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Vancomycin-Resistant *Enterococcus faecalis* from a Wastewater Treatment Plant in Tabriz, Iran

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Abstract: The aim of this study was to determine the resistance pattern and the type of resistance genes of vancomycin-resistant *Enterococcus faecalis* from Gharamalek wastewater treatment plant in Tabriz, Iran. Following filtering of sewage samples, approximately 300 colonies grew on specific media, of which 53 were randomly selected and purified using 0.45 µm membranes. The membranes were placed on culture media containing antibiotics to isolate the vancomycin-resistant *Enterococcus*. Biochemical tests, antibiogram and determining minimum inhibitory concentration of antibiotics with E-test including vancomycin were performed. Polymerase Chain Reaction (PCR) was carried out to determine the type of resistance genes. All tested samples were found to be *E. faecalis*. Antimicrobial susceptibility tests indicated multidrug resistance in the samples, with 98% of them highly resistant to vancomycin. The highest frequency was of *vanA* (96%), followed by *vanB* (4%); *vanC* was not seen among the tested samples. The results confirmed that the risk of exposure to antibiotic-resistant pathogens from the evaluated urban wastewater is considerable.

Key words: Antimicrobial resistance, antibiotics, PCR, urban wastewater, sludge

INTRODUCTION

Enterococcus sp., the fermentative gram-positive cocci, are part of human and animal intestine normal flora. Their clinical importance was neglected in the past; however, with gaining resistance to antibiotics, they have received attention (Oprea *et al.*, 2004). They are regarded as the main reason for nosocomial infections as well as the second common cause of endocarditis, bacteremia and urinary system infections since 1970 (Flannagan *et al.*, 2003). Glycopeptidic antibiotics such as vancomycin and teichoplanin are the front line of treatment for serious infections caused by gram-positive cocci in the US. However, the excessive use of vancomycin in the past two decades has resulted in *Enterococcus* sp., that are resistant to glycopeptides and therefore, has brought about major problems in the treatment of nosocomial infections caused by these microorganisms (Shankar *et al.*, 2002). The importance of Vancomycin-Resistant *Enterococcus* (VRE) is emphasized when a patient with vancomycin-resistant *Staphylococcus aureus* infection has concurrent infection

of vancomycin-resistant *Enterococcus faecalis*. Samples of *S. aureus* along with *E. faecalis*, resistant to high levels of vancomycin, have been isolated from wounds on diabetic feet, suggesting the transmission of resistance genes between these microorganisms.

Many studies have shown the spreading capability of these strains in different environments carrying their resistance genes. *In vivo* studies suggest that enterococcal sex pheromones play a role in transmission of vancomycin resistance from *E. faecalis* to *S. aureus* (Stosovic *et al.*, 2004). Conjugated transposones of *E. faecalis* can also transmit the resistance to *S. aureus* (Flannagan *et al.*, 2003). These microorganisms share *vanA*, the main genetic factor of resistance to vancomycin in *S. aureus* (Shankar *et al.*, 2002). Vancomycin resistance genes of *Enterococcus* sp., include *vanA*, *vanB*, *vanC1* and *vanC2/C3*. *vanA* and *vanB* are located on transposones Tn 1546 and Tn 1547 either on plasmids or chromosomes, where *vanC1* and *vanC2/C3* are located only on chromosomes. Polymerase Chain Reaction (PCR) has been used to detect the types of resistance genes.

Transmission of resistance genes among *Enterococcus* sp., has been reported in urban wastewater systems. Samples of VRE in wastewater were first seen in the UK in 1993, followed by other cases including its detection in fecal samples of pigs and birds in a small town in Germany (Stosovic *et al.*, 2004). Since vancomycin resistance genes are transmittable between microorganisms, environmental repositories like municipal wastewater system can have a significant role in the spreading of resistant *Enterococci* to healthcare facilities including hospitals. The purpose of this study was to determine the resistance pattern and the type of resistance genes of vancomycin-resistant *Enterococcus faecalis* from a wastewater treatment plant in Tabriz, Iran.

MATERIALS AND METHODS

Sample collection: Sampling was carried out in September and October 2007 from Gharamalek municipal sewage treatment plant in Tabriz, Northwest Iran. Samples were collected from incoming sewage, sludge and outgoing sewage.

Isolation of bacteria: 1:10 dilutions of samples with phosphate-buffered saline were prepared and filtered using 0.45 μm membranes (Millipore Corporation, USA). Filters were then removed and samples were transferred to Brain Heart Infusion agar medium (DIFCO, USA) and kept at 37°C for 24 h. Filters were again placed in M *Enterococcus* agar plates (DIFCO, USA) containing 8 $\mu\text{g mL}^{-1}$ vancomycin and incubated at 42°C for 48 h followed by another incubation on bile esculin agar (DIFCO, USA) at 45°C for 24 h. Finally, samples were purified from filters on blood agar (Shankar *et al.*, 2002).

Determining biochemical identity: After purifying the cultures, the identity of species was determined by conventional biochemical tests including growth in presence of 6.5% NaCl, L-pyrrolidonyl-L-naphthylamide and growth in 45°C (Schouten *et al.*, 2000).

Antimicrobial susceptibility testing: Antimicrobial resistance pattern was determined using vancomycin (30 μg), streptomycin (10 μg), chloramphenicol (30 μg), gentamycin (120 μg), amikacin (30 μg), ampicillin (10 μg), ciprofloxacin (5 μg), tetracycline (30 μg) and erythromycin (15 μg) diffusion disks (BBL, SensiDisk, USA) as well as broth microdilution using vancomycin and teichoplanin disks (E-test, AB BIO DISK, Sweden) to

determine the Minimum Inhibitory Concentration (MIC) according to the guidelines of the National Committee for Clinical Laboratory Standards (Schouten *et al.*, 2000).

DNA extraction: Motanolysin method was used to extract the whole DNA content. Briefly, 2 mL of suspension from 24 h bacterial culture in BHI broth was centrifuged with 14000 rpm for 10 min. Then, 1 mL of lysis buffer (1 mmol NaCl, 10 μmol Tris HCl, 5 mmol EDTA, 0.5% Triton X-100) was added to the substrate. The followings were then added consecutively and the solution was incubated at each stage at 37°C for 1 h: 10 mg mL⁻¹ lysosyme, 50 units of motanolysin, 20 mg mL⁻¹ proteinase K, 20% sarcosil and 25% SDS. Finally, the obtained solution was extracted first with phenol and chloroform-isopropanol and then only with chloroform. Following centrifuge, the supernatant was collected (Van Horn *et al.*, 1996).

PCR: PCR assay was performed with the specific primers to assess the vancomycin resistance genes including *vanA*, *vanB*, *vanC1* and *vanC2.3*. Standard and positive controls included *E. faecalis* ATCC 51299 for *vanA*, *E. faecium* ATCC V583 for *vanB* and *E. gallinarum* ATCC BM4174 for *vanC*. PCR assay was performed separately for each primer. Then, electrophoresis of PCR products was carried out on 1% agarose gel and the results were evaluated after staining.

RESULTS AND DISCUSSION

Sampling was performed for a total of three times from the sewage treatment plant. Following filtering of sewage samples, approximately 300 isolates grew on specific media, of which 53 were randomly selected and purified. Biochemical tests showed that all of the isolates were *E. faecalis*.

Ninety eight percent of species had high-level resistance to vancomycin (MIC ≥ 4); only one species had a MIC of ≤ 4 . Antibiogram of the nine antibiotics evaluated demonstrated a high-level multidrug resistance in the tested samples (Table 1). Except for the cases of chloramphenicol (21%) and tetracycline (15%) which samples showed a considerably lower resistance to them compared to others, 94% of sample species were resistant to the tested antibiotics.

According to the results of PCR, the highest frequency was of *vanA* followed by *vanB* (Fig. 1). In two cases which did not contain *vanA*, both had a MIC of ≤ 8 for teichoplanin indicating *vanB*. The distribution of PCR

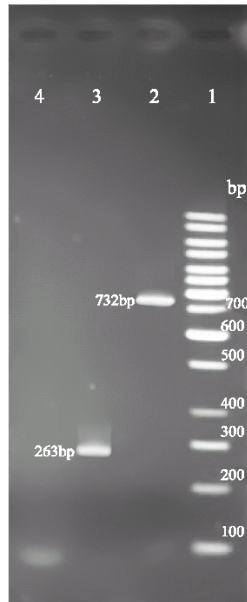


Fig. 1: The results of PCR indicate the presence of *vanA* and *vanB*, but no *vanC*, among the tested samples

Table 1: Antibiogram results in the tested samples with evaluated antibiotics

Antimicrobial agent	Resistance (%)
Vancomycin	100
Streptomycin	100
Erythromycin	98
Amikacin	98
Ampicillin	98
Gentamycin	94
Ciprofloxacin	94
Chloramphenicol	21
Tetracycline	15

Table 2: Distribution of *vanA* and *vanB* in the tested samples (n = 53)

Vancomycin resistance gene	Positive n (%)	Negative n (%)
<i>vanA</i>	51 (96)	2 (4)
<i>vanB</i>	8 (15)	45 (85)
<i>vanC1</i>	0 (0)	53 (100)
<i>vanC2,3</i>	0 (0)	53 (100)

Table 3: Distribution of *vanA* and *vanB* according to the source of samples taken

Source of sampling	No. of samples with <i>vanA</i>	No. of samples with <i>vanB</i>
Incoming	13	2
Sludge	18	0
Outgoing	20	0
Total	51	2

results for the evaluated samples are presented in Table 2 and the same according to the source of sampling are presented in Table 3.

In the present study, a selective method was used for the isolation of *Enterococcus* sp., with high resistance to vancomycin. This method provides suitable isolation of

such species from an urban wastewater system and facilitates the phenotype and genotype assessments. All of the isolated species in the present study were *E. faecalis*. Earlier studies also showed a higher prevalence of *E. faecalis* in wastewater systems compared with other species (Harwood *et al.*, 2001; Méndez-Alvarez *et al.*, 2000).

Although multidrug resistance was seen to be high with the tested antibiotics, the samples showed a relatively lower resistance to chloramphenicol and tetracycline in comparison with other antibiotics such as ampicillin and gentamycin. The reason for this observation might be the lower clinical use of these two antibiotics, which results in a lower amount of resistance plasmids in the swage system (Murray, 2000; Lukášová and Šustáčeková, 2003).

vanA phenotype is determined by acquired high-level resistance to both vancomycin and teichoplanin, where *vanB* phenotype is determined by resistance only to vancomycin. *vanC* phenotype is determined by low-level resistance to vancomycin and susceptibility to teichoplanin. In this study, only two species did not contain *vanA* and instead had *vanB* genotype; MIC of ≤ 8 for teichoplanin demonstrated the susceptibility of both samples confirming *vanB* phenotype as the range of MIC in species with *vanB* varies between 4-256. *vanB* is less frequent than *vanA* and is considered as a second factor for the spread of the phenotype (Murray, 2000). *vanC1* and *vanC2,3*, the intrinsic resistance factors of *E. gallinarum* and *E. calciflavus*, were not seen in the present study as neither of these species were found among the studied samples. Novais *et al.* (2005) in a similar study isolated 31 samples from municipal hospital and river wastewaters in Portugal and found that 25 cases were *E. faecalis*. Performing PCR revealed that 27 cases had *vanA*, four cases had *vanB* and only one case had *vanC*. In the study of Iversen *et al.* (2002) in Sweden, VRE samples from an urban wastewater treatment plant were isolated using M Enterococcus agar specific medium were containing $8 \mu\text{g mL}^{-1}$ vancomycin. From 35 VRE samples identified, 24 isolates (69%) were *E. faecalis* and most of the samples had multidrug resistance. PCR of the samples revealed that 30 isolates had *vanA* and five had *vanB*. Regarding the results of these studies and the present study, the use of membrane filtering and M Enterococcus agar medium containing $8 \mu\text{g mL}^{-1}$ of vancomycin seems to be a standard effective method for isolation of highly-resistant *Enterococcus* from an urban swage system.

Despite being differentiated by their metabolic activity, the two main *E. faecalis* and *E. faecium* species can cause similar diseases. Although *E. faecium*

has steadily proved the most resistant species to various antibiotics to date among *Enterococcus* sp., *E. faecalis* yields the highest prevalence in the clinical settings, probably due to having the highest prevalence in the environment as well as containing more virulence factors. Regarding the frequency of resistance genes to vancomycin, many studies as well as the present study show that *vanA* owns the highest frequency, one reason being its high transferring capability of its transposone. *vanB* follows *vanA* as the second important gene responsible for the transmission of the disease. Present results confirmed that the risk of exposure to antibiotic-resistant pathogens from the evaluated urban wastewater is considerable. Therefore, measures for reducing or eliminating the microorganisms from the swage system in the wastewater treatment plants should be implemented in order to prevent the spread of the resistance species to healthcare facilities.

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