Iran J Public Health, Vol. 45, No.6, Jun 2016, pp.806-813

Original Article

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

Mona NASAJ [1], Seyed Masoud MOUSAVI [1], Seyed Mostafa HOSSEINI [1], *Mohammad Reza ARABESTANI [1, 2]

1. *Dept. of Microbiology, Faculty of Medicine, Hamadan University of Medical Sciences, Hamadan, Iran*
2. *Brucellosis Research Center, Hamadan University of Medical Sciences, Hamadan, Iran*

***Corresponding Author:** Email: Mohammad.arabestani@gmail.com

(Received 10 Sep 2015; accepted 15 Dec 2015)

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* isolated 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_1* 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

important 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 1,024 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 (*Cyl*), 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 *hyl* 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_1 (aggregation substance), *gelE* (*gelatinase*), *hyl* (*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%). *Enterococcus* 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 ºC for 5 min, followed by amplification by 30 cycles of denaturation at 95 ºC for 30 sec, annealing at 52.5 ºC for 30 sec and elongation at 72 ºC for 1 min, and a final extension at 72 ºC for 10 min. The *E. faecalis* ATCC 29212 and *E. faecium* BM4147 were used as quality control strains.

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 according 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
asa_1	F: GCACGCTATTACGAACTATGA R: TAAGAAAGAACATCACCACGA	375	13
hyl	F: ACAGAAGAGCTGCAGGAAATG R: GACTGACGTCCAAGTTTCCAA	276	13
esp	F: AGATTTCATCTTTGATTCTTGG R: AATTGATTCTTTAGCATCTGG	510	13
ddl E. faecalis	F: ATCAAGTACAGTTAGTCTTTATTAG R: ACGATTCAAAGCTAACTGAATCAGT	941	12
ddl E. faecium	F: TTGAGGCAGACCAGATTGACG R: TATGACAGCGACTCCGATTCC	658	12
vanA	F: GGGAAAACGACAATTGC R: GTACAATGCGGCCGTTA	732	12
vanB	F: ATGGGAAGCCGATAGTC R: GATTTCGTTCCTCGACC	635	12
vanD	F: TGTGGGATGCGATATTCAA R: TGCAGCCAAGTATCCGGTAA	500	14

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_1 by PCR

Identify of three virulence determinants in all *E. faecalis* and *E. faecium* isolates was performed by Multiplex PCR *(esp, asa_1)* 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_1, 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 *hyl*, 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_1 positive), *E. faecium* C68 (*hyl* 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 Surgeryandcardiaccare 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
Synercid	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 VRE_{fs} and 49 of VRE_{fm} strains (96.7%) had high-level resistance to vancomycin and teicoplanin carried the *vanA* gene. Two VRE_{fm} 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.

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

Among the *E. faecalis* strains, the asa_1 gene was the most prevalent factor, followed by the *esp* and *hyl* genes; additionally, in *E. faecium* strains, the asa_1 gene was the highest prevalence and *hyl* gene has the lowest frequency, followed by the *esp* gene.

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	R	S	I	R	S	R	S	R
MIC (μg/ml)	8≤	32≥	4≤	8-16	32≥	8≤	32≥	4≤	32≥
Number	18	49	16	2	49	166	6	166	9
Percent (%)	26.9	73.1	24	3	73	95	5	95	5

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_{Vanco} and MIC_{Teico} results (P≤0.001) in strains of *E. faecalis* and *E. faecium.*

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* is 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 VRE_{fm} 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 (*hyl*). The extracellular surface protein *(esp),* encoded by the chromosomal *esp* gene, found on pathogenicity island in multidrug-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 *hyl* 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_1, (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_{fs} strains (26, 35). S Jahangiri et al. (11) did not found gene asa_1 in either 49 of VRE_{fm} strains or 17 of VSE_{fm} strains.

Hyaluronidase, coded by the chromosomal gene *hyl*, is a degradative enzyme associated with tissue damage that influence on the hyaluronic acid (hyaluronate, HA) (33). We found the *hyl* 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 *hyl* gene among 71% of the United Kingdom VRE_{fm} isolates; but it was in contrast to another study (11), who detected gene *hyl* 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. faecium* 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.

Acknowledgement

The authors declare that there is no conflict of interests.

References

1. Xu H, Tian R, Chen D, Xiao F, Nie ZY, Hu YJ, et al. (2011). Nosocomial spread of hospital-adapted CC17 vancomycin-resistant *Enterococcus faecium* in a tertiary-care hospital of Beijing, China. *Chin Med J*, 124 (4):498-503.
2. Vagnerova I, Sauer P, Kolar M, Slepickova S, Hubacek J, Faber E, et al (2006). Sources and pathways of spread of vancomycin-resistant en-

terococci in hemato-oncological patients. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub*, 150 (1):117-20.
3. Batistão DWdF, Gontijo-Filho PP, Conceição N, Oliveira AG, Ribas RM (2012). Risk factors for vancomycin-resistant enterococci colonization in critically ill patients. *Mem Inst Oswaldo Cruz*, 107(1):57-63.
4. Hegstad K, Giske CG, Haldorsen B, Matuschek E, Schønning K, Leegaard TM, et al (2014). Performance of the EUCAST disk diffusion method, the CLSI agar screen method, and the Vitek 2 automated antimicrobial susceptibility testing system for detection of clinical isolates of enterococci with low-and medium-level VanB-type vancomycin resistance: a multicenter study. *J Clin Microbiol*, 52 (5):1582-9.
5. Sharifi Y, Hasani A, Ghotaslou R, Varshochi M, Hasani A, Aghazadeh M, et al (2012). Survey of virulence determinants among vancomycin resistant Enterococcus faecalis and Enterococcus faecium isolated from clinical specimens of hospitalized patients of North west of Iran. *Open Microbiol J*, 6:34-39.
6. Ji GY, Son BR, Kim JW (2014). Development of a Novel Immunochromatographic Assay for Rapid Detection of VanA Ligase-Producing Vancomycin-Resistant Enterococci. *J Microbiol Biotechnol*, 24:427-30.
7. Song JY, Cheong HJ, Seo YB, Kim IS, Heo JY, Noh JY, et al (2013). Clinical and microbiological characteristics of vancomycin-resistant enterococci with the VanD phenotype and vanA genotype. *Jpn J Infect Dis*, 66 (1):1-5.
8. Feizabadi MM, Sayadi S, Shokrzadeh L, Parvin M,YadegaryniA D (2008). Increase in prevalence of vancomycin resistant isolates of *Enterococcous faecium* at Labbafinejad hospital. *Clin Infect Dis*, 3 (2):123-26.
9. Mira M-U, Deana M, Zora J, Vera G, Biljana M, Biljan R (2014). Prevalence of different enterococcal species isolated from blood and their susceptibility to antimicrobial drugs in Vojvodina, Serbia, 2011-2013. *Afr J Microbiol Res*, 8 (8):819-24.
10. Kariyama R, Mitsuhata R, Chow JW, Clewell DB, Kumon H (2000). Simple and reliable multiplex PCR assay for surveillance isolates of vancomycin-resistant enterococci. *J Clin Microbiol*, 38 (8):3092-5.
11. Jahangiri S, Talebi M, Eslami G, Pourshafie MR (2010). Prevalence of virulence factors and antibiotic resistance in vancomycin-resistant *Enterococcus faecium* isolated from sewage and clinical samples in Iran. *Indian J Med Microbiol*, 28 (4):337-41.
12. Dutka-Malen S, Evers S, Courvalin P. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. *J Clin Microbiol,* 1995;33(1):24-7.
13. Creti R, Imperi M, Bertuccini L, Fabretti F, Orefici G, Di Rosa R, et al (2004). Survey for virulence determinants among *Enterococcus faecalis* isolated from different sources. *J Med Microbiol*, 53 (p1):13-20.
14. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Third Informational Supplement. CLSI document M100-S23. Wayne, PA: Clin Labora Stand Institu. 2013.
15. Mulla S, Patel KG, Panwala T, Rewadiwala S (2012). Prevalence of enterococci with higher resistance level in a tertiary care hospital: a matter of concern. *Nat J Med Rse*, 2 (1):25-27.
16. Ghalandarzadeh DZ, Javadpour S, Kargar M (2013). Frequency of vana & vanb genes in vancomycin-resistant enterococci isolated from clinical specimens at shahid mohammadi hospitals bandar abbass. *J Microbiol Wrd*, 6 (114):23-33.
17. Mohammadi F, Tabaraie B, Sadeghifard N, Ghafoorian S, Maleki A, Davoodian E, et al (2011). Evaluation of drug resistance frequency among *Entrococci faecium* and *E. faecalis* strains and detection of VanA/B genes in vancomycin resistance isolated by PCR method in ilam and kermanshah hospitals. *J Ilam Univ Med Sci,* 19:1-8.
18. Rafiei Tabatabaei S, Karimi A, Navidinia M, Fallah F, Tavakkoly Fard A, Rahbar M (2012). A study on prevalence of vancomycin-resistant enterococci carriers admitted in a children hospital in Iran. *Ann Biol Res*,3 (12):5441-45.
19. Daikos GL, Bamias G, Kattamis C, Zervos MJ, Chow JW, Christakis G, et al. (2003). Structures, locations, and transfer frequencies of genetic elements conferring high-level gentamicin resistance in *Enterococcus faecalis* isolates in Greece. *Antimicrob Agents Chemother*, 47 (12):3950-3.
20. Khair H, VanTassell P, Henderson J, Warren DK, Marschall J (2013). Vancomycin resistance has no influence on outcomes of enterococcal bacteriuria. *J Hosp Infect*, 85 (3):183-8.

21. Hoseinizadeh A, Abtahi H, ShojaPour M, Akbari M, Nazari R , Sofian M (2012). Prevalence and antimicrobial susceptibility pattern of vancomycin resistant enterococci isolated from clinical sample of educational hospitals in Arak. *Arak Med Univ J*, 15 (6):11-6.
22. Nateghian A, Robinson J, Arjmandi K, Vosough P, Karimi A, Behzad A, et al. (2011). Epidemiology of vancomycin-resistant enterococci in children with acute lymphoblastic leukemia at two referral centers in Tehran, Iran: a descriptive study. *Int J Infect Dis*, 15 (5):e332-5.
23. Shaghaghian S, Pourabbas B, Alborzi A, Askarian M, Mardaneh J (2012). Vancomycin-Resistant *Entrococci* colonization in chronic hemodialysis patients and its risk factors in southern Iran (2005-2006). *Iran Red Crescent Med J*, 14 (10):686-691.
24. Praharaj I, Sujatha S, Parija SC (2013). Phenotypic & genotypic characterization of vancomycin resistant *Enterococcus* isolates from clinical specimens. *Indian J Med Res*, 138(4): 549–556.
25. Resende M, Caierão J, Prates JG, Narvaez GA, Dias CA, d'Azevedo PA (2014). Emergence of vanA vancomycin-resistant *Enterococcus faecium* in a hospital in Porto Alegre, South Brazil. *J Infect Dev Ctries*, 8 (2):160-7.
26. Comerlato CB, Resende MCCd, Caierao J, Caierão J, d'Azevedo PA (2013). Presence of virulence factors in *Enterococcus faecalis* and *Enterococcus faecium* susceptible and resistant to vancomycin. *Mem Inst Oswaldo Cruz*, 108 (5):590-5.
27. Sharifi Y, Hasani A, Ghotaslou R, Aghazadeh M, Milani M, Bazmany A (2013). Virulence and antimicrobial resistance in Enterococci isolated from urinary tract infections. *Adv Pharm Bull*, 3 (1):197-201.
28. Kafil HS, Mobarez AM, Moghadam MF (2012). Multidrug resistant and most virulent *Enterococcus faecium* (strain 2653), isolated from hospitalized patient wound in Iran. *Schol J Med*, 2 (3):36-9.
29. Shankar V, Baghdayan AS, Huycke MM, Lindahl G, Gilmore MS (1999). Infection-derived *Enterococcus faecalis* strains are enriched in *esp*, a gene encoding a novel surface protein. *Infect Immun*, 67 (1):193-200.
30. Eaton TJ, Gasson MJ (2001). Molecular Screening of *Enterococcus* Virulence Determinants and Potential for Genetic Exchange between Food and Medical Isolates. *Appl Environ Microbiol*, 67 (4):1628-35.
31. Medeiros AW, Pereira RI, Oliveira DVd, Martins PD, d'Azevedo PA, Van der Sand S, et al. (2014). Molecular detection of virulence factors among food and clinical *Enterococcus faecalis* strains in South Brazil. *Braz J Microbiol*, 45 (1):327-32.
32. Leavis HL, Willems RJ, Top J, Spalburg E, Mascini EM, Fluit AC, et al (2003). Epidemic and non-epidemic multidrug-resistant *Enterococcus faecium*. *Emerg Infect Dis*, 9 (9):1108-1115.
33. Terkuran M, Erginkaya Z, ÜNAL E, Güran M, Kızılyıldırım S, Ugur G, et al. (2014). The relationship between virulence factors and vancomycin resistance among Enterococci collected from food and human samples in Southern Turkey. *Ankara Univ Vet Fak Derg*, 61:133-40.
34. Worth L, Slavin M, Vankerckhoven V, Goossens H, Grabsch EA, Thursky KA (2008). Virulence determinants in vancomycin-resistant *Enterococcus faecium* vanB: clonal distribution, prevalence and significance of esp and hyl in Australian patients with haematological disorders. *J Hosp Infect*, 68 (2):137-44.
35. Kowalska-Krochmal B, Dworniczek E, Dolna I, Bania J, Wałecka E, Seniuk A, et al (2011). Resistance patterns and occurrence of virulence determinants among GRE strains in southwestern Poland. *Adv Med Sci*, 56 (2):304-10.
36. Rice LB, Carias L, Rudin S, Vael C, Goossens H, Konstabel C et al (2003). A potential virulence gene, hylEfm, predominates in *Enterococcus faecium* of clinical origin. *J Infect Dis*, 187 (3):508-12.
37. Duprè I, Zanetti S, Schito AM, Fadda G, Sechi LA (2003). Incidence of virulence determinants in clinical *Enterococcus faecium* and *Enterococcus faecalis* isolates collected in Sardinia (Italy). *J Med Microbiol*, 52 (Pt 6):491-8.
38. Lund B, Edlund C (2003). Bloodstream isolates of Enterococcus faecium enriched with the enterococcal surface protein gene, esp, show increased adhesion to eukaryotic cells. *J Clin Microbiol*, 41 (11):5183-5.
39. Van Wamel WJ, Hendrickx AP, Bonten MJ, Top J, Posthuma G, Willems RJ (2007). Growth condition-dependent Esp expression by *Enterococcus faecium* affects initial adherence and biofilm formation. *Infect Immun*, 75 (2):924-31.