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Detection of *Van*-Positive and Negative Vancomycin Resistant Entrococci and their Antibacterial Susceptibility Patterns to the Newly Introduced Antibiotics

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Abstract: The aim of this study was to determine the types and frequencies of VRE in terms of the existence of *Van* genes and to investigate the efficacy of newly introduced antibiotics. Totally 297 enterococcal strains were isolated from patients' specimens. Minimum inhibitory concentration of resistant isolates to vancomycin and tecoplanin were determined by E test method. Simultaneous detection of *Van* genes and species identification was preformed using multiplex PCR. Sensitivity patterns of VRE isolates to several antibiotics were determined by disk diffusion (Kirby-Bauer) method. One hundred and four (35%) of the isolates were VRE of which 12.5, 10.5 and 7% from urine, blood and stool samples were detected, respectively. Resistant isolates were sensitive to tigecycline and linezolid and resistant to ciprofloxacin and amikacin. The isolates which were resistant to ciprofloxacin, amikacin and gentamicin also showed cross-resistance to the other tested antibiotics. *VanA* is the predominant gene of *Van*-positive isolates in Iran. Meanwhile, the prevalence of *Van*-negative intermediate VRE in *E. fecalis* is markedly increased. These findings lend support to the hypothesis that due to frequent vancomycin administration in our clinics the acquisition of *Van* genes as well as selection of resistant mutant isolates could be facilitated. Rational prescription of vancomycin and wisely administration of newly introduced antibiotics like tigecycline and linezolid is warranted.

Key words: Vancomycin-resistant enterococci, MIC, tigecycline, linezolid

INTRODUCTION

More than 30 different species of entrococci have been identified (Liassine *et al*., 1998; Kuriyama *et al*., 2003; Pangallo *et al*., 2008) but most human enterococcal infections are caused by *E. faecalis* and *E. faecium* (Palladino *et al*., 2003). Entrococci have been recognized as an important cause of infective endocarditis for almost a century. These important nosocomial pathogens represent the third leading cause of bacteremia and the second leading cause of UT infections in hospitals throughout the world (Liassine *et al*., 1998; Kuriyama *et al*., 2003; Matsumoto *et al*., 2004). Wide range of antibiotics includes β-lactam, macrolides, aminoglycosides and glycopeptides have been used to treat entrococcal infections (Liassine *et al*., 1998). A treatment regimen with a cell wall active agent (either a β-lactam drug or a glycopeptide such as vancomycin) in combination with aminoglycosides is recommended and practiced (Palladino *et al*., 2003). Entrococcal intrinsic resistance against cephalosporins and semisynthetic penicillin and penicillins are well known. However, acquired resistance against aminopenicillins and glycopeptides antibiotics is a subject of considerable concern (Kuriyama *et al*., 2003; Pangallo *et al*., 2008). The emergence and spread of glycopeptide (vancomycin and teicoplanin) resistance in enterococci has become a significant clinical issue and Vancomycin-Resistant Enterococci (VRE) are now an important universal problem in hospitals worldwide (Palladino *et al*., 2003; Chou *et al*., 2008), because of the reduced number of treatment options for disease management. The seven gene clusters, *VanA*, *VanB*, *VanC1*, *VanC2/3*, *VanD*, *VanE* and *VanG* causing glycopeptide resistance have been identified in enterococci. The *VanA* gene cluster encodes proteins that confer high-level resistance to both vancomycin and teicoplanin and the *VanB* gene product provides moderate to severe resistance to vancomycin but not to tecoplanin (Palladino *et al*., 2003). The *VanC1* and *VanC2/3* isolates exhibit low level resistance to vancomycin only and are specific to some species of VRE such as *E. gallinaram*, *E. casseliflavus* and *E. flavescens*. The other genes of resistance have been detected rarely (Palladino *et al*., 2003; Matsumoto *et al*., 2004). The resistant genotypes of *VanA* and *VanB* are clinically the most important because they limit therapeutic options (Palladino *et al*., 2003; Chou *et al*., 2008).

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Many studies have been conducted in Europe and the USA on the prevalence, incidence, epidemiology and risk factors of VRE, however, the data from the Middle East are not that much. In addition, the majority of the reported data in literature regarding the epidemiology of VRE also emphasize the detection of *Van*-positive isolates that exhibit high resistance to glycopeptides antibiotics. However, our preliminary study in screening of entrococcal strains isolated from the patients' samples revealed that the majority of VRE have not acquired *Van* gene and their resistance to glycopeptides antibiotics are elevated. Therefore, the purpose of this study was to determine the prevalence of *Van*-positive and *Van*-negative VRE and their corresponding important clinical impacts. In addition, antibacterial susceptibility patterns of VRE to newly available antibiotics were also determined in order to introduce alternative antibiotics of choice.

MATERIALS AND METHODS

Isolation of entrococci: Totally 297 enterococcal strains were isolated from the blood, urine, stool, ear exudates, burn site, vaginal swab, sputum and wound collected from patients, between May 2005 to March 2008 in Nemazee hospital, affiliated to Shiraz University of Medical Sciences, Shiraz, Iran. This hospital is tertiary setting facilities with one thousand beds. All the isolates were identified to species level using a strategy that included Gram's stain, motility assessment; catalase production, growth in 6.5% NaCl, L-pyrrolidonyl-b-naphthalamide hydrolysis, assimilation of (xylose, mannitol, arabinose; sorbitol), bile and esculin growth and hydrolysis, pigment production, leucine aminopeptidase activity and acidification of methyl-a-D-glucopyranoside, all as described earlier by Turenne *et al*. (1998).

Screening test for vancomycin resistance in enterococci
VRE screening test: Pure single colonies of the clinical isolates were applied to esculin agar medium containing 6 μg mL^{-1} vancomycin (Sigma Chemical, St. Louis, Mo). All black colonies growing on the esculin agar medium were identified based on biochemical test described earlier (Turenne *et al*., 1998). Colonies confirmed as VRE were then the subject of second screening plate method, described below.

VRE reconfirmation test: Single colonies of resistant entrococci isolated on esculin agar plates were inoculated in Brain Heart Infusion (BHI) broth containing 3 μg mL^{-1} vancomycin and incubated in ambient conditions to reach the turbidity equivalent to 0.5 MacFarland standards. The BHI agars supplemented with 6 μg mL^{-1} vancomycin were then inoculated by spotting 1-10 μL suspension of the bacteria. The final inoculums were 10^5 to 10^6 cfu $spot^{-1}$ and incubated at 35°C in ambient air for 24 h. The presence of more than one colony or a haze of growth indicates the resistance. *Enterococcus faecalis* (ATCC 29212) and *E. faecalis* (ATCC 51299) as negative and positive controls were tested. The results were interpreted as recommended by Clinical Laboratory Standards Institute (NCCLS, 2004).

DNA extraction: Pure entrococci colonies on BHI agar were inoculated into 3 mL brain heart infusion shaken overnight. Cells were harvested by 8000 g for 5 min. DNA extraction was carried out based on the standard protocol for Gram positive bacteria with some modification (Ligozzi and Fontana, 2003). The pellet suspend into 400-600 μL lysis solution containing lysozyme (5 mg mL^{-1} Sigma Chemical, St. Louis, Mo.), 10 mM EDTA, 10 mM tris hydrochloride (pH 8.0), achromopeptidase (3.3 mg mL^{-1}, Sigma Chemical, St. Louis, Mo) and incubated at 37°C for 45 min. The resulting suspension was heated at 95°C for 10 min and immediately transferred on ice. The lysate once extracted with phenol/chloroform and once with phenol/chlorofom/isomel alcohol precipitated in absolute ethanol at -20°C overnight. DNA was collected by centrifugation at 12000 g for 15 min and washed with ethanol 70% and dried at room temperature. The dried DNA was dissolved in 50 μL distilled water. The quantity of DNA was measured with Nanodrop (NanoDrop Technologies,Wilmington, Delaware USA) and adjusted to 500 ng μL^{-1}.

Vancomycin-resistance genotyping: DNA of all the resistant isolates was subjected to multiplex PCR to detect the presence of *VanA*, *VanB*, *VanC1*, and *VanC2/VanC3* genes. Primers and sequence of primers and size of the expected amplicons are shown in Table 1. The primers were obtained from TIB MOLBIOL Syntheselabor GmbH (Berlin, Germany), 1.5 mM $MgCl_2$; 200 μM each dATPs, dCTPs, dGTPs, and dTTPs; 50 mM KCl, 10 mM Tris-HCl, 1 U *Taq* polymerase (Fermentas, Lithvania), 2500 ng DNA in 5 μL volume were added to the reaction mixtures as follows: 5 pmol of each *VanA* primer, 2.5 pmol of the *VanB*, *VanC1*, *VanC2/C3* and *rrs* primers; 5 pmol of the *E. faecalis*-specific primers and 1.25 pmol of the *E. faecium*-specific primers in total 25 μL volume. Polymerase chain reaction was optimized under pre-denaturation at 94°C for 8 min followed by 30 cycles at 94°C for 70 sec, 54°C for 65 sec, 72°C for 95 sec and a final extension step at 72°C for 10 min. A *VanA*-positive strain (*E. faecium* ATCC 51559) and *E. faecalis* ATCC 51299

Table 1: Primers used in this study

Genes	Size of amplified product (bp)	Primers (oligonucleotide sequence)	References
VanA	1,030	5'-CATGAATAGAATAAAAGTTGCAATA-3'	Clark *et al*. (1993)
		5'-CCCCTTTAACGCTAATACGATCAA-3'	
VanB	433	5'-GTGACAAACCGGAGGCGAGGA-3'	Clark *et al*. (1993)
		5'-CCGCCATCCTCCTGCAAAAAA-3'	
VanC1	822	5'-GGTATCAAGGAAACCTC-3'	Dutka-Malen *et al*. (1995)
		5'-CTTCCGCCATCATAGCT-3'	
VanC2/C3	484	5'-CGGGGAAGATGGCAGTAT-3'	Satake *et al*. (1997)
		5'-CGCAGGGACGGTGATTTT-3'	
E. faecalis	941	5'-ATCAAGTACAGTTAGTCTTTATTAG-3'	Dutka-Malen *et al*. (1995)
		5'-ACGATTCAAAGCTAACTGAATCAGT-3'	
E. faecium	658	5'-TTGAGGCAGACCAGATTGACG-3'	Cheng *et al*. (1997)
		5'-TATGACAGCGACTCCGATTCC-3'	
Rrs (16S rRNA)	320	5'-GGATTAGATACCCTGGTAGTCC-3'	Van de Klundert and
		5'-TCGTTGCGGGACTTAACCCAAC-3'	Vliegenthart (1993)

(*Van B*) were used as positive genotypes. In each set of PCR reactions, the *rrs* gene (16S rRNA) with 320 bp in size was also included as an internal control (Table 1). Products were electrophoresed in 1.5% agarose, stained by ethidium bromide and video image were obtained by gel documentation (Uvtec, Sigma, Germany) system.

Antibacterial susceptibility testing: Susceptibility of the VRE isolates to the seven antibiotics including vancomycin (30 µg), teicoplanin (30 µg), gentamicin (120 µg), amikacin (30 µg), linezolid (30 µg), tigecycline (15 µg), quinopristin/dalfopristin (15 µg) ciprofloxacin (5 µg), were determined according to Kirby-Bauer method using Mast Co., (Mast Co., Merseyside, UK) standard disc. The MICs of the resistant isolates to vancomycin and tecoplanin were also determined by E-test (AB Biodisk, Solna, Sweden). MICs breakpoints for vancomycin and tecoplanin were determined according to the manufacturer's recommendation. American Typing Culture Collection of *E. faecalis* (ATCC 19433) was used as controls for MICs determination. The data analysis was performed by SPSS, version 15 using Fisher exact test and $p<0.05$ was considered significant.

RESULTS AND DISCUSSION

Two hundred ninety seven enterococci were isolated from the patients' specimens consisting of *E. faecalis* 180 (60%) and *E. faecium* 113 (38%) and *E. gallinarium* 4 (2%). Out of 297 entrococci, 98 (33%), 90 (30%) and 57 (19%) were isolated from the blood, urine and stool samples, respectively. Totally, 104 isolates (35%) were vancomycin resistant based on agar esculin and BHI agar screening methods. Frequencies of different species of enterococci and sources of the specimens are shown in Table 2. As demonstrated in Table 3, the results of multiplex PCR assay revealed the isolates with *VanA*-positive genes among others (*VanA*, *VanB*, *VanC1*, *VanC2/C3*) are predominant. *VanB* and *VanC2/C3* were not detected while *VanC1* and *VanC1+B* were each observed in only 1 (1%) of vancomycin resistant isolates. A spectrum of different types of *Van* genes with their corresponding species, the intermediate resistant strains along with the controls is shown in Fig. 1. The resistant isolates predominately consisted of *E. faecalis* (62.5 %) and *E. faecium* (35.5 %), but the frequency of *Van*-positive (*VanA*) gene in *E. faecium* was more noticeable. Tigecycline and linezolid were more effective against VRE, compared to ciprofloxacin and amikacin *in vitro*. Statistical

Table 2: Sources and frequencies of vancomycin sensitive and resistant isolates of entrococci

	No. of specimens	Frequency and percentage of resistant species			
Sources	(%)	*E. faecalis*	*E. faecium*	*E. gallinarium*	Total
Urine	98 (33)	23 (8.0)	11 (3.5)	2 (1)	36 (12.5)
Blood	90 (30)	21 (7.5)	10 (3.0)		31 (10.5)
Stool	57 (19)	10 (3.5)	11 (3.5)		20 (7.0)
Burn site	20 (7)	3 (1.0)	3 (1.0)		6 (2.0)
Vagina	12 (4)	2 (0.5)	2 (1.0)		5 (1.5)
Wounds	9 (3)	2 (0.5)			2 (0.5)
Sputum	6 (2)	2 (0.5)			2 (0.5)
Ear exudates	5 (2)	2 (0.5)			2 (0.5)
Total	297 (100)	65 (22.0)	37 (12.0)	2 (1)	104 (35.0)

Table 3: Glycopeptide susceptibility of 104 *Van*-positive and *Van*-negative VRE

		MIC (µg mL^{-1})			
Genes (%)	Species	Vancomycin	Tecoplanin	No. of isolates	Total
VanA	*E. faecium*	>256	>32.0	20	32 (30.5)
		256	32.0	8	
		256	16.0	4	
	E. faecalis	256	32.0	4	5 (5)
		256	16.0	1	
VanC1	*E. gallinarium*	8	1.0	1	1 (1)
VanC1+B	*E. gallinarium*	256	0.5	1	1 (1)
Van-negative	*E. faecalis*	8	0.5	32	60 (57.5)
		16	0.75	19	
		24	0.75	9	
	E. faecium	8	0.125	3	5 (5)
		16	0.5	1	
		24	0.5	1	
Total					104 (100)

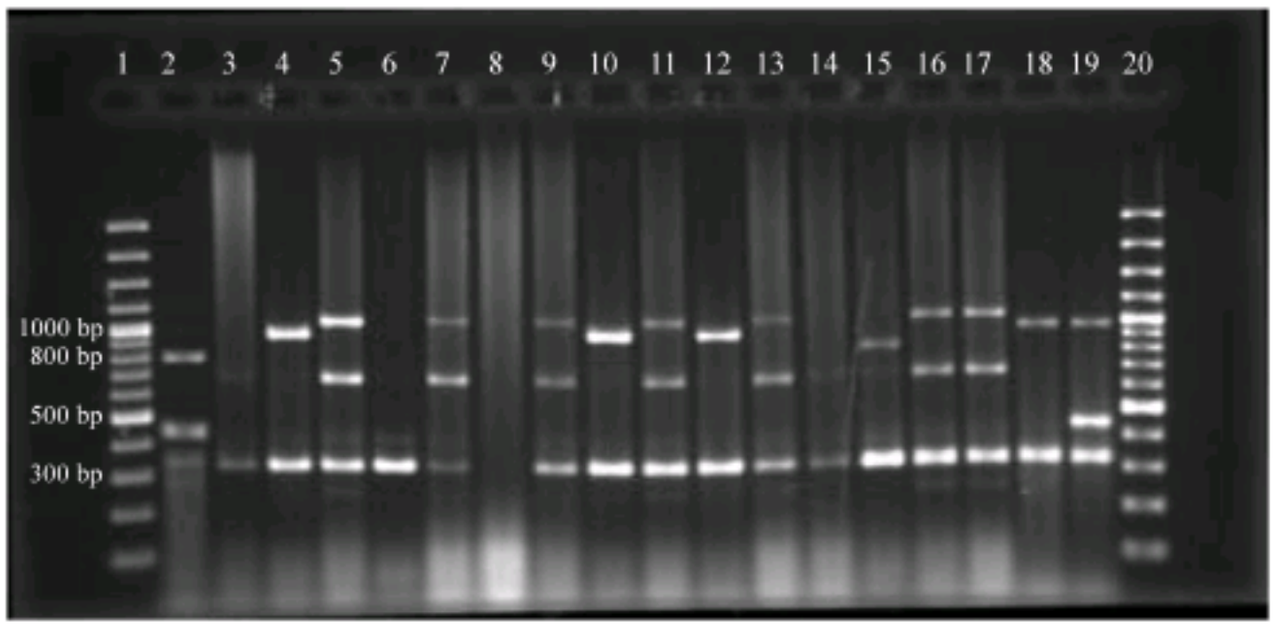

Fig. 1: Agarose gel electrophoresis of amplified *VanA*, *VanB*, *VanC1*, *VanC+B* of *E. faecium*, *E. faecalis* and *E. gallinarium* and their corresponding species by multiplex PCR. Lane 1 and 20, 100 base pairs DNA ladder, (Fermentas, Lithuania), lane 2 *E. gallinarium* (patient, *Van C1+B*), lane 3, 14 intermediate resistant *E. faecium* (patients), lane 4, 10, 12 and 18 intermediate resistant *E. faecalis* (patients) lanes 5 *E. faecium* ATCC 51559 (*VanA*), lane 6 Pediococcus isolates (rrs internal control), lanes 7, 9, 11, 13, 16, and17 *VanA*-positive *E. faecium* (patients), lane 8 control negative (no DNA template), lane 15 *E. gallinarium* (patient, *VanC*) lane 19, *E. faecalis* ATCC 51299 (*Van B*)

Table 4: Patterns of antibiotics susceptibility for 104 *Van*-positive and intermediate resistant *Van*-negative isolates

Species N	Genotype	Pattern	TGC	QD	LZD	TEC*	p-value	CIP	AK	GM*	p-value
E. faecalis	*Van*-negative	S	41 (69)	7 (11)	47 (80)	55 (92)	0.0001	3 (5)	8 (13)	33 (54)	0.049
(N = 65)		R	-	49 (83)	-	-		34 (55)	48 (81)	25 (42)	
		IR	19 (31)	4 (6)	13 (20)	5 (8)		23 (40)	4 (6)	2 (4)	
	VanA-positive	S	5 (100)	3 (60)	4 (80)	-		-	-	-	
		R	-	2(40)	-	5 (100)		5 (100)	5 (100)	5 (100)	
		IR	-	-	1 (20)	-		-	-	-	
E. faecium	*Van*-negative	S	4 (80)	3 (60)	4 (80)	5 (100)	0.0000	-	4 (80)	3 (60)	0.037
(N = 37)		R	-	1 (20)	-	-		5 (100)	1 (20)	-	
		IR	1(20)	1 (20)	1 (20)	-		-	-	2(40)	
	VanA-positive	S	29 (90)	25 (78)	21 (66)	-		-	-	4 (13)	
		R	-	5 (16)	-	28 (87)		31 (97)	31 (97)	28 (87)	
		IR	3 (10)	2 (6)	11 (34)	4 (13)		1 (3)	1 (3)	-	
E. gallinarium	*VanC1*-positive	S	-	-	-	1 (100)		-	-	1 (100)	
(N = 1)		R	-	1 (100)	-	-		1 (100)	1 (100)	-	
		IR	1 (100)	-	1 (100)	-		-	-	-	
E. gallinarium	*VabC1+B*-positive	S	1 (100)	1 (100)	1 (100)	-		-	-	-	
(N = 1)		R	-	-	-	1 (100)		1 (100)	1 (100)	1 (100)	
		IR	-	-	-	-		-	-	-	
Total		S	80 (77)	39 (37)	77 (74)	61 (59)		3 (3)	12 (11)	34 (33)	
(N = 104)		R	-	58 (56)	0	34 (32)		77 (74)	85 (82)	43 (41)	
		IR	24 (23)	7 (7)	27 (26)	9 (9)		24 (23)	7 (7)	27 (26)	

Antibiotics: TGC: Tegicycline, QD: Quinopristin/dalfopristin, LZD: Linezolid, TEC: Tecoplanin; CIP: Ciprofloxacin, AK: Amikacin, GM: Gentamicin. *Statistical difference between intermediate resistant isolates (*Van*-negative) and *Van*-positive VRE for sensitivity to tecoplanin and gentamicin was significant

difference between intermediate resistant isolates (*Van*-negative) and *Van*-positive VRE for sensitivity to tecoplanin and gentamicin was significant (Table 4). Decreased sensitivity of entrococci to quinopristin/ dalfopristin was detected *in vitro*. Cross-resistance of vancomycin resistant *E. faecium* containing *VanA* gene to the tested antibiotics was investigated and shown in Table 5. All the isolates resistant to ciprofloxacin also showed cross-resistance to the other tested antibiotics.

Table 5: Cross-resistance of *VanA*-positive *E. faecium* to the tested antibiotics

		No. of isolates and percent (%) resistant to					
Antibiotics	n	VA	GM	AK	CIP	TEC	QD
VA	32		28 (88)	31(97)	32 (100)	30 (94)	5 (16)
GM	28	28 (100)		28 (100)	28 (100)	26 (93)	5 (18)
AK	31	31 (100)	28 (90)		31 (100)	29 (94)	5 (16)
CIP	31	31 (100)	29 (94)	31 (100)		30 (97)	5 (16)
TEC	28	28 (100)	24 (86)	27 (96)	28 (100)		4 (14)
QD	5	5 (100)	5 (100)	5 (100)	5 (100)	4 (80)	

VA: Vancomycin, GM: Gentamicin. AK: Amikacin, CIP: Ciprofloxacin, TEC: Tecoplanin, QD: Quinopristin/dalfopristin

Enterococci comprise 1% of the human intestinal micro flora. Despite *E. faecalis* and *E. faecium* are the most common species isolated from the human feces, they are also the most common agents recovered from enterococcal infectious diseases. In the present study, 180 (60%) *E. faecalis* and 113 (38%) *E. faecium* were isolated from the patients' samples. Similar results were also obtained in other studies for clinical isolates in Iran. (Feizabadi *et al*., 2004; Emaneini *et al*., 2008), European countries (Liassine *et al*., 1998; Kuriyama *et al*., 2003) and United States (Gordon *et al*., 1992). Enterococcal infections have received much attention after the emergence of isolates resistant to glycopeptide antibiotics (CDC, 1993; Cetinkaya *et al*., 2000). In Europe, enterococci have been isolated from livestock, small animals and healthy people (van den Braak *et al*., 1998; Cetinkaya *et al*., 2000). In contrast, the community reservoir seems to be absent in the USA, where VREs pose an alarming problem in hospitals (McDonald *et al*., 1997).

Several PCR protocols have been developed to identify enterococcal species and to detect glycopeptide resistance genotypes (Dutka-Malen *et al*., 1995; Woodford *et al*., 1997). They found 95% agreement between genotypic and phenotypic methods (Woodford *et al*., 1997). In this investigation, primers concentration, annealing temperature, amplification cycles with pure and defined concentration of template DNA were carefully adjusted in order to optimize a multiplex PCR assay that allows simultaneous detection of *Van* genes and the bacteria species. Our optimized PCR method has 100% agreement in term of species identification with phenotypic method and could detect two main human species and their associated *Van* genes. The only limitation was the absence of primers to detect *E. gallinarium*. Precise and quick identification of resistant entrococci could help clinicians to timely administer appropriate antibiotics which may be lifesaving.

Sixty five (62.5%) of the resistant isolates were *Van*-negative with multiplex PCR assay. These isolates showed low level of resistance to vancomycin (MICs = 8-24 μL mL^{-1}). We adhered to the criteria recommended by Clinical Laboratory Standard Institute (CLSI) to isolate resistant bacteria. To reduce the chance of vancomycin sensitive isolates to be recovered, after the first agar screening method, second selective procedures in two steps were followed. Therefore, the resulting isolates could be true intermediate VRE. The possible mechanism for the emerging of these intermediate VRE could be due to the high mutation frequency in ligase enzyme joining the two molecules of D-alanyl-D-alanine which is then added to UDP-N-acetylmuramyltripeptide to form the UDP-N-acetylmuramyl-pentapeptide. The UDP-N-acetylmuramyl-pentapeptide, when incorporated into the nascent peptidoglycan (transglycosylation), permits the formation of cross-bridges (transpeptidation) and contributes to the strength of the peptidoglycan layer (Eliopoulos, 1997). It has been hypothesized that a high mutation frequency in mutant isolates could happen due to its inability to repair mismatches (Schaaff *et al*., 2002). Mutant isolates, therefore, may not produce very strong D-alanyl-D-alanine termini where it is the target site for vancomycin to block cross-link formation in sensitive isolates. Alternatively, mutant isolates could emerge due to produced remarkably thickened cell wall with an increased proportion of glutamine nonamidiated mucopeptides. Presence or absence of glucose or glutamine that influence cell wall thickness have been proven in vancomycin intermediate resistant staphylococcus (Gemmell, 2004). Similar mechanism of resistance may contribute to the emerging vancomycin intermediate resistant entrococci. Continuous administration of vancomycin for prophylactic, empiric or treatment purposes could exert high pressure on sensitive isolates. As a consequence, this pressure can accelerate selection of both *Van*-positive (acquired resistant) and *Van*-negative (resistant mutant) entrococci from the pool of microflora of urogenital and gastrointestinal organs of colonized and other infected sites of patients (Talon *et al*., 2001). Experimentally vancomycin resistant mutants have been isolated by *in vitro* stepwise passage of *S. aureus* in media containing increasing concentration of vancomycin (Arthur *et al*., 1993). It has been well established that the transfer of *VanA* genes could happen via gene exchange through of mobile genetic elements such as transposon, plasmid or integrons (Arthur *et al*., 1993; Arthur and Courvalin, 1993). Emerging of vancomycin resistant *Staphylococcus aureus* (VRSA) identical to *VanA* of *E. fecalis* suggests the transfer of *VanA* gene from *E. fecalis* to *S. aureus* (Chang *et al*., 2003; Flannagan *et al*., 2003). Unfortunately, the transfer of *VanA* gene to methicillin resistant *Staphylococcus aureus* (MRSA) could have adverse clinical and nosocomial consequences. In the present study, *VanA* isolates mainly consisted of *E. feceium*. Similar data were reported earlier in other parts of Iran (Feizabadi *et al*., 2004; Emaneini *et al*., 2008) (11, 12), however, the reports from Europe and USA are different in terms of the genotype and prevalence of the resistant isolates (Malani *et al*., 2002; Libisch *et al*., 2008). Nevertheless, in consistent with some earlier international reports, *E. feceium* acquired resistance was of higher frequency as compared to *E. faecalis* (Hryniewicz *et al*., 1998; Wu *et al*., 2004).

Difference in rates of acquisition of *Van* resistance genes may lead us to speculate that *E. feceium* might have more efficient gene capturing system such as integron. These types of integron are well known in acinetobacter (Wu *et al*., 2004; Turton *et al*., 2005). To prove this hypothesis, conducting appropriate genetics studies such as PCR detecting assay, determining the sequence of integron and *in vitro* conjugation experiments could be informative. Nevertheless, regardless of the mechanisms of vancomycin resistances, the emerging of VRE could have negative impacts on clinics which might be reflecting in both management and the cost of patients' treatments.

There were significant statistical differences between sensitivities of *Van*-positive and *Van*-negative intermediate VRE to gentamicin and tecoplanin which might be due to concordant transfer of *VanA* and gentamicin resistance genes in *VanA* isolates (Perlada *et al*., 1997; Nelson *et al*., 2000). In addition, almost all the isolates with *VanA* gene showed cross-resistance to ciprofloxacin and amikacin, indicating these antibiotics may not be suitable for the treatment of *VanA* positive patients. Present VRE were sensitive to the two newly introduced antibiotics (tigecycline and linezolid). However, the availability and cost of treatment of the patients with new antibiotics must be taken into account. These antibiotics are not yet easily available to the clinicians and the cost of treatment using them is much higher than that with vancomycin (Townsend *et al*., 2006). Therefore, short term strategy to control resistant isolates should be based on prudent administration of conventional and relatively effective antibiotics and implementation of strict control measures in the hospitals while in the long run, clinical administration of the newly introduced effective anti-VRE antibiotics is mandatory. Recently, it was shown that 5.3% of our hospital personnel carry MRSA in their noises (Askarian *et al*., 2009). Similar situations may stand for VRE which may urge us to increase control measures seriously. Entrococci is normal flora of the intestine; therefore, VRE colonization of hospital personnel can act as a potential source for resistance transmission especially via infected hands. It is advisable to determine the suitability of antibiotics for the treatment of specific infections before prescription of the newly introduced antibiotics in clinics. Quinopristin/dalfopristin is an example of such a case, which is suggested to be an anti-VRE antibiotic but is not used in our clinics. Only 37% of VRE isolates were found to be sensitive to it i*n vitro* (Table 4). In this study, it was revealed that *Van*-negative VRE is the predominant type and *VanA* is the principal type for *Van*-positive isolates in Iran. Based on the present results two effective antibiotics against VRE could be tigecycline and linezolid.

CONCLUSION

Considering the above findings, we can conclude that the rational use of conventional effective antibiotics, periodical surveillance studies in order to monitor changes in enterococcal resistance patterns and preventive measures against the spread of genetically-related resistant isolates can alleviate the situation. Furthermore, concomitant control of MRSA and VRE along with prudent and programmed administration of the newly effective introduced antibiotics such as tigecycline and linezolid are also recommended.

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