the world, especially in patients with hemato-oncological diseases and hospitalized in intensive care units (2). Currently, nine types of vancomycin-resistance have been described in *Enterococci*, eight of these types correspond to acquired resistance, i.e., *vanA*, *vanB*, *vanD*, *vanE*, *vanG*, *vanL*, *vanM* and *vanN*; one type-*VanC*- have been identified in species of *Enterococcus gallinarum* and *E. casseliflavus–E. flavescens* intrinsically resistant to low levels of vancomycin but remain susceptible to teicoplanin and have been found in human intestinal tract (3, 4).

Among vancomycin-resistance genotypes in *Enterococci*, *vanA* and *vanB* possess greatest clinical significance (5). The *vanA* genotype is the most commonly genotype in VRE worldwide, and is associated with the transfer of a considerable amount of vancomycin resistance from *Enterococci*, particularly, vancomycin-resistant *E. faecium* (VREF) to *Staphylococcus aureus* (1, 6). The *vanA*-type genes play an
importance role in inducing resistance against both teicoplanin and vancomycin, while the level of resistance to vancomycin in strains sheltering vanB-type genes are not constant but somewhat variable (MICs, 4 to 10.24 mg/l). Moreover, remain susceptible to teicoplanin (MICs ≤ 2 mg/l) in vitro (4). The low-level resistance against such antibiotics as vancomycin vancomycin (vancomycin MIC, 64–128 mg/ml) and susceptibility or intermediate resistance to teicoplanin (teicoplanin MIC, 8–16 mg/ml) is one of the main features of the vanD phenotype (7).

Although these organisms lacking strong virulent factors, but enterococcal infection is intricated by the intrinsic resistant / tolerant to many important antimicrobial agents including cephalosporin, lincomycin, cotrimoxazole, and low levels of penicillin and aminoglycosides, and as well as by the ability to acquire resistance to penicillins, chloramphenicol, tetracyclines, aminoglycosides (high-level) and vancomycin either by mutation, or by acquisition of plasmids or transposons (8, 9). Thus, the incidence of antimicrobial resistant Enterococci, especially VRE is a persisting clinical problem in public health care facilities in all geographical areas (9). Cytolysin (Cyt), gelatinase (GelE) and aggregation substance (Agg) are virulence factors identified in E. faecalis that can influence the relationship between parasite and host, on other hand, esp and bhl have been found in both E. faecalis and E. faecium (10, 11). “These virulence factors may causes increasing persistence of enterococci in the nosocomial environment, and consequently inter and intra-hospitals dissemination” (10).

Regarding the virulence determinants in clinical isolates of Enterococci that may result in promoting emergence of infections and persistence of this organism in nosocomial settings and consequently leads to increased resistance (5), this study was undertaken to determine the antimicrobial susceptibility patterns and virulence factors including esp (enterococcal surface protein), asa, (aggregation substance), gelE (gelatinase), bhl (hyaluronidase) in clinical isolates of E. faecalis and E. faecium.

Materials and Methods

Identification of enterococcal isolates

One hundred and seventy-five E. faecalis and sixty-seven E. faecium isolates were collected from different clinical specimens submitted in three teaching hospitals located in Hamedan/ western Iran, from Dec 2012 to May 2014. The origins of isolates were as follows: urine 200 (82.6%), tracheal 17 (7%), blood 8 (3.3%), wound 6 (2.5%), abscess and lower respiratory tract 6 (2.5%), and body fluids 5 (2.1%). Enterococci genus were identified using routine microbiological methods (9) then, PCR targeting D-alanine- D-alanine ligases specific for E. faecalis (ddl E. faecalis) and E. faecium (ddl E. faecium) was used to confirm phenotypic speciation (12).

DNA extraction

Enterococci chromosomal DNA was extracted by boiling method. Briefly, 3-5 clones of overnight bacterial culture was suspended in 500 µl of sterile distilled water, boiled for 10-15 min, and then centrifuging at 14000 g for 5 min to pellet cell debris (13).

Detection of E. faecalis and E. faecium species by PCR

PCR reactions of ddl E. faecalis and ddl E. faecium genes were performed with previously designed primers (Table 1), with some modification on Kariyama’s protocol (12) using Eppendorf and Biorad thermocycler (ASTEC Co., Japan) in a final volume of 20 µl containing 2 µl template DNA, 1 µl of each primer, 10 µl of Master Mix, 6 µl of sterile distilled water. The optimum conditions of PCR for both genes were as follows: an initial denaturation at 95 °C for 5 min, followed by amplification by 30 cycles of denaturation at 95 °C for 30 sec, annealing at 52.5 °C for 30 sec and elongation at 72 °C for 1 min, and a final extension at 72 °C for 10 min. The E. faecalis ATCC 29212 and E. faecium BM4147 were used as quality control strains.

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Antibiotic susceptibility testing
Antibiotic susceptibility testing of 175 E. faecalis strains and 67 E. faecium strains was performed using Kirby-Bauer disk diffusion method on Muller-Hinton agar in accordance to the Clinical and Laboratory Standards Institute (CLSI) guidelines (14). Antimicrobial agents used in this study were included: vancomycin (30 µg), teicoplanin (30 µg), tetracycline (30 µg), erythromycin (15 µg), norfloxacin (10 µg), ciprofloxacin (5 µg), nitrofurantoin (300 µg), quinopristin-dalfopristin [synercid (15 µg)] (Mast co., UK), chloramphenicol (30 µg), linezolid (30 µg), gentamicin (10 µg), and ampicillin (10 µg) (HiMedia Mumbai Co., India).

Determination of minimum inhibitory concentration (MIC) of the glycopeptide antibiotics i.e. vancomycin and teicoplanin (Sigma-Aldrich, Poole, Co., UK) for E. faecalis and E. faecium isolates was done using microdilution broth method and Cation Adjustment Muller Hinton Broth (CAMHB) medium according to the CLSI guidelines (14). The E. faecalis ATCC 29212 (Vancomycin sensitive), E. faecalis ATCC 51299 (vanB positive), E. faecalis E206 (vanA positive) were used as quality control strains for performing antimicrobial tests.

Table 1: Primers used in this study

| Gene targets | Primer sequences (5' to 3') | amplicon / product size (bp) | Reference |
|--------------|-----------------------------|-----------------------------|-----------|
| asa1         | F: GCACGCTATTACGAACACTATGA  | 375                         | 13        |
|              | R: TAAGAAAGAACATCACCACGA    |                             |           |
| hyl          | F: ACAGAAGAGCTGCAGGAAATG    | 276                         | 13        |
|              | R: GACTGACGTCCAAAGTTTCAA    |                             |           |
| esp          | F: AGATTTTCATCTTTGATCTTTGG  | 510                         | 13        |
|              | R: AATTGATTCTTTTACACATGG    |                             |           |
| ddl E. faecalis | F: ATCAAGTGACATGTCGGATGAC    | 941                         | 12        |
|              | R: ACGTTCAAAGCTGACTGAATG    |                             |           |
| ddl E. faecium | F: TTGAGGGCAAGCCAGATTGACG   | 658                         | 12        |
|              | R: TTAGACCGAGCTCCGATTCC     |                             |           |
| vanA         | F: GGGAAACCGACAATGTC        | 732                         | 12        |
|              | R: GTACATGTCGGGCGCTTA       |                             |           |
| vanB         | F: ATGGGAAGCGCGATAGTC       | 635                         | 12        |
|              | R: GATTTGTTTCCTGGACC        |                             |           |
| vanD         | F: TGTGGGATGCGATATTCAA      | 500                         | 14        |
|              | R: TGCAAGCAGTATCGGTAA       |                             |           |

Detection of van determinants
Isolates with a vancomycin and teicoplanin Minimum Inhibitory Concentration (MIC) of 2 µg/ml were analyzed by PCR for the presence of the genes encoding the vancomycin-resistance determinants vanA, vanB, vanD using specific primers (Table 1). The PCR reaction was performed in a volume of 20 µl and contained: 2 µl template DNA, 1 µl of each primer, 10 µl of Master Mix, 6 µl of sterile distilled water on a Eppendorf and Biorad thermocycler (ASTEC Co., Japan) with an initial denaturation at 94 °C for 3 min, 30 cycles of amplification (denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min, and extension at 72 °C for 1 min), and a final extension at 72 °C for 7 min (12).

Detection of virulence genes esp, hyl, and asa, by PCR
Identify of three virulence determinants in all E. faecalis and E. faecium isolates was performed by Multiplex PCR (esp, asa,) and single PCR (hyl) using specific primers for each gene (Table 1), with some modification on Vankerckhoven’s protocol. The first 25 µl of PCR mixture contained 3 µl of template DNA (1µl of plasmid DNA, 2 µl of
chromosome  DNA), 1µl of each primer for genes esp and asa, 12.5 µl of Master Mix, and 5.5 µl of sterile distilled water; the second 20 µl PCR mixture contained 2 µl of template DNA, 1 µl of each primer for byl, 10 µl of Master Mix, and 6 µl of sterile distilled water. The PCR conditions included a pre-denaturation step at 95 ºC for 10 min, followed by 30 cycles of 1 min at 94 ºC, 1 min at 56 ºC and 1 min at 72 ºC. A final extension step was performed at 72 ºC for 10 min (13). The E. faecalis ATCC 29212 (asa, positive), E. faecium C68 (byl and esp positive) were used as quality control strains.

Statistical analysis
Data were analyzed statistically using Chi-Square test and difference was considered significant at P<0.05 by SPSS software version 19 (Chicago, IL, USA).

Results

Enterococci isolates
Of 280 enterococcal isolates, 190 (67.8%) isolates were identified as E. faecalis, 75 (26.8%) as E. faecium and 15 (5.4%) as Enterococcus spp., using biochemical methods. Overall, 175 (62.5%) E. faecalis strains (5.62%) and 67 E. faecium strains (24%) were confirmed by the PCR method. A total of 38 strains (13.5%) have remained to the Enterococcus genus. The most of isolates 95 (39.3%) were collected from internal ward, followed by Outpatient ward with 72 (29.8%), Nephrology 26 (10.7%), ICU 22 (9.1%), Emergency 21 (8.7%), Burn 3 (1.2%), and Surgery and Cardiacare 3 (1.2%).

Antimicrobial susceptibility testing
Resistance to the majority antibiotics except for chloramphenicol, tetracycline, and quinopristin-dalfopristin was higher in E. faecium isolates than E. faecalis isolates. However, they showed good rate of sensitivity to linezolid (100%), nitrofurantoin and chloramphenicol (74.6%). All isolates of E. faecalis were susceptible to nitrofurantoin. None of the Enterococcus isolates was resistant to linezolid. The susceptibility patterns of E. faecium and E. faecalis to antibiotics are presented in Table 2. Of 175 isolates of E. faecalis, resistance of 9 and sensitivity of 166 isolates to vancomycin and teicoplanin were confirmed by microdilution broth method.

Table 2: Antibiotic resistance behavior of Enterococci isolates with disk diffusion

| Antimicrobial agent | Percent of E. faecalis isolates (n=175) | Percent of E. faecium isolates (n=67) | Total |
|---------------------|----------------------------------------|--------------------------------------|-------|
|                     | S          | I         | R  | S       | I         | R  | S | I   | R  |
| Vancomycin          | 95         | 0         | 5  | 24      | 0         | 76 | 75.2 | 0 | 24.8 |
| Teicoplanin         | 95         | 0         | 5  | 27      | 0         | 73 | 76  | 0 | 24  |
| Ampicillin          | 96.6       | 0         | 3.4 | 39.3    | 0         | 62.7 | 80.2 | 0 | 19.8 |
| Tetracycline        | 9.7        | 2.3       | 88 | 21      | 6         | 73  | 12.8 | 3.3 | 83.9 |
| Ciprofloxacin       | 36.6       | 24        | 39.4 | 0       | 19.4      | 80.6 | 26.5 | 22.7 | 50.8 |
| Norfloxacin         | 63         | 4         | 33 | 0       | 16.4      | 83.6 | 45.5 | 7.4 | 47.1 |
| Erythromycin        | 26.3       | 11.4      | 62.3 | 0       | 13.4      | 86.6 | 19  | 12  | 69  |
| Sycnerd             | 4.6        | 0         | 95.4 | 24      | 6         | 70  | 10  | 1.6 | 88.4 |
| Chloramphenicol     | 54.3       | 13.1      | 32.6 | 74.6    | 15        | 10.4 | 60  | 13.6 | 26.4 |
| Gentamicin          | 56         | 8         | 36 | 1.5     | 6         | 92.5 | 41  | 734 | 51.6 |
| Nitrofurantoin      | 100        | 0         | 0  | 74.6    | 0         | 25.4 | 93  | 0   | 7   |
| Linezolid           | 100        | 0         | 0  | 100     | 0         | 0    | 100 | 0   | 0   |

Of 67 isolates of E. faecium, 51 strains were resistant to vancomycin by disk diffusion method, but resistance of 49 strains to vancomycin was confirmed by Microdilution Broth. Two strains of E. faecium determined as resistant strains by disk diffusion using Microdilution Broth were identified as intermediate strains, corresponded with identification vanB gene in these two strains (Table 3).
Analysis of vanA-vanB-vanD types vancomycin resistance genes

All VRE₃ and 49 of VRE₄ strains (96.7%) had high-level resistance to vancomycin and teicoplanin carried the vanA gene. Two VRE₄ isolates (3.3%) had moderate-level resistant to vancomycin with MIC=8 µg /ml and were insensitive to teicoplanin, carried the vanB gene, vanD gene was identified in none of VRE strain.

Table 3: Results of MIC for glycopeptides antibiotics of vancomycin and teicoplanin in E. faecium and E. faecalis strains

| Antimicrobial agent | E. faecium | E. faecalis |
|---------------------|------------|------------|
|                     | Teicoplanin| Vancomycin | Teicoplanin | Vancomycin |
| Sensitivity Status  |            |            |            |            |
| S                   | 8≤         | 4≤         | 8≤         | 8≤         |
| R                   | 32≥        | 32≥        | 32≥        | 32≥        |
| MIC (µg/ml)         |            |            |            |            |
| Number              | 18         | 16         | 2          | 166        |
| Percent (%)         | 26.9       | 73.1       | 3          | 95         |
|                     | 24         | 73         | 95         | 5          |
|                     | 49         | 166        | 23         | 5          |
|                     | 175        | 166        |            |            |

Results of statistical analysis

Using SPSS software, there were significant correlations between the disk diffusion agar and broth microdilution methods for vancomycin and teicoplanin antibiotics (P≤0.001); and between PCR and MIC of VRE. The prevalence of vancomycin-resistant compared to susceptible strains were resistant to greater number of antibiotic classes.

Prevalence of virulence genes in E. faecalis and E. faecium strains

Among the E. faecalis strains, the asa₁ gene was the most prevalent factor, followed by the esp and bhl genes; additionally, in E. faecium strains, the asa₁ gene was the highest prevalence and bhl gene has the lowest frequency, followed by the esp gene.

Discussion

In the present study, of 280 enterococcal isolates, 175 isolates were identified as E. faecalis, 67 as E. faecium and 38 as Enterococcus spp. The prevalence of E. faecalis strains were reported 76% and 55.5% respectively (15, 9). In Iran, the prevalence of E. faecalis and E. faecium strains were reported 77.5% and 22.5%, 70.4% and 18.5%, 85.3% and 10.8%, 28% and 71%, respectively (8, 16-18). VRE strains are resistant to different classes of antibiotics simultaneously. Rising high-level resistance to penicillin, ampicillin and aminoglycosides has been demonstrated in recent years, especially in vancomycin-resistant E. faecium strains. Multidrug-resistant strains of Enterococci, especially E. faecalis and E. faecium are serious problems in treatment patients with enterococcal infections due to improper use of antibiotics (19). In the current study, 85% of strains of E. faecalis and all E. faecium strains had multidrug resistance, but strains of vancomycin-resistant compared to susceptible strains were resistant to greater number of antibiotic classes.

In the current study, the prevalence of vancomycin resistance was found 24%, which was consistent with some previous (9, 20). The prevalence of vancomycin resistance in Enterococcus strains were reported 23.3% and 29%, respectively. Similar studies in Iran reported the prevalence of vancomycin resistance in Enterococcus strains as 24.10%, 16.9%, 14.6%, 25%, 22%, respectively (16, 18, 21-23). According to typing results of genotypes for resistance to vancomycin, vanA-type was the most common genotype seen among VRE strains obtained in Hamadan and vanB type was the second. Majority of the VRE isolates (96.8%) have the vanA gene, 3.3% of VRE isolates with vanC, and vanB genotype was identified in any VRE strains (24).

All VRE₃ strains (100%) carrying the vanA gene; but vanB-C-D-E-G genotypes were not reported (25). The frequency of genes vanA and vanB among VRE strains were identified 85% and 15%, 62.5% and 37.5%, 69.23% and 15.38% respective-
ly (16). Increase in the prevalence of VRE, especially *E. faecium*, in different countries has been attributed mainly to the incidence and diffusion of *vanA* and *vanB* positive VRE, which exhibited some virulence factors such as Esp (*esp*), cytolysin (*cyl*), and hyaluronidase (*bhl*). The extracellular surface protein (*esp*), encoded by the chromosomal *esp* gene, found on pathogenicity island in multi-drug-resistant pathogenic lineages of both *E. faecalis* and *E. faecium* strains. Esp is a cell wall-associated protein which contribute to the colonization and persistence of *E. faecalis* strains in ascending infections of the urinary tract. In addition, Esp may participate in biofilm formation, and may also be involved in antimicrobial resistance (26, 27). Blood and wound infections caused by *Enterococci* strains are mediated by Esp protein (28). In a study, the gene *esp* was detected only in *E. faecalis* strains (29). However, incidence of the gene *esp* in clinical *E. faecium* are increasing compared to clinical *E. faecalis* isolates (30).

In the present study, prevalence of the genes *esp* and *bhl* were significantly higher among ampicillin-resistant VRE FM isolates (53.7%, 37.3%) compared to ampicillin-susceptible VRE FM isolates (19.4%, 22.4%). This finding is in accordance with the reported results of other related studies (32-34). In our study, the frequency of the gene *asa*, (which encodes aggregation substance) among *E. faecalis* was as high as 97 percent and among *E. faecium* strains was as high as 100 percent. This gene has a high incidence in *E. faecalis*, as well. Results of studies on clinical *E. faecium* isolates are contradictory. Previous studies detected this virulence factor among 5%, 65% of VRE FM and 2.7%, 60% of VRE FM strains (26, 35). S Jahangiri et al. (11) did not found gene *asa*, in either 49 of VRE FM strains or 17 of VSE FM strains. Hyaluronidase, coded by the chromosomal gene *bhl*, is a degradative enzyme associated with tissue damage that influence on the hyaluronic acid (hyaluronate, HA) (33). We found the *bhl* gene among 49.3% of VRE FM isolates and 22.4% of VSE FM isolates, which is in accordance to Rice et al. results (36), who detected the *bhl* gene among 71% of the United Kingdom VRE FM isolates; but it was in contrast to another study (11), who detected gene *bhl* among 80% of VSE FM isolates and in 28.5% of VRE FM isolates. Most of *esp*-positive isolates were resistant to more than 3 antibiotics (11, 37). Laud B et al. (38) demonstrated that the strong correlation between the carriage of gene *esp* and antimicrobial resistance could be due to the higher conjugation frequencies in strains carrying the *esp* gene than strains lacking this gene. *E. faecium* strains carrying the gene *esp* were resistant to more than 90% of the antibiotics tested and 64% of *E. faecalis* strains were resistant to vancomycin (5).

Considering these results, the gene *esp* facilitates *E. faecium* isolates ability to acquire antibiotic resistance genes. The expression level of gene *esp* depending on growth conditions constantly vary between strains of *E. faecium* and is associated with initial connection and biofilm formation (39).

**Conclusion**

Due to increasing resistance rate of *Enterococci* to most common antibiotics, applying the preventive and control measures are required.

**Ethical considerations**

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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The authors declare that there is no conflict of interests.

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