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Original Article

Antibiotic Susceptibility in *Enterococci* Isolated from Patients in Kerman, Southeastern Iran

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Abstract

Background: *Enterococci faecalis* are predominating species of *Enterococci* causing nosocomial infections. Acquisition of resistance to antibacterial agents, and ability to transfer the resistant genes made them clinically important. This study was performed to determine the frequency of isolation of different species of *Enterococci*, and the antibacterial resistance pattern of the isolated species.

Methods: Enterococcal species were isolated from clinical samples. *In vitro* susceptibility of the isolates to 10 antibacterial agents was tested by standard methods and β-lactamase production was detected using starch-iodide method.

Results: 100 E*nterococci* were isolated from 585 different clinical samples. 73% of the isolates were *E. faecalis,* 13% *E. faecium* and 14% which were not identified as either one, were regarded as other enterococcal species. Highest rate of resistance (98% or more) was found for oxacillin and penicillin while vancomycin and chloramphenicol were among the most active agents. Resistance to antibacterial agents was more common for *E. faecium* and β-lactamase production was found in 81% of the isolates.

Conclusion: *E. faecalis* was the dominant species, with the higher rate of β-lactamase production. *E. faecium* was more resistant to antibacterial agents as compared to other isolates. 80% of the isolates had multiple drug resistance phenotypes (MDR). Low–level resistance to vancomycin (intermediate reaction in disk diffusion method, minimum inhibitory concentrations range ≥4–16 μg/ml) and presence of MDR isolates is very important and should be considered as an danger alarm for serious enterococcal infections.
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Keywords ● *Enterococci* ● antibacterial resistance ● beta-lactamase

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Introduction

Enterococci are Gram positive cocci that are ubiquitous in nature and occur in soil, surface water as well as treated and untreated waters, plants, vegetables and also represent a natural part of the intestinal flora in both humans and animals.[1,2,3] They are opportunistic pathogens capable of causing life threatening infections such as acute endocarditis, urinary tract infections, intra-abdominal and wound

infections in human.[4] *Entrococci* are important pathogens in hospital setting, exhibit high morbidity and mortality in patients with bacterimia and severe basic disease.[5] Using molecular typing methods, it was confirmed that these bacteria have the ability to transfer the resistant genes to other opportunistic bacteria.[5]

The genus of *Enterococci* comprise as many as 19 species of which *E. faecalis* and *E. faecium* still account for the majority of human infections.[5,6] Recent studies have revealed a significant increase in the isolation of more unusual species such as *E. durans, E. hirae, E. gallinarum* and *E. casseliflavus* from clinical samples.[7] In Europe, the food chain has been suspected as a potential source of colonization and infection in human.[8] Consumption of meat has been associated with colonization of gastro-intestinal tract of human with enterococci especially with vancomycin resistant strains.[8]

The increasing resistance to antibacterial agents such as penicillins, aminoglycosides, trimethoprim, and also to glycopeptides such as vancomycin and teicoplanin, created an increasingly worrisome problem in clinical practice.[3,9,10] Furthermore, *Enterococci* have different mechanisms for the transfer of antibiotic resistance genes, to other more pathogenic gram positive bacteria such as *Staphylococcus aureus* which is very important clinically.[11] Since some species such as *E. casseliflavus*, and *E. gallinarum* are less commonly associated with clinical infections and are inherently resistant to glycopeptides, screening for resistant strains by clinical laboratories are recommended in order to identify them to the species level.[5,12] Isolation and species identification of *Enterococci* including their resistance to antibacterial agents are reported from Iran, but these are mainly from Tehran, and Shiraz.[13-16] The present study describes the isolation, species identification, β-lactamase production and the resistance pattern of this bacterium against vancomycin and several other antibacterial agents in Kerman, Southeast of Iran.

Materials and Methods

Bacterial source

In this study the *Enterococci* were only isolated from urinary tract infections or fecal flora but not from any 50 other clinical samples tested. A total number of 585 samples were tested for the isolation of the *Enterococci*. They comprised 400 isolates from urine samples of patients with urinary tract infections (UTI), 135 samples of fecal flora from inpatients and outpatients, and 50 different clinical samples from blood and wounds. All samples were initially cultured on *Enterococci* isolation media (SF broth, Difco Laboratories, Detroit, USA). They were sub-cultured on blood agar plates 24 to 48 hrs after incubation at 37 ˚C. Catalase test was performed on all Gram-positive cocci suspicious of being *enterococci*. Growth in the presence of 40% bile (4% Oxgall), esculin hydrolysis and growth in the presence of 6.5% NaCl was performed for the final identification of *Erterococci*.[17] For identification of species, bacteria were tested for their ability to ferment mannitol and/or raffinose and growth in the presence of 0.4% potassium tellurite.[17]

Antibiotic sensitivity testing

Antibacterial resistance patterns of *Enterococci* to 10 antimicrobial agents were performed with ampicillin (Am), amoxicillin-clavulanic acid (Amx-clav), oxacillin (Ox), chloramphenicol (Ch) ceftizoxime (Ct), ciprofloxacin (Cip), gentamicin (Gm), penicillin G (Pg), trimethoprim-sulfamethoxazole (Sxt) and vancomycin (Va) using standard disk diffusion method (Kirby–Bauer sensitivity test) susceptibility tests.[18] All tests were performed on Muller–Hinton agar (Oxoid Co, Hampshire, UK), and the results read after 24 hrs of incubation at 37 ˚C. The zone diameter measured around each disk was considered as resistant, intermediate, or sensitive according to the zone size diameters, with the ranges provided by the disk manufacturer.[18] Minimum Inhibitory concentrations (MICs) were determined for multiple–drug–resistant (MDR) isolates resistant to three or more antibacterial agents simultanously. The antibacterial agents tested were Am, Gm and Sxt obtained from Jabber-Eben Hyan Co (Tehran, Iran), cloxacillin from Farabi Laboratory (Tehran, Iran), ciprofloxacin from Razak Pharm Co (Karaj, Iran), and vancomycin from Qualimed (Cox Laboratories, France). Stock solutions of antibiotics were prepared in an appropriate solvent and were kept frozen at -20 ˚C until used. MICs was determined by the agar dilution method using Muller-Hinton agar, and 10^4 CFU of bacteria was used to spot the medium containing different concentrations of antibacterial agents. MICs were recorded when no visible growth was observed on the spotted agar plate after 24 hrs of incubation at 35 ˚C.[18] β-lactamase test was performed to determine the presence of β-lactamase enzyme by the isolates using starch-iodide method as described previously.[19]

Statistical analysis

The difference in susceptibility patterns was analyzed by the Chi-square or two-tailed

Table 1: Minimum inhibitory concentrations (MICs) of antibiotics for 80 *Enterococci* with multiple drug resistance (MDR: resistance to ≥3 antibacterial agents) phenotype.

Drugs	Number (%) of the isolates with MICs relative to each agent							
	1 µg/ml	2 µg/ml	4 µg/ml	8 µg/ml	16 µg/ml	32 µg/ml	64 µg/ml	128 µg/ml
Am	0 (0)	23 (28.7)	17 (21.2)	14 (17.5)	6 (7.5)	8 (10)	5 (6.2)	7 (8.7)
Ox	0 (0)	0 (0)	0 (0)	0 (0)	33 (41.2)	24 (30)	23 (28.7)	0 (0)
Va	19 (23.7)	32 (40)	8 (10)	7(8.7)	14 (17.5)	0 (0)	0 (0)	0 (0)
Sxt	1 (1.2)	10 (12.5)	25 (31.2)	8 (10)	10 (12.5)	10 (12.5)	8 (10)	8 (10)
Gm	0(0)	28 (35)	24 (30)	4 (5)	5 (6.2)	14 (17.5)	5 (6.2)	0 (0)
Cip	18 (22.5)	33 (41.2)	29 (36.2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Am= ampicillin, Ox= oxacillin, Va= vancomycin, Sxt= trimethoprim sulfamethoxazole, Gm= gentamicin, Cip= ciprofloxacin

Table 2: Comparison between antibacterial resistance pattern and β-lactamase production (β-lac) in 100 *Enterococci* isolated from urinary tract infections (UTIs, n=50), or fecal flora (FF, n=50)

E from	β-lac No. (%)	Number(%) of isolates resistant to							
		Am	Pg	Ct	Ch	Va	Sxt	Gm	Cip
UTIs	45 (90)	4 (8)	49(98)	41 (82)	12 (24)	15 (30)	18 (36)	39 (78)	32 (64)
FF	36 (72)	12 (24)	49 (98)	44 (88)	11 (22)	8 (16)	48 (96)	18 (36)	40 (80)
P value	0.0022	0.029	1.0	0.4	0.91	0.097	0.000	0.007	0.07

Am= ampicillin, Pg= penicillin G, Ct= ceftizoxime, Ch= chloramphenicol, Va= vancomycin, Sxt= trimethoprim sulfamethoxazole, Gm= gentamicin, Cip= ciprofloxacin.

Table 3: Comparison between antibacterial resistance pattern and β-lactamase production (β-lac) in the *E. faecalis, E. faceium* and other *Enterococcus* isolates from urinary tract infections (n= 50) or fecal flora (n= 50).

Species	β-lac n(%)	Number (%) of isolates resistance* to							
		Am	Pg	Ct	Ch	Va	Sxt	Gm	Cip
E. Faecalis	66 (90.4)	5 (6.8)	72 (98)	61 (83.6)	17 (23.3)	18 (24.7)	48 (57.9)	41(57.9)	49(65.9)
E. Faecium	4 (30.7)	8(61.5)	2(93.8)	9(87.5)	3 (31.29)	3 (68.8)	10 (87.5)	9 (62.5)	10 (81.2)
Others	11 (78.6)	3 (21.4)	12 (85)	14 (100)	3 (21.4)	0 (0)	8 (57.1)	7 (50)	13 (92.9)
P<	0.00002	0.000	0.000	0.002	0.13	0.0007	0.03	0.76	0.43

*Resistance isolates: Are the isolates with resistance plus those isolates with intermediate response. For abbreviations see Table 1.

Fisher exact test. A $P<0.05$ was considered as statistically significant.

Results

The highest rate of resistance was found in the case of Ox (100%), and Pg but only 16% of the isolates were found to be resistant to Am. The MIC ranges for Am and Ox was 2–128 µg/ml and16–64 µg/ml, respectively (Table 1). Similarly, the isolates exhibited high rate of resistance to Ct and Sxt (66%), whereas resistance to Gm (30%, MIC range 2-64 µg/ml) and Cip (9%, MIC range 1-4 µg/ml) were not as high as other antibacterial agents used. None of the isolates were resistance to Va, and for Ch low resistance (3.3%) were obtained. These two antibiotics were the most active antibiotics against our isolates. Grossly, isolates from fecal samples were more resistant to antibacterial agents compared with other isolates, especially in the case of Sxt, Cip and Am (Table 2). Among the *Enterococcus* species, *E. faceium* exhibited a higher rate of resistance to antibacterial agents (Table 3), and by disk diffusion method 68.8% of the isolates showed intermediate reaction to Va. Resistance to three or more antibacterial agents was regarded as multiple drug resistance and was found in 80% of the isolates (39 isolate from UTIs and 41 from fecal flora). No statistically significant difference was found between the numbers of MDR isolates from different species of *Enterococci.*

Using starch iodide method, 81 isolates (80%) were found to produce the β-lactamase enzyme (Tables 2, and 3). The frequency of β-lactamase production in UTIs, and fecal flora were 92%, 72%, respectively. Production of β-lactamase was significantly higher in the *E. faecalis* compared with *E. faecium or* other *Enterococci* species (P<0.00001).

Discussion

As a measure of infection control, it is essential to differentiate *Enterococci* from other Gram positive bacteria inherently resistant to vancomycin.[12] In this study *Enterococci* were differentiated from other Gram positive bacteria by standard biochemical tests.[17] From 100 *Enterococci* isolated, 73% were identified as *E. faecalis* and 13% as *E. faecium.* In a report from 27 European countries and also similar reports from, America, Brazil and Iran, the most frequent species isolated from clinical environmental or food samples was *E. faecalis* with the ratio of *E. faecalis/E. aecium as* 6.1;

7.0; 5.2; and 4.0, respectively.[1,3,16,20] The ratio of 5.5 in present study was similar to those reported from European countries, and America.

More frequent antibacterial resistance in *E. faecium* compared with other *Enterococci* which was found in this study is similar to other studies.[9, 20, 21] Table 1 shows a high frequency of resistance of *Enterococci* to Pg (98%) with complete resistance to Ox (100%, MIC range 16-64 µg/ml), whereas, the isolates were highly sensitivite to Amx-clav and partially sensitive to Am (84%, MIC range 2-64 µg/ml). These are in agreement with the results reported by Kaufhold, Ferrieri, Murray and their colleagues who showed a high level of Pg resistance in *Enterococci*.[4,5]

Trimethoprim–sulfamethoxazole (Sxt)–resistant *Enterococci* have been isolated worldwide.[10] In a surveillance program on the bacteria isolated from UTI across Canada, the resistance of *Enterocci* to Sxt was reported to be 17.9% with MIC ranging from 0.06-128 µg/ml.[22,23] In contrast to their finding a much higher rate of resistance to Sxt (66%, with the MIC ranging from 1 to ≥128 µg/ml) was found in the present study. Resistance exhibited by Gm was also high in our study and only 42% of the isolates were sensitive to this antibiotic. However, the isolates of this area had low resistance to Gm (MIC range 2 to 64 µg/ml) indicating less frequent usage of this drug could be the cause.[4] Fluroquinolones like ciprofloxacin are of special interest because of their potential use in the treatment of infections caused by resistance *Enterococci*.[20] In a report from 27 European countries, resistance to Cip in *E. faecalis* and *E. faecium were* reported to be 6% and 22% respectively. In a study carried out in Tehran, Cip resistance for *Enterococci* was reported to be 42%.[16] In this study, only 9% of the isolates were resistance to Cip with 63% exhibiting intermediate response by the disk diffusion method with MIC range of ≥4 which is an indication of resistance.[18] Therefore, high resistance to Cip could be due in fact to the indiscriminative usage of Cip, especially for the treatment of UTI, in this region.

Resistance of *Enterococci* to glycopeptides poses an increasing problem in clinical practice in many countries around the world.[9] The prevalence of vancomycin resistant *E. faecium* in health care institutions across United States is reported to be 15% with high MIC of up to 512 µg/ml.[21] In our isolates we did not found any vancomycin resistant *Enterococci*. Indeed those isolates with intermediate reactions in the disk diffusion method had the MIC range of 4 to 16 µg/ml. According to the recommendation of National Committee for Clinical Laboratory Standards (NCCLs), these isolates could not be considered as resistant isolates to vancomycin because the range of MIC for resistant strains is ≥32 µg/ml.[9]

Conclusion

E. faecalis was the dominant species isolated while *E. faecium showed* higher frequency of resistance to antibacterial agents. Multiple drug resistant isolates comprise 80% of the isolates and simultaneous resistance to 3, 4 and ≥5 antibacterial agents were observed in 33%, 30%, and 17% of the isolates respectively. Due to the emergence of many multidrug resistant *Enterococci*, periodic screening should be performed for the *Enterococci* isolated from patients. This screening should be specially considered for antibacterial resistance in high risk patients to prevent infection with vancomycin–resistant strains.

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