Frequency of Vancomycin-Resistant *Enterococci* isolated from clinical samples of Shahid Mohammadi hospital through the E-test method

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Original Article

Abstract

Introduction: *Enterococci* are gram-positive coccoid bacteria that are present in the normal flora of the gastrointestinal tract in human, and many mammals and birds, as well as the environment. Vancomycin-resistant *Enterococci* (VRE) are a major and rising problem in hospitals throughout the world. The present study aimed to investigate the frequency of strains of *Enterococci* and the pattern of drug sensitivity in clinical samples.

Methods: This cross-sectional study was conducted on 54 samples of *Enterococcus* in 2012. The diagnostic kit of RapID STR System was used for identification of *Enterococcus* species. Antibiotic sensitivity was determined by Kirby-Bauer disk diffusion method according to CLSI instructions. E-Test was used to determine Vancomycin MIC.

Results: Of the 54 isolates of *Enterococci*, the obtained strains included 38 *E. faecalis* (70.40%), 10 *E. faecium* (18.50%), 3 *E. hirae* (5.55%), one *E. mudtii* (1.85%), one *E. durans* (1.85%), and one *E. avium* (1.85%). Thirteen strains had vancomycin MIC > 32 µg/mL. They had the highest resistance to gentamicin and cephalexin by 70.40% and the lowest resistance to linezolid by 3.70%. The highest resistant strain was obtained from urine (76.9%) and internal ward (46.1%).

Conclusion: The substantial abundance of VRE isolated from the study area necessitates the performance of controlling measures.

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Introduction:

The word *Enterococcus* is originated from the Greek words of enteron meaning intestine and kokkus meaning grain (1). *Enterococci* are fermentative gram-positive cocci which are present in the colon as the normal flora in almost equal number of *E. coli*. They are also distributed in the environment where they can survive for a long time given their high tolerance to dryness (2-3). *Enterococci* have little potential for disease and lack strong toxins and notable pathogens, however, they can cause diseases such as bacteremia, endocarditis, and infections of surgical wounds, urinary tract, newborns, central nervous system, abdomen, and pelvis (2, 4-6). The main problem of nosocomial

enterococcal infections is the emergence of multi-resistance to antibiotics (2). According to the national nosocomial infections surveillance system (NNIS), *Enterococcus* is the fourth causing agent of hospital infections (the third cause of bacteremia and the second cause of urinary tract infections) (7). About 90% of enterococcal infections in humans are caused by *E. faecalis* and the remaining 10% by *E. faecium* (8). These strains are considered fecal. Other strains are seen with a very low percentage in clinical samples. It has been suggested recently that specific subsets of this species can be considered as environmental rather than fecal, in particular *E. casseliflavus*, *E. gallinarum*, and *E. mudtii* (9).

Vancomycin was produced in 1958 for the treatment of staphylococcal infections, but was not significantly utilized until the late 1970s and outbreak of methicillin-resistant *Staphylococcus aureus* (10). Vancomycin was first used in clinical cases in 1972 (11). Vancomycin-resistant *Enterococci* were first seen in 1986 in Europe and in 1987 in USA, and then throughout the world, with an increasing detection thereafter (12). The mechanisms of resistance of *Enterococci* to antimicrobial agents, *i.e.* intrinsic (low-level resistance to penicillin, cephalosporins, and aminoglycosides) and acquired (resistance to glycopeptides, high concentrations of aminoglycosides), are now of great importance and attention (7). No single factor shows a good activity against even glycopeptides-sensitive members in this genus (13). In clinical patients, treatment with a combination of agents active on the cell wall, such as penicillin or glycopeptide with aminoglycoside or cephalosporins with bactericidal activity, can be effective (13,14). Production of vaccines and immunotherapy are currently raised for the treatment of enterococcal infections (15-17). This study aimed to determine the prevalence of vancomycin-resistant *enterococci* in clinical samples of Shahid Mohammadi Hospital, in Bandar Abbas and to determine the pattern of antibiotic resistance in 2012.

Methods:

This descriptive study was performed on 54 patients admitted to Shahid Mohammadi Hospital of Bandar Abbas City for one year. There was no limit on age, gender, and cause of admission to be enrolled in this study. All samples were transferred to the laboratory of Microbiology, Faculty of Medicine of Bandar Abbas. They were then incubated on nutrient agar medium (Merck) for 24 hours at 37 °C. The grown bacteria were examined with gram stain and catalase test, and were confirmed through the tests of Bile Aesculin agar (Merck), TSB containing 6.5% salt (Merck), and growth at 45 °C. The genus of *Enterococcus* was identified with RapID STR System kit (Remel Company). The panel of this kit includes 10 wells and 14 tests. The tests are based on substrate analysis and reaction of the resulting products with indicators. Hemolysis should also be reported in addition to 14 tests performing for this kit. As a result, a total of 15 tests were evaluated. Since the diagnostic kit of RapID STR System cannot differentiate *Streptococcus durans*, *E. hirae*, *E. casseliflavus*, and *E. mundtii*, these strains were identified through the motion and growth test at 45 °C (18). The resistance phenotype was determined through the disc diffusion method according to CLSI guidelines using the following discs; vancomycin 30 mg, ampicillin 10 mg, linezolid 30 mg, imipenem 10 mg, ticoplanin 30 mg (Mast Company), and trimethoprim (1.25) + sulfamethoxazole (23.75), ceftizoxime 30 mg, gentamicin 10 mg, and cephalexin 30 mg (Himedia Company). Opacity of 0.5 McFarland was used to inoculate into Mueller Hinton agar (Mast). In this study, MIC was determined through E-Test or MIC Test Strip. After placing the E-Test on Mueller Hinton plate, the plates were incubated at 37 °C for 18 hours. The lowest concentration of antibiotic in the presence of which no growth was occurred was defined as MIC, and according to the Company's guidelines, the minimum inhibitory concentrations greater than 32 μg/mL, 6-12 μg/mL, and less than 4 μg/mL were interpreted as resistant, intermediate, and sensitive, respectively. Strains of *E. faecalis* ATCC 29212 and *E. faecium* BM4147 were used as negative and positive controls, respectively. MIC was determined for all samples. Statistical analysis was performed with SPSS-19 using chi-square test and Fisher's exact test when necessary, and $p < 0.05$ was considered statistically significant.

Results:

Within a year, 54 different strains of *Enterococci* were obtained from clinical samples of patients who were 53.7% male and 46.3% female, with an age range of 9 months to 96 years, an average age of 52.27±3.119 years, and median of 57 years. The mean length of stay was 8 days with a maximum of 59 days and a minimum of 1 day.

Table 1. Type and number of clinical samples collected (%)

Clinical sample	Urine	Wound	Perioneal fluid	Blood	Trachea
Frequency	42 (77.80%)	8 (14.80%)	2 (3.70%)	1 (1.85)	1 (1.85%)

The majority of samples were obtained from urine (42) and the least from blood and peritoneal fluid (1) (Table 1).

The results of strain identification using kits and supplementary tests are shown in Table 2.

Table 2. The identifiled *Enterococcus* species

Enterococcus species	Number	Frequency
E. faecalis	38	70.40%
E. faecium	10	18.50%
E. hirae	3	5.55%
E. durans	1	1.85%
E. avium	1	1.85%
E. mundtii	1	1.85%

Regarding the pattern of antibiotic resistance, the highest resistance was against gentamicin and cephalexin and the lowest against linezolid (Table 3).

Table 3. Enterococci antimicrobial resistance pattern

Antibiotic	Resistant	Semi-sensitive	Sensitive
Linezolid	3.70%	0	96.30%
Ampicillin	18.50%	0	81.50%
Ticoplanin	20.40%	0	79.60%
Imipenem	20.40%	0	79.60%
Vancomycin	24.10%	0	75.90%
Ceftizoxime	57.40%	3.70%	38.90%
Cotrimoxazole	63%	0	37%
Cephalexin	70.40%	0	29.60%
Gentamicin	70.40%	0	29.60%

Based on the pattern of resistance to antibiotics, the prevalence of multi-drug resistance (MDR) was obtained, and the highest strains (33.33%) were resistant to three drugs (Fig. 1).

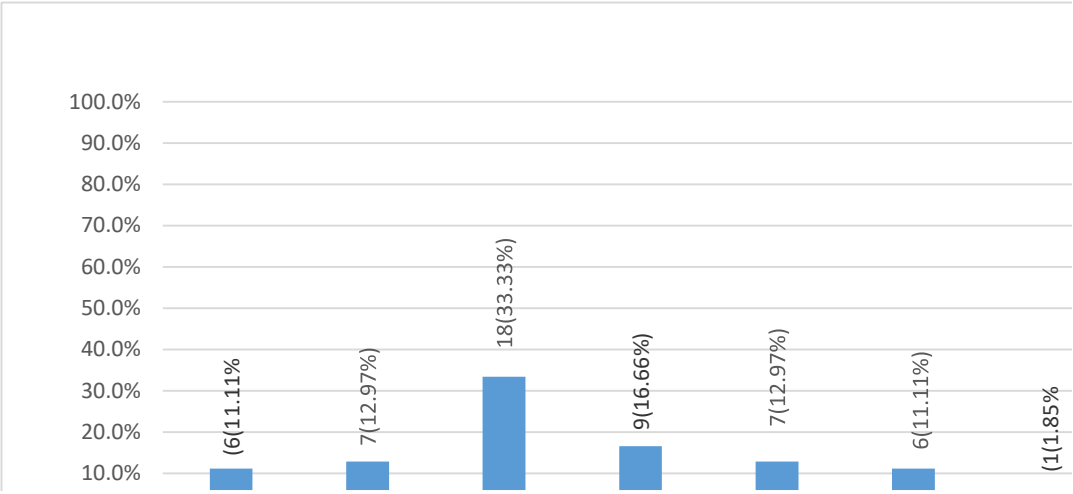

Figure 1. Frequency of multi-drug resistant strains of *Enterococcus*

No significant relationship between strains of *Enterococci* and vancomycin resistance was found based on Chi-square statistical analysis (p=0.08).

Strains of vancomycin-resistant *Enterococcus* included *E. faecalis* with 6 strains (46.15%), *E. faecium* with 4 strains (30.78%), *E. avium* with one strain (7.69%), *E. durans* with one strain (7.69%), and *E. hirae* with one strain (7.69%).

Regarding vancomycin-resistant strains in hospital wards, 6 strains (46.15%) were from the

internal ward, 5 strains from the outpatient department (38.47%), one strain (7.69%) from the burning ward, and one strain (7.69%) from the intensive care unit.

The most frequent resistant strains were from the urine samples with a frequency of 10 (76.93%).

Strains with high-level resistance to vancomycin have grown throughout the plate. Among the resistant strains, the minimum inhibitory concentration (MIC) was 48 in one strain, 64 in one strain, more than 256 in the remaining resistant strains which were grown throughout the plates (Table 4).

Table 4. Measurements of MIC by Vancomycin E-test

MIC (mg/mL)	MIC <4 µg/mL	MIC 8-12 µg/mL	MIC >32 µg/mL
Frequency	41	0	13

Conclusion:

Studies performed in Iran have shown that *Enterococci* are the first cause of urinary tract infection among gram-positive cocci and the third cause of urinary tract infection in women in Iran after *Escherichia coli* and *Klebsiella pneumoniae* (19).

In our research, as expected, the most isolated strains were *E. faecalis* (70.4%) followed by *E. faecium* (18.5%). In most studies also, *E. faecalis* and *E. faecium* have been reported as the first and second causes, and the other strains were more or less similar to this study.

In a study by Fatholahzadeh *et al*. in 2006, the identified strains were *E. faecalis* (38%), *E. faecium* (25%), *E. mundtii* (25%), and *E. raffinosus* (12%) (20). In the study of Eini *et al*. in children with urinary tract infection, the strains were reported as *E. faecalis* (58%), *E. faecium* (30%), *E. mundtii* (9%), *E. avium* (1%), *E. hirae* (1%) and *E. raffinosus* (1%) (21).

The study of Eini *et al*. conducted in Tehran showed that *E. faecalis* and *E. faecium* accounted for the highest percentage, because these strains are fecal and other strains were environmental.

Although we are faced with a relative difference in the incidence, there exist similar strains in hospitals, except for *E. raffinosus* which was not found in the samples. However, regarding its small percentage, if sample collection duration and sample size increased, perhaps we would find this strain as well. In a study by Schouten *et al*. in Europe, the prevalence of *Enterococcus* strains was reported as follows: *E. faecalis* (83%), *E. faecium* (13.6%), *E. gallinarum* (1.20%), *E. durans* (0.71%), *E. casseliflavus* (0.53%), *E. avium* (0.46%), *E. hirae* (0.12%), *E. mundtii* (0.05%), and *E. raffinosus* (0.02%) (22). Schouten *et al*. conducted a study in 27 countries of Europe and found a greater variety of strains; this can be attributed to the extensive study performed in various European countries with large size sample. Perhaps if the number of samples and hospitals was reduced, the variety of the viruses would be decreased. Among 180 clinical samples, Mohammadi et al. found 128 strains of *E. faecalis* (71.1%) and 52 strains of *E. faecium* (28.9%) (23). In a study by Nouhi et al., out of 76 samples, 59.2%, 38.1%, and 2.7% were *E. faecalis*, *E. faecium*, and *E. gallinarum*, respectively (24). Strains of *Enterococcus* from clinical samples in a study by Guiney and Uriwin included 84.4% *E. faecalis*, 14.8% *E. faecium*, and 0.8% other species including *E. casseliflavus*, *E. avium*, and *E. durans* (25).

Unlike the results of the present study and a majority of research performed in Iran and abroad, Oskoee and Farrukh reported 17 strains of *E. faecium* and 15 strains of *E. faecalis* out 32 of strains (26). In their study, the number of strains of *E. faecium* and *E. faecalis* had a slight difference, and as mentioned earlier, the number of *E. faecalis* could be increased if the sample size increased.

The size of samples obtained from hospital wards varies in different studies, but urine samples usually formed the majority of clinical samples (18-23). In the present study, the rate of VRE was about 24%; while 34% of samples in the study of Ghafarpassand *et al*. (2007) performed on clinical samples of Shahid Beheshti Hospital were resistant to vancomycin (7). VRE prevalence in the studies of Stephen Fluit, and Hanbergor and Nilson was 28%, 10% and less than 1%, respectively. Resistance to vancomycin was reported 15.5% in the study of Rafee *et al*. in Mofid Children's

Hospital (27). Unlike the present study, Salah *et al*. carried out their research on renal patients and strains of *E. faecalis* in 2008 through the disc diffusion method and found that 100% of strains were susceptible to vancomycin (26). In the study Fatholahzadeh *et al*. in 2006, about 7% of the isolates were resistant to vancomycin (20). Harris et al. reported a VRE prevalence of 10% (21).

Increased frequency of vancomycin resistant *Enterococci* isolated from clinical samples is consistent with increasing use of antibiotics to which *Enterococci* are naturally resistant. In particular, consumption of cephalosporins and quinolones which are largely effective against gram-negative bacteria has significantly increased. These antibiotics may pave the way to the advent of *Enterococcus* as hospital-acquired pathogen. It is difficult to proof this hypothesis, but circumstantial evidence is compelling. For example, many controlled studies have shown that nosocomial enterococcal infections are related to treatment with beta-lactams antibiotic (imipenem) and quinolone (25).

In this study, the greatest resistance was observed against cephalexin and gentamicin, and linezolid and imipenem were the best treatment options. In a study by Ghasemi *et al*. in Shahid Beheshti Hospital and Shabihkhani Maternity Hospital in 2008 carried out on strains of *E. faecalis* isolated from clinical samples, resistance to gentamicin, ampicillin, imipenem, and vancomycin was reported as 38.7%, 11.3%, 10.4%, and 4.7%, respectively. Multi-drug resistant phenotype was observed in 37.7% of strains (28). In this study, gentamicin and cephalexin had the highest rate of resistance, but vancomycin had not the least resistance. In most studies, same as the present research, linezolid was the least resistant (linezolid resistance pattern was not reported by Ghasemi et al.). In the study of Ghafarpassand *et al*. in 2007 on clinical samples in Shahid Beheshti Hospital, 60%, 44%, 34%, and 33% of the strains were resistant to ampicillin, gentamicin, vancomycin, and linezolid, respectively (7). Resistance to ampicillin, vancomycin, and linezolid in the study of Ghafarpassand et al. was higher than the present research which can be due to difference in the consumption of drugs in hospitals, the resistance rate of the organism in the hospital, and hospital environments. In this study, the rate of vancomycin-resistant *enterococci* was high, and requires controlling and preventive measures.

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