Original Article

STUDY OF ENTEROCOCCAL SUSCEPTIBILITY PATTERNS ISOLATED FROM CLINICAL SPECIMENS IN TABRIZ, IRAN

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ABSTRACT

Objectives: To identify the prevalence of enteroccoci species in clinical specimens, to determine their susceptibilities to some antibiotics for treatment, and to detect the *vanA*-specific 377-bp fragment from the genomic DNA of all vancomycin resistant enteroccoci (VRE).

Methodology: One hundred thirty seven isolates of enterococci species were obtained from samples of patients who were referred to microbiology laboratory of two hospitals in Tabriz from March 2001 to April 2002. After identification of enterococcal species by biochemical methods, the antibiotic susceptibility of isolates was determined by standard disk diffusion test according to NCCLS. MIC tests for vancomycin were also carried out for VRE strains by macro-dilution method. The *vanA*-specific 377-bp fragment was amplified from the genomic DNA of all VRE by PCR.

Results: The isolates were found to consist of *E. faecalis* (90.5%), *E. faecium* (5.84%) and *Enterococcus* species (3.66%). According to susceptibility data obtained, six (4.38%) out of 137 isolates were found to be VRE with MIC e"32ìg/ml. The *vanA* gene fragments of *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus gallinarum* and *Enterococcus durans*, were amplified from isolates and were detected.

Conclusion: Finding of this study shows an emergence of VRE along with increased rate of resistant enterococci in Tabriz.

KEYWORDS: Vancomycin resistant enterococci, Antibiotic resistance, *VanA* gene, Tabriz.

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INTRODUCTION

Enterococci are gram-positive, opportunistic bacteria that inhabit the gastrointestinal tracts of humans and many animals. Enterococci are the second most frequently reported cause of surgical wound infections and nosocomial urinary tract infections and the third most frequently reported cause of bacteremia.[1,2] Resistance to environmental conditions such as heat or desiccation allow prolonged survival and poor compliance with hand-washing procedures by health care workers results in the rapid spread of enterococci in hospitals.[3,4] Moreover,

strains of enterococci have acquired resistance to essentially most of the antimicrobial agents over the past three decades. Nosocomial infections with entrococci are major concern at many hospitals and have rapidly increased in many countries worldwide including Iran.[5-8] Resistance of these microorganisms against the first choice therapy (aminopenicillin/ aminoglycoside) required alternative treatment with the glycopeptides, vancomycin or teicoplasmid.

The prevalence of vancomycin resistance in enterococci (VRE) has dramatically increased in the last few years.[9] Six types of vancomycin resistance have been reported in enterococci (*VanA, VanB, VanC, VanD, VanE and VanG*). The resistance to vancomycin is inducible and is encoded by the *vanA* gene cluster, which is carried on transposons.[2] Transfer of resistance can occur via conjugative plasmids. Enterococci as reservoirs of antibiotic resistance genes, tend to transfer their resistance genes to the other bacteria among them methicillin- resistant *Staphylococcus aureus*.[10] Monitoring the antibiotic resistance of enterococci isolated from clinical specimens is a useful tool to get information about prevalence of VRE and will be essential for controlling the spread of bacterial resistance.

The aim of this study was to determine the species distribution and drug susceptibility patterns of clinical enterococcal isolates at two hospitals in Tabriz, Iran.

METHODOLOGY

Samples collection: In cross sectional study during March 2001 to April 2002, a total of 137 enterococcal isolates were recovered from clinical specimens (Urine, ascetic fluid, wound, catheter, blood, bone marrow) of patients from two clinical microbiology laboratories of Emam and Sina hospitals in Tabriz, Iran. Only one isolate per patient was included in the study. All isolates were stored in brucella glycerol broth at -20°C until tested.[11]

Identification of strains: Isolates were identified to the genus and species level based on the standard biochemical and microbiological methods such as: morphologic appearance on Gram-stain (gram positive cocci forming short chains), catalase negative, ability to hydrolyze esculin in the presence of bile, growth in the presence of 6.5% NaCl at 45ºC, as well as commercially available kit (API 20 Strep, biomerieux, France).[11]

Antimicrobial susceptibility testing: Susceptibility to antimicrobial agents for all enterococci isolates was determined by the standard disk diffusion method and confirmed according to the NCCLS guidelines current at the time of study on Muller-Hinton agar incubated for 24 hr at 37ºC.[12] The following antibiotic disks (BBL) were used: oxacillin (1µg), penicillin (10 units), ampicillin (10µg), amoxicillin/ clavulanic acid(30µg), gentamicin (10µg), erythromycin (15µg), vancomycin (30µg), trimethoprim-sulfamethoxazole (23.75µg sulfamethoxazole, 1.25µg trimethoprim), nalidixic acid (30µg), ciprofloxacin (5µg), chloramphenicol (30µg) and nitrofurantoin (300µg). The values of minimum inhibitory concentration (MIC) of each VRE isolates for vancomycin were determined by the broth macrodilution method according to NCCLS guidelines.[13] Susceptibility test results were assessed after 24-48 hr incubation at 37ºC. The control strains used in this work were *Staphylococcus aureus* ATCC 29213 and *E. faecalis* ATCC 29212 for susceptibility test. Isolates with MIC of <4µg/ml were considered susceptible and with MIC of >16µg/ml were recorded as resistant.

vanA gene: All VRE strains were grown for 24 hour at 37 ± 0.5°C in BHI with 5% sheep blood. DNA was extracted using sodium dodecyl sulphate- proteinase K method modified with N-cetyl-N, N, N-trimethylammonium bromide (CTAB).[14] The following oligonucleotides were used as primers for amplification of the 377-bp fragment of the *vanA* gene: *vanA* 1 (5 -TCT GCA ATA GAG ATA GCC GC-3) and *vanA* 2 (5 -GG AGT AGC TAT CCC AGC ATT-32).[15](Primers were obtained from TIB MOLBIOL, Berlin, Germany). The total volume

of PCR mix was 25µl, including: sterile redistilled H_2O 17.05µl, 10X PCR buffer 2.5µl, dNTP mix (10mM) 0.5µl, $MgCl_2$ (50mM) 0.75µl, forward primer (25µM) 0.5µl, reversed primer (25µM) 0.5µl, Taq DNA polymerase (5U/µl) 0.2µl, template DNA 3µl. Negative controls contained all components except template DNA.

The two primers and other reagents were prepared according to the manufacturer's recommendations. PCR reactions were performed with an automated thermal cycler (Eppendorf mastercycler gradient, Germany) with the PCR cycling conditions (initial cycle at 94°C for 4 min, 30 cycles of denaturation at 94°C for 40 sec, annealing at 57°C for 40 sec, and extension at 72 °C for 40 sec, final cycle extension at 72 °C for 7 min).[16]

Gel electrophoresis was performed for 60-120 min in a 1.2% agarose gel at 75 V. DNA profiles were visualized by means of ultraviolet (UV) light after ethidium bromide staining on a UV transiluminator. The gels were photographed using a gel documentation system (UVP, USA) for the analysis of bands. *E. faecium*, a *vanA*-negative strain, and *vanA*-positive strain *E. faecium* were used as reference strains.

RESULTS

Collection of strains: A total of 137 strains were collected: 120 (87.59%) isolated from urine; five (3.65%) were from ascetic fluid; four (2.92%) were from blood; three (2.19%) were from wounds; three (2.19%) were from Catheter; and two (1.46%) were from bone marrow. Distribution of Enterococcus isolates is shown in Table-I. A total of six VRE strains isolated during the investigation period, were identified to the species level as follows: One *E. faecalis*, three *E. faecium*, one *E. gallinarum* and one *E. durans*. The Susceptibility data obtained in vitro for 137 isolates with 13 antibiotic substances are shown in Table-II. Multidrug-resistant isolates were found in all of isolates. Of 137 enterococci only six (4.38%) isolates were resistant to vancomycin (MICs >16Aµg/ml,). Four (66.7%) out of six isolates were from urine specimens (one *E. faecalis*, two *E. faecium*, one *E. durans*,). The other two VRE were isolated from blood (16.65% *E. gallinarum*) and catheter (16.65% *E. faecium*). In addition to vancomycin, all of the VRE isolates (n = 6) demonstrated resistance to a wide variety of other antimicrobial agents such as penicillin, oxacillin, ampicillin, imipenem, nalidixic acid, ciprofloxacin, trimethoprim-sulfamethoxazole, gentamicin, and erythromycin. They were susceptible to amoxicillin-clavulanic acid, chloramphenicol and nitrofurantoin.

Table-I : Identification of Enterococcus isolates to the species level

Species	*No.*	*%*
E. faecalis	124	90.5
E. faecium	8	5.84
E. gallinarum	3	2.2
E. hirae	1	0.73
E. durans	1	0.73
Total	137	100

vanA gene: The *vanA*-specific 377-bp gene fragments of *E. faecalis*, *E. faecium*, *E. durans*, and *E. gallinarum*, were amplified from isolates and the results obtained for some strains are shown

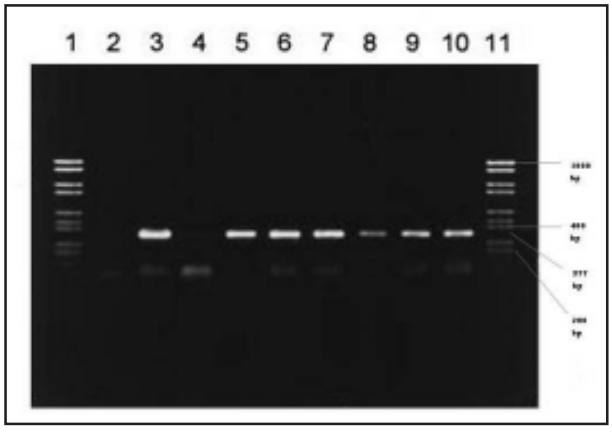

Fig-1: Detection of the van A gene in VRE from clinical specimens. (A) Lane 1 and 11, Size marker (100bp DNA Ladder); Lane 2, blank; Lane 3, *E. faecium* (positive control); Lane 4, *E. faecium* (negative control); Lane 5, *E. faecalis* VRE 1; Lane 6, 7, 8 *E. faecium* VRE 2, 3, 4; Lane 9, *E. gallinarum* VRE 5; Lane 10, *E. durans* VRE 6; The main band of the positive control strain and the VRE strains represents the vanA –specific 377 –bp fragment.

Table-II: Antibiotic resistance patterns of enterococci (n= 137) isolated from clinical specimens

Antibiotic(s)	Susceptible		Resistant	
	N	%	N	%
Ampicillin	58	42.34	79	57.66
Penicillin G	3	2.2	134	97.8
Oxacillin	0	0	137	100
Amoxicillin\clavulanic acid	100	100	0	0
Imipenem	113	82.5	24	17.5
Vancomycin	131	95.62	6	4.38
Gentamicin	43	31.4	94	68.6
Erythromycin	12	8.76	125	91.24
Ciprofloxacin	54	39.42	83	60.58
Nalidixic acid	0	0	137	100
Chloramphenicol	128	93.44	9	6.56
Nitrofurantoin	87	63.5	50	36.5
Trimethoprim-sulfamethoxazole	8	5.84	129	94.16

in figure. The positive control strain *E. faecium* contained the typical 377-bp fragment, and the negative control strain *E. faecium* did not.

DISCUSSION

Resistance is known to arise where use of antimicrobial agent is high and spread of these resistance bacteria is easy. Because of the limited therapeutic options and lack of enough information and programs to control rapid spread of Enterococci species, the mortality of the enterococcal infection is on the rise, so comprehensive data concerning the susceptibility patterns of enterococcal isolates is needed to control spread of these resistant bacteria. The distribution of enterococcal species observed in this study was similar to the previous reports and *E. faecalis* (90.5%) was the species more frequently isolated from clinical samples.[7,17,18]

The resistant rate to ampicillin (57.66%) in enterococcal isolates in this study was close to the resistance rate to ampicillin in Ireland (51%).[19] However resistance rate to ampicillin reported by Mathur et al in India (66%) is higher than our result.[20] Since ampicillin is the drug of choice in the treatment of enterococcal infections, the relatively high resistance of isolates in this study to ampicillin is of great concern especially in the case of endocarditis treatment. The finding that all of isolates were susceptible to amoxicillin/clavulanic acid is of great importance, and is similar to that reported in West Indies.[21] Although there are reports of 46% and 8.16% enterococci resistance to amoxicillin / clavulanic acid from India.[17,22] Chloramphenicol was the third most active antibiotic (93.44%) against our isolated enterococci, which is similar to findings of other studies.[7,23,24]

The prevalence of gentamycin resistance in enterococcal isolates in present study (68.6%) was higher than that found in Ireland (60%)[19] so limiting the success of such associated antibiotic therapy. The high-level resistance to aminoglycosides is of great concern, since it eliminates synergy with cell-wall active antibiotics, a combination commonly used for the treatment of enterococcal endocarditis. The prevalence of high-level gentamicin resistance in enterococci was comparable to that reported in the antimicrobial surveillance program in Europe.[9]

In this study 91.24% of isolates were resistant to erythromycin which is higher than what was reported from other countries such

as India (85%) and Lebanon (59%).[20,23] Of the total of 137 enterococcal isolates, 83 (60.58%) were resistant to ciprofloxacin which was similar to the findings of Miskeen et al (55.78%)[17] and Rudy et al[25] but was much lower than the result obtained in India (88%).[20].The prevalence of ciprofloxacin resistant enterococci have been reported to be 3.14%, 10% and 34% in French, Japanese and Lebanon studies respectively.[23,26,27] These results indicate diverse geographic distribution of ciprofloxacin resistant enterococci. According to literature nitrofurantoin is one of the effective antibiotics on enterococci species[18,25] but the results of this study showed 36.5% of resistance to nitrofurantoin, for which the possible common prescription of this drug for urinary tract infections could be the reason. The increase of resistance to nitrofurantoin in this study also corresponds to the finding of other workers in Tehran.[7]

The concomitant resistance observed to penicillin, oxacillin, co-trimoxazole, doxycycline, nalidixic acid, and some other antibiotics confirms that resistance of enterococci to multiple antibiotics is common as it is also observed in other parts of the world.[7,21,25]

The reported incidence of vancomycin-resistant enterococci (VRE) isolated in hospitals throughout the world,[2,7,9,23] has been also observed in Tabriz (4.38%). This result is in agreement with the enterococcal vancomycin resistance rates in some of the European countries and Canada but less than that reported in Turkey (11.7%), Latvia (14.3%)[9] and Tehran (10.6%).[7]

Presence of *vanA* gene cluster, on some of our isolates can provide transfer of vancomyvin resistance via conjugative plasmids not only to enterococci species but also to other bacteria such as *Staphylococcus aureus* so we expect the increase of the number of VRE in the future. The resistance rate to vancomycin (4.38%) is a serious threat that necessitates using surveillance studies, infection control and monitoring of antibiotic sensitivity among hospital isolated strains.

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