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Original Article

Characterization of Vancomycin Resistant *Enterococcus faecium*

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Abstract

Background: To determine the species distribution, updated drug susceptibility patterns and genes conferring resistance in clinical vancomycin resistant enterococcal (VRE) isolates.

Methods: Clinical enterococcal isolates collected during 7 months, from September 2005 to April 2006 from hospitalized patients and outpatients were studied. Twenty five VRE were isolated from 450 enterococci samples (5.6%). VRE isolates were subjected to antibiotic susceptibility tests. Genotype of these isolates was determined by PCR.

Results: All of the isolates were *E. faecium* and carried the *vanA* gene. Antibiotic susceptibility tests showed that the isolates were resistant to ampicillin 25(100%), ciprofloxacin 25(100%), gentamicin 24(96%), erythromycin 25(100%), tetracyclin 10(40%) and chloramphenicol 2(8%).

Conclusion: VRE strains were resistant to three antibiotics and were susceptible to new antibiotics linezolid and dalfopristin-quinupristin. Switching to treatment with these antibiotics would relieve the problem for a short time.

Keywords: *Vancomycin*, Enterococcus faecium, *Iran*

Introduction

Enterococci are important causes of nosocomial infections that are often recovered from clinical samples of patients at many hospitals throughout the world including Iranian hospitals (1-3). Previously, due to the low pathogenicity of enterococci, these organisms had not being treated, however nowadays this organisms is the main focus of antimicrobial therapy (4). These bacteria are often resistant to multiple antibiotics, thus limiting the number of therapeutic options available to the physician (5). Until recently vancomycin has been the drug of last resort against multiresistant enterococci (6) but since the first reports of vancomycin- resistant enterococci (VRE) that began to appear in the late 1980s, VRE now ranks third among antimicrobial resistant nosocomial infections (7). Important characteristic of some of the enterococci is their intrinsic or the ability to acquire the vancomycin resistance genes (8). At present, six gene clusters conferring glycopeptide resistance have been sequenced from enterococci (*vanA* to *vanG*), with *vanA* being the most commonly encountered in clinical enterococci in Europe (9). Two resistance genotypes, *vanA* and *vanB*, are considered to be of main importance because they are transferable (10). *E. faecium* with *vanA* genotype is considered as the most prevalent VRE among infected patients, environment and sewage treatment plants (10).

In developed countries, nationwide surveillance programmes such as the National Nosocomial Infections Surveillance (NNIS) System (11) monitor the prevalence of bacterial pathogens and their antimicrobial resistance patterns and periodically publish reports. Unfortunately, in some countries, including Iran, such national surveillance programmes are absent. Epidemiological studies like SENTRY (12) have demonstrated that for controlling the spread of resistance in a geographical region, data regarding susceptibility patterns of bacteria from a geographical region are essential

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(13). Moreover, updated bacterial susceptibility data are particularly necessary to physicians.
The aims of this study were to determine the species distribution, drug susceptibility patterns and genes conferring resistance in clinical vancomycin resistant enterococcal isolates at three hospitals and outpatients in Tehran from September 2005 to April 2006.

Materials and Methods

Specimen's collection Cases were patients admitted to three major hospitals (Milad, Shariati, Amiraalam) and outpatients in Tehran. The studies were carried out over a period of 7 mo, from September 2005 to April 2006. Only one isolate per patient was included in the study.

Species identification Identification of strains to the genus level was performed by using the following characteristics:
growth and blacken of bile-esculin agar; growth in the presence of 6.5% NaCl; absence of catalase; and presence of pyrrolidonyle arylamidase; 0.04% telurite reduction, arabinose utilization, arginine dehydrolase activity, methyl-α-d-glucopyranoside acidification, motility, and pigmentation using Facklam's recommendations (14). The final identification of *Enterococcus* species was based on PCR results as described previously (15).

Determination of antibiotic susceptibility
The isolates were primarily identified as enterococci, subsequently tested for resistance to vancomycin (30 μg) by the disk diffusion agar method. Vancomycin resistant enterococci were also tested with teicoplanin (30 μg) gentamicin (120 μg), erythromycin (15μg), ciprofloxacin (5 μg), tetracycline (30μg), chloramphenicol (30 μg), from (Bio-Rad, Hercules, CA, USA), dalfopristin-quinupristin (15 μg) and linezolid (30 μg) from (Mast Diagnostics Ltd, Bootle, Mersey Side, UK). MICs of vancomycin and teicoplanin, were determined by the Etest (AB Biodisk, Solna, Sweden) method on Mueller-Hinton agar according to the manufacturer's instructions. All plates were incubated at 37° C for 20 h. MIC results were interpreted according to Clinical and Laboratory Standards Institute guidelines (16). Both antibiotics were tested in the range 0.25-256 μg/ml.

DNA extraction DNA was extracted by mutanolysin method. Briefly, enterococci cells were grown in BHI medium for 16-18 h. Then cultures were harvested at 10000 g for 10 min. One ml of lysis buffer (1M NaCl, 10 mM Tris-HCl pH 8.0, 5mM EDTA, 0.5% Triton-X100) was added and thoroughly mixed. Ten mg of lysozyme were added and the culture was incubated at 37° C for 1 h, then 50U of mutanolysin were added and incubated at 37° C for 1 h, and 10μl of proteinase K (20mg/ml) and 100μl of 20% (w/v) sarkosyl solution were added and incubated at 37° C for 1 h. DNA was extracted and purified using two phenol/chloroform purification steps, ethanol precipitation, and suspension in a buffer containing Tris-HCl, EDTA, and RNase (17).

PCR Identification of *van* genotypes (*vanA* and *vanB*) for each VRE isolates was performed by separate PCR as described previously (15). Primer sequences (*vanA*: 5'-CATGAATAGAATAAAAGTTGCAATA-3', 5'-CCCCTTTAACGCTAATACGATCAA-3' *vanB*: 5'-GTGACAAACCGGAGGCGAGGA-3', 5'-CCGCCATCCTCCTGCAAAAAA-3') were derived from the published sequences of the genes (15). PCR assay was performed in a total volume of 25 μl containing 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM each dNTPs), 0.5 U of *Taq* DNA polymerase (HT Biotechnology, Cambridge, United Kingdom) and each primer (40 pmole). The PCR cycle was done as follow; initial denaturation at 94° C for 3 min, 30 cycles of 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. *E. faecalis* V583, *E. faecium* BM4147 *E. faecalis* ATCC29212 were used as the reference strains.

Results

A total of 450 isolates were obtained from clinical samples in the three main hospitals and outpatients. Isolates were from clinical samples including 380(85%) from urine, 25(5.5%) from wounds,

20(4.5%) from blood cultures, 10(2%) from body fluid, 10(2%) from sputum and 5(1%) from abscess. Amongst the different sites, most VRE isolates were obtained from urine 22(88%) (Table 1) and one (4%) isolate from each of wound, pulmonary secretion and abscess samples. The rates of isolation of enterococci from different wards were in the following order: transplants 1(4%), women surgery 4(16%), men surgery 6(24%), pediatric 1(4%) and Intensive care units 7(28%). The remaining isolates were recovered from other wards 4(16%) and outpatients 2(8%).

All of isolates obtained from all sites were *E. faecium*. The results of the PCR assay with the VRE isolates were in accordance with the phenotypic characterization. The *vanA* gene was the glycopeptide resistance determinant found in all of the isolates (Fig.1). No *vanB* gene was detected.

Most vancomycin resistant isolates studied were also resistant to teicoplanin 21(84%), ampicillin 25 (100%), erythromycin 25(100%), gentamicin 24 (96%), and ciprofloxacin 25(100%). Resistance to tetracycline 10(40%) and chloramphenicol 2(8%) were low. All isolates were susceptible to linezolid, and dalfopristin-quinupristin (synersid). According to MIC results all of tested enterococcal isolates were highly resistant to vancomycin. The vancomycin MIC for all of isolates were ≥256 and ≥128 µg/ml. MIC for 5(20%) isolates was ≥128 µg/ml and the results for the remaining showed the MIC was ≥256 µg/ml. However, the range of MIC for tiecoplanin was 4-256. The tiecoplanin MIC results for 20(80%) isolates were 4-48 µg/ml and for 5(20%) were ≥256 µg/ml. The results of the glycopeptide susceptibility tests were almost in agreement with the resistance genotypes.

Table 1: Clinical sources of 450 nosocomial isolates of enterococci and the 25 VRE resistant isolates

Isolates	Source and number of samples (%)					
	urine	**blood**	**wound**	**abscess**	**body fluid**	**sputum**
Total enterococci	380 (85)	20 (4.5)	25(5.5)	5(1)	10 (2)	10 (2)
VRE	22 (88)	0	1 (4)	1 (4)	0	1 (4)

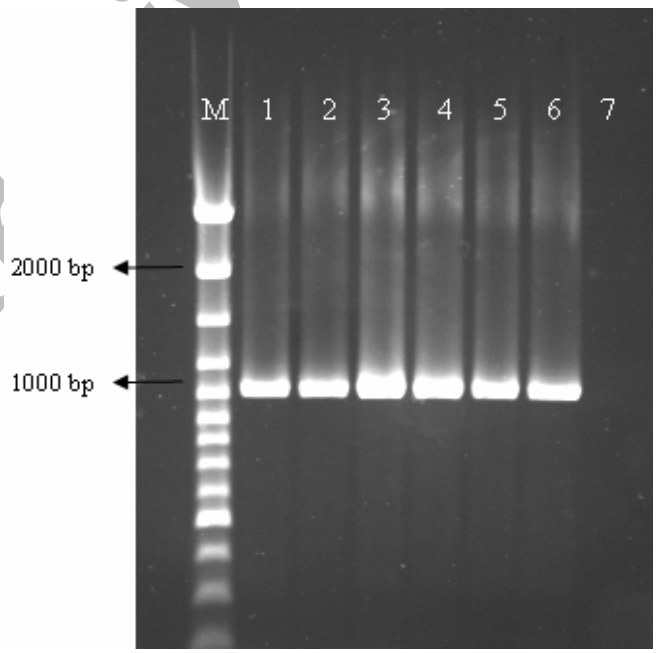

Fig. 1: The representative image is showing 1000bp amplicons of *vanA* gene. The positive and negative control is shown in lane 6 and 7 respectively.

Discussion

Failure of antibiotic therapy is a serious and growing issue that results in increased hospital costs and patient mortality (18). Infection with enterococci is endemic at Tehran hospitals, with 16.5% of isolates being resistant to at least three drugs (19), and multi drug-resistant strains of *E. faecalis* and *E. faecium* have caused serious problems in Iran due to inappropriate use of antibiotics. Nevertheless in previous study there were a few reports from VRE incidence in Iran (19). Comparison of our results with other studies in Iran, suggests that the incidence of VRE in Iran is higher. Moreover, our study demonstrated that the prevalence of vancomycin resistant enterococci was 5.6% which is less than prevalence of these species found in the united state and Europe (7, 20). Although the resistance of enterococci to vancomycin in hospital settings is a fairly recent phenomenon, VRE are now responsible for a large subset of nosocomial infections. This upward trend in resistance is alarming: VRE itself is now a major and largely untreatable infection, and VRE can pass the vancomycin resistance genes to the highly virulent methecillin-resistant *Staphylococcus aureus* (MRSA) (21).

Our results present that all of isolates in this study were *Enterococcus faecium*. *E. faecalis* and *E. faecium* are the predominant enterococcal species identified in clinical microbiology laboratories. Historically, these laboratories report that 80 to 90% of enterococci are *E. faecalis*, whereas *E. faecium* accounts for 5 to 10% of enterococci. This finding is of potential concern, as *E. faecium* is more commonly associated with vancomycin resistance than are the other enterococci (4).

In our study, all VRE isolates harbored *vanA* gene and showed high level vancomycin (100%) and teicoplanin (84%) resistance, a typical characteristic of VanA phenotype. It was interesting to see that the 16% of our teicoplanin-susceptible isolates also harbored *vanA* instead of *vanB* gene. Similar observation has also been made by other investigators (22). These strains can mislead the selection of antibiotics.

In agreement with previous findings (10, 23), the majority of our isolates were resistant to at least three of tested antimicrobial agents besides vancomycin. This associated resistance may contribute to the maintaining of vancomycin-resistant enterococci. Most of isolates were susceptible to tetracycline and chloramphenicol. This might be resulted from the limited usage of these antibiotics. All *E. faecium* isolates showed susceptibility to dalfopristin-quinupristin and linezolid.

Additional concern in the treatment of enterococcal infections in Iran is warranted because gentamicin resistance among our enterococcal isolates has also been shown to be much higher than the enterococci studied in other geographical areas (24). Aminoglycosides, particularly gentamicin have been in widespread use for at least three decades in Iran. They are still in use for treatment of very different infections in both hospitalized patients and outpatients

Our report draws attention to the importance of the awareness of physicians in identifying vancomycin resistant enterococci during treatment of patients and underscores the need for devising a national strategy to control the spread of resistance in Iran. In view of the fact that reports of updated susceptibility data from Iran are sparse, we believe that our data, in conjunction with comprehensive surveillance data from other cities of Iran, will further strengthen the reliability of ongoing global surveillance programmes in developed countries and thus will enhance attempts at limiting the spread of bacterial resistance worldwide.

In conclusion, the first choice therapy with β-lactams and aminoglycosides cannot be used due to resistance. The situation may worsen should VRE strains emerge. Switching to newer antibiotics linezolid and dalfopristin-quinupristin would only relieve the problem for a short time.

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The authors declare that they have no Conflict of Interests.

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