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Distribution of Enterococcal Species and Detection of Vancomycin Resistance Genes by Multiplex PCR in Tehran Sewage

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ABSTRACT

Background: Enterococci are important because of their role as the leading cause of nosocomial infections which have a significant role in the dissemination and persistence of antimicrobial resistance genes. **Methods**: In this study, we determined the distribution of enterococcal species in the sewage treatment plants in Iran. Furthermore, we improved a rapid and specific PCR method using primers (*sodA* and *ddl* genes) for identification of enterococci spp. **Results and Conclusion**: A total number of 712 enterococci spp. were isolated and the results showed that 56%, 24%, 12%, 4%, 2%, 1% and 1% isolates were *E. faecium, E. hirae, E. faecalis, E. gallinarum, E. casseliflavus, E. mundtii* and other enterococcal spp., respectively. The use of species-specific PCR was in agreement with the biochemical tests. Furthermore, multiplex PCR was developed to study the presence of vancomycin resistant genes in *E. faecium* or *E. faecalis*. The multiplex PCR appeared to be a useful, rapid and specific method for detecting and discriminating genotypes for vancomycin-resistant *Enterococcus*. *Iran. Biomed. J. 11 (3): 161-167, 2007*

Keywords: *Enterococcus*, Vancomycin, PCR, Multiplex PCR, *sod*A

INTRODUCTION

The major niche of enterococci is the gastrointestinal tract of human and animal where they make up a significant portion of the normal flora [1]. They are released into the environment via the sewage where they can survive for a long period of time [2, 3].

Enterococci, nowadays, represent the second leading cause of nosocomial urinary tract infections and the third leading cause of nosocomial bacteremia [4, 5]. They include 20 species, but most human enterococcal infections are caused by *E. faecalis* and *E. faecium* [4, 5]. A few cases of human infections caused by other enterococci spp. such as *E. durans*, *E. gallinarum* and *E. casseliflavus* have also been reported [4].

Biochemical identification tests to the species level of enterococci are not routinely performed in the clinical laboratories which are very laborious and require time-consuming steps. This, therefore, explain a possible underestimation of the frequency of various enterococcal species in the infections. In order to overcome the problems associated with the biochemical testing, the use of molecular methods for the identification of enterococcal species has been suggested [6]. Several genes coding heat shock protein 60, elongation factor EF-Tu, D-Ala: D-Ala ligase and manganese-dependent superoxide dismutase have been recommended for the molecular identification of the enterococcal species [6-8]. The use of PCR for identification of genus and species of enterococci has been reported previously [6-8]. However, we report for the first time the distribution of enterococci in Tehran (Iran) sewage followed by a multiplex PCR for rapid identification of *Enterococcus* spp. and vancomycin resistant genes, simultaneously.

MATERIALS AND METHODS

Sample collection. Samples were collected ten times from 2004 to 2005 from four different urban sewage treatment plants located in different parts of Tehran including Shoosh (south), Jonoob (south), Sahebgharanyeh (north) and Ekbatan (west). At every sewage treatment plant, sampling was carried out on incoming raw sewage (n = 282), outgoing treated sewage (n = 221) and sludge (n = 209). All

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of the samples were collected in the sterile 250 ml bottles and were kept refrigerated and analyzed within 3 h.

Isolation of enterococci. For achieving isolated colonies, various sample dilutions were filtered with 0.45 µm membrane (Millipore Corporation, Bedford, MA, USA). Outgoing treated sewage was vortexed and filtered directly. The samples taken from sludge and incoming raw sewage were diluted 5-folds with phosphate-buffered saline before filtration as described before by others [2, 3]. The membranes were subsequently transferred in the m*Enterococcus* agar (Becton Dickinson and Co., Sparks, MD, USA) and incubated at 37°C for 48 h. The membranes with well isolated colonies were transferred to bile esculin agar plates and then incubated at 44°C for 2 h. The black isolated colonies were then selected for Gram-staining, growth at 6.5% NaCl, Pyrrolidonyl aminopeptidase (PYR) [9] and catalase tests [10, 11]. The total number of enterococci was recorded and the presumed *Enterococcus* was defined as the isolates that grew at 44°C and in 6.5% NaCl, esculin and PYR positive and catalase negative.

Phenotyping of enterococcal isolates. Isolates exhibiting *Enterococcus* characteristics were identified to the species level using the following biochemical tests: acid production of L-arabinose, lactose, D-sorbitol, D-mannitol, L-sorbose, glucose, methyl-α-D-glucopyranoside, arginine dihydrolase, motility, hippurate hydrolysis, haemolysis, pigmentation, tetrazolium 0.01% and tellurite 0.04% reduction [10, 11].

Antimicrobial susceptibility testing. The susceptibility tests were performed with disk diffusion method and interpreted according to the guidelines from the Clinical and Laboratory Standards Institute [12]. The following antibiotics were purchased from BD BBL (Becton, Dickinson and Company, Sparks, MD, USA); vancomycin (30 µg), tetracycline (30 µg), gentamicin (120 µg), erythromycin (15 µg), ciprofloxacin (5 µg) and chloramphenicol (30 µg). Minimum inhibitory concentration (MIC) of the vancomycin resistant enterococci (VRE) isolates was determined by using Etest (AB Biodisk, Solna, Sweden). *E. faecalis* (ATCC 29212) and *E. faecalis* (ATCC 51299) were used as the quality control strains.

PCR and multiplex PCR. The genes encoding D-alanine-D-alanine ligases specific for *E. faecium* (*ddl* $_{E. faecium}$) and for *E. faecalis* (*ddl* $_{E. faecalis}$) and the vancomycin resistance genes, *vanA* and *vanB*, were detected by a modified multiplex PCR assay using the primers listed in Table 1. The classical PCR was done using primers (superoxide dismutase genes) specific for *E. faecium* (*sodA* $_{E.faecium}$), *E. faecalis*

Table 1. Primers used in this study.

species	Sequence (5'-3')	Reference	Product size (bp)
E. faecium	TTGAGGCAGACCAGATTGACG TATGACAGCGACTCCGATTCC	14	658
E. faecalis	ATCAAGTACAGTTAGTCT ACGATTCAAAGCTAACTG	14	941
vanA	CATGAATAGAATAAAAGTTGCAATA CCCCTTTAACGCTAATACGATCAA	14	1030
vanB	GTGACAAACCGGAGGCGAGGA CCGCCATCCTCCTGCAAAAAA	14	433
E. faecium	CGAATTTAAATTCAGCAATTGA CTTTCCTTCCATCAATGGAG	This report	359
E. faecalis	ATGTGACTAACTTAAACGCAG AATCTTGGTTTGGTGTTGAA	This report	347
E. gallinarum	TTACTTGCTGATTTTGATTCG TGAATTCTTCTTTGAAATCAG	6	189
E. hirae	TAAATTCTTCCTTAAATGTTG CTTTCTGATATGGATGCTGT	6 This report	186
E. casseliflavus	GCTAGTTTACCGTCTTTAACG TTAGCAGACTTTTCTTCTGTAC	6 This report	253
E. mundtii	CAGACATGGATGCTATTCCATCT AGGTTTCTTGCCTTCCATCAAT	6 This report	301

Table 2. Prevalence of enterococci species according to the site of collection.

Species / Sampling time	*E. faecium*	*E. hirae*	*E. faecalis*	*E. gallinarum*	*E. casseliflavus*	*E. mundtii*	*other*	No.
E 2004	3	-	-	-	-	-	-	3
J 2004	12	2	2	4	-	-	-	20
S 2004	9	-	6	-	-	2	3	20
Sh 2004	42	-	2	-	6	-	-	50
Sh 2004	37	17	17	-	1	-	2	74
J 2004	32	-	4	9	1	2	-	48
S 2005	26	3	5	2	-	-	-	36
E 2005	102	66	18	4	-	3	1	194
S 2005	22	27	11	-	1	2	-	63
E 2006	117	55	17	6	7	2	-	204
Total	402	170	82	25	16	11	6	712

E,Ekbatan; J, Jonoob; Sh, Shoosh; S, Sahebghranyeh sewage treatment plants.

(*sodA* $_{E.\ faecalis}$), *E. hirae* (*sodA* $_{E.\ hira}$), *E. casseliflavus* (*sodA* $_{E.\ casseliflavus}$), *E. gallinarum* (*sodA* $_{E.\ gallinarum}$) and *E. mundtii* (*sodA* $_{E.\ mundtii}$) listed in Table 1. The following VRE strains carrying *vanA* and *vanB* genes were used as quality control strains; *E. faecalis* V583 (*vanB*) and *E. faecium* BM4147 (*vanA*). Species identification of the isolates was done using PCR with genus and species-specific primers (Table 1). For DNA extraction, one isolated colony from each plate was transferred into 200 µl distilled water and boiled at 100°C for 15 min [13]. The mixture was centrifuged and 10 µl of supernatant was used as the DNA template in the PCR mix (for PCR and multiplex PCR) containing 10× PCR buffer, *taq* DNA polymerase (0.5 U) (HT Biotechnology, Cambridge, United Kingdom), each primer (1.6 µM), $MgCl_2$ (1.2 µM) and each dNTP (0.64 µM). The PCR cycles for the isolates were as follow: an initial denaturation at 95°C for 4 min, with 30 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 1 min and elongation at 72°C for 1 min and final extension at 72°C for 7 min [14]. PCR products were electrophoresed on a 1.5% agarose gel in a 0.5 X Tris-borate-EDTA buffer and stained in ethidium bromide.

RESULTS

Prevalence and antibiotic resistance. A total of 712 isolates from four different sewage treatment plants in Tehran were identified to the species level using the standard biochemical tests and PCR. Six enterococcal species: *E. faecium, E. hirae, E. faecalis, E. gallinarum, E. casseliflavus* and *E. mundtii*, were isolated from Tehran sewage using PCR (Fig. 1 and Table 2). The classical PCR using conserved *sodA* gene which catalyzes the dismutation of superoxide showed that in some cases distinct bands were not evident for *E. hirae* and *E. gallinarum*. On the other hand, a distinct DNA band for *E. mundtii* was observed (Fig. 1). These organisms covered 99% of the total isolated enterococci. Other rare isolates such as *E. raffinosus, E. dispar* and *E. avium* with low frequency (1%) were also obtained. *E. faecium* were the most frequently identified *Enterococcus* spp. (53%) followed by *E. hirae* (24%) and *E. faecalis* (12%). *E. faecium* was isolated from sewage in every

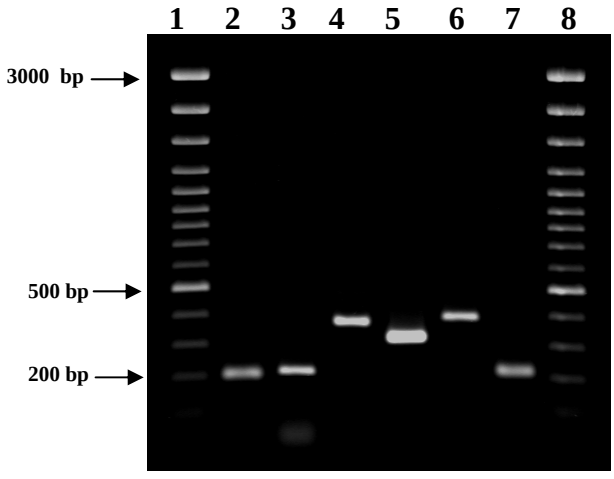

Fig. 1. PCR for identification of different enterococcal spp. PCR products were loaded on 1.5% agarose gel. 100 bp DNA ladder (lanes 1 and 8), *E. hirae* (lane 2), *E. gallinarum* (lane 3), *E. faecalis* (lane 4), *E. mundtii* (lane 5), *E. faecium* (lane 6) and *E. casseliflavus* (lane 7).

Table 3. Antibiotic resistance according to the every sample in *E. faecium, E. hirae* and *E. faecalis* isolates (%) no isolates was detected.

Species	*E. faecium*						*E. hirae*						*E. faecalis*					
Sampling time	V	Tet	Gm	E	Cip	C	V	Tet	Gm	E	Cip	C	V	Tet	Gm	E	Cip	C
E 2004	0	10	0	0	67	33	-	-	-	-	-	-	-	-	-	-	-	-
J 2004	0	50	0	0	17	42	0	0	0	0	0	0	0	50	0	0	0	0
S 2004	0	0	0	67	29	0	-	-	-	-	-	-	0	83	0	50	0	0
Sh 2004	0	19	2	64	10	17	-	-	-	-	-	-	0	50	0	0	50	0
Sh 2004	8	16	5	32	30	5	0	0	0	0	0	0	0	36	0	0	0	0
J 2004	0	41	0	34	28	19	-	-	-	-	-	-	0	25	0	25	25	0
S 2005	0	27	0	38	12	12	0	0	0	0	0	0	0	20	0	0	20	0
E 2005	11	16	10	70	38	9	0	5	0	5	2	0	0	6	6	6	17	0
S 2005	18	18	18	55	41	14	0	0	0	0	7	0	0	73	0	0	18	9
E 2006	1	22	1	57	15	0	0	0	0	2	4	0	0	59	6	12	12	6

Species	*E. gallinarum*						*E. casseliflavus*						*E. mundtii*					
Sampling time	V	Tet	Gm	E	Cip	C	V	Tet	Gm	E	Cip	C	V	Tet	Gm	E	Cip	C
E 2004	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
J 2004	0	0	0	0	25	0	-	-	-	-	-	-	-	-	-	-	-	-
S 2004	-	-	-	-	-	-	-	-	-	-	-	-	0	0	0	0	0	0
Sh 2004	-	-	-	-	-	-	0	17	0	67	17	0	-	-	-	-	-	-
Sh 2004	-	-	-	-	-	-	0	0	0	0	0	0	-	-	-	-	-	-
J 2004	0	0	0	0	0	0	0	0	0	0	0	0	0	50	0	0	0	0
S 2005	0	50	0	50	0	0	-	-	-	-	-	-	-	-	-	-	-	-
E 2005	0	25	0	25	0	0	-	-	-	-	-	-	0	67	33	67	67	33
S 2005	-	-	-	-	-	-	0	0	0	100	0	0	-	-	-	-	-	-
E 2006	0	33	0	17	33	0	0	43	0	43	14	14	0	0	0	0	50	0

V, vancomycin; Te, tetracycline; Gm, gentamicin; E, erythromycin; Cip, ciprofloxacin; C, chloramphenicol; The symbol (-) denotes no isolates was detected. E, Ekbatan; J, Jonoob; Sh, Shoosh; S, Sahebghranyeh treatment plants.

sampling, *E. faecalis* in 9, *E. hirae* in 6, *E. gallinarum* in 5, *E. mundtii* and *E. casseliflavus* were isolated in 4 out of 10 sampling.

Antimicrobial susceptibility tests showed that the highest level of resistance was observed with erythromycin, tetracycline and ciprofloxacin (Tables 3). *E. avium* was susceptible to all of the antibiotics tested here (data not shown). The antibiotic resistance to three major isolates is shown in Table 3. *E. faecium, E. faecalis* and *E. hirae,* showed resistance to more than one antibiotic examined. The level of antibiotic resistance to the least common isolates, *E. gallinarum*, *E. casseliflavus* and *E. mundtii*, was less evident (Table 3).

Antibiotic resistance study showed that all VRE were *E. faecium*. All 19 VRE isolates were simultaneously resistant to erythromycin, amikacin and ciprofloxacin. The highest level of resistance was observed with ampicillin (95%), gentamicin (89%) and chloramphenicol (53%). Sixteen percent of the VRE isolates were resistant to all of the antibiotics examined here. The frequency of VRE strains isolated from the three sewage treatment plants was similar to an average of 3% VRE from the total enterococcal isolations.

Multiplex PCR. Since distinct DNA bands were not observed following amplification of *sodA* gene, the gene coding for D-Ala:D-Ala ligase (*ddl)* was used in the multiplex PCR. The multiplex PCR was performed and *vanA* gene was detected in all of the 19 VRE strains. These isolates were highly resistant to vancomycin with MIC≥256 mg/l. The *vanB* gene was also detected in 6 of 19 isolates (32%) (Fig. 2). VRE strains (36%) were isolated from Ekbatan treatment plant (west part of Tehran), 21% and 16% were isolated from Sahebgharanyeh (north) and Shoosh (south) treatment plants, respectively.

DISCUSSION

For the first time we have determined the prevalence of enterococci population in the sewage in Iran. Similar to the report by the European study group [3], it was shown here that *E. faecium* was the most common enterococcal isolate followed by *E. hirae*. The presence of *E. faecium* in every sampling and sewage treatment plant indicated the capability of this organism to survive and resist in the sewage. Furthermore, it was shown that all of the VRE strains were *E. faecium*, that have important role to disseminate and establish antibiotic resistance within the enterococcal populations.

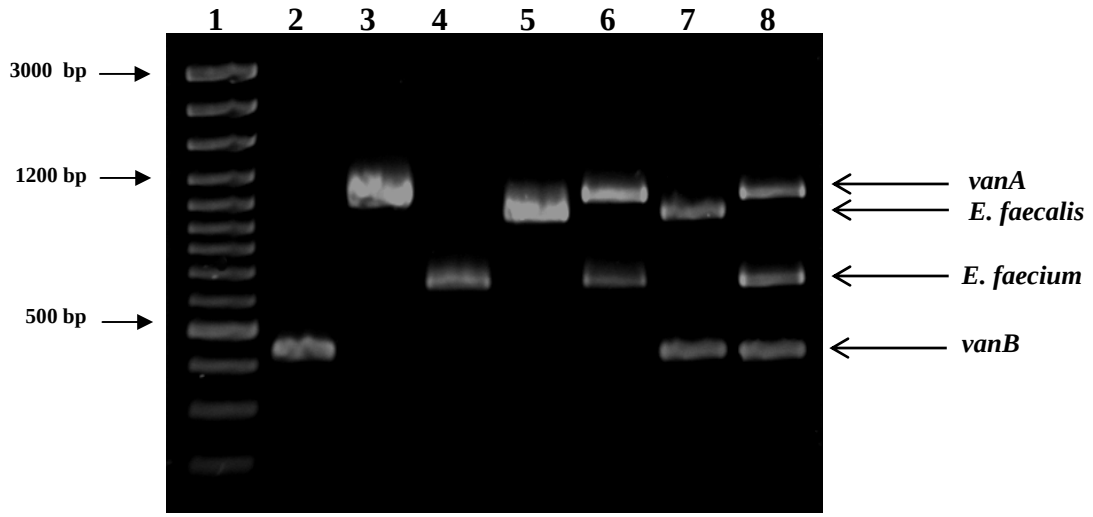

Fig. 2. Multiplex PCR for detection of *vanA* and/or *vanB* genes, and *E. faecalis* or *E. faecium*. PCR products were loaded on 1.5% agarose gel. 100 bp DNA ladder (lane 1), positive control *vanB* gene alone (lane 2), positive control *vanA* gene alone (lane 3), *E. faecium* control without *van* genes (lane 4), *E. faecalis* control without *van* genes (lane 5), *E. faecium* BM4147 carrying *vanB* gene (lane 6), *E. faecalis* V583 carrying *vanA* gene (lane 7), and *E. faecium* sample carrying both *vanA and vanB* genes (lane 8).

Contrary to the report by others [15], we couldn't isolate certain rare species of enterococci such as *E. durans* and *E. cecorum* in the sewage [15]. This could be due to i) climate differences and ii) bacterial composition in the sewage treatment plants in Tehran, that could justify the differences observed in our study and the studies done in Europe [15].

No other strains beside *E. faecium* showed high level of resistance to vancomycin. *E. faecalis* and *E. gallinarum* strains only showed intermediate resistance to vancomycin as determined by disk diffusion assay. However, upon performance of MIC, it was shown that these strains were vancomycin susceptible. The frequency of vancomycin resistance *E. faecium* isolated in our study was about 3% from the total isolates. The prevalence of the VRE obtained from the sewage treatment plants varies in different countries. In Spain, for example, much lower frequency VRE (0.4%) has been observed in the waste water [16]; in New Zealand [17], France [13] and USA [1], the percent of VRE isolation from broilers, human fecal and farm wastewater has been 6%, 4% and 6%, respectively. Up to 20% of VRE, however, has been reported in Portugal from hospital sewage samples [18]. Kuhn and her colleagues [3] showed that the frequency of VRE isolated from animals, humans and the environment in different European regions was in the range of 8 to 11%. Collectively, the data suggest that the frequency of VRE varies in different geographical regions and the site of sample collection.

Several investigators have reported the isolation of vancomycin resistant *E. faecium, E. faecalis* and *E. hirae* [3, 15]. We, however, could only isolate vancomycin resistant *E. faecium* in our samples which may suggest that the transfer of vancomycin resistant genetic elements to other enterococcal spp., which has been reported extensively elsewhere [19], is uncommon in Iran. Furthermore, the antibiotic resistance pattern of the VRE was very similar among the isolates obtained from the different sewage treatment plants. For example, *E. hirae* was almost susceptible to all of the antibiotics examined.

Identification of enterococci by using biochemical tests is a tedious process that requires a numerous tests and long times. The use of molecular techniques could, therefore, enhance the identification process. A multiplex PCR assay was developed in this study to enable the identification of vancomycin resistant genes in *E. faecium* and *E. faecalis*.

Variations in the sequences of *sod*A genes among enterococcal spp. have been reported [6]. The *sod* gene coding for superoxide dismutase was selected as the target for the classical PCR. The results showed that for enterococcal spp. distinct bands could not be observed; therefore, the gene coding for D-Ala:D-Ala ligase (*ddl)* was used for further analysis in our multiplex PCR. The result showed that distinct DNA bands could be viewed when multiple primers for *ddl* genes for *E. faecium/E. faecalis* and *vanA/vanB* genes were included in a single PCR mixture.

VanA and *vanB* genes are two of the most common vancomycin resistant genes which are transferable and are of significant importance in the pathogenicity of the enterococci. *VanA* confers high-level resistance to vancomycin and teicoplanin and *vanB* confers low-level resistance to vancomycin and no resistance to teicoplanin [2]. Using the multiplex PCR in a single assay we observed that all VRE strains carry *vanA* gene. We also found that *vanB* gene was more prevalent in our samples than reported by in other countries [1, 2, 4, 18].

Jackson *et al.* [6] reported the use of 7 different mixtures of primers for identification of over 20 species of enterococci. It has been suggested that the reason they were not able to perform a single multiplex PCR is because of the inhibitory activity of the primers in the mixture [6]. In the present study, we also observed that the primers for the enterococci genus *Ent* gene inhibit *Sod*A gene in a PCR reaction. Therefore, similar to other studies [6] we were not able to perform a single multiplex PCR for all of the enterococcal spp. However, the results of the multiplex for *E. faecium* and *E. faecalis* using *ddl* were satisfactory. Furthermore, a single annealing temperature (52°C) was found for DNA amplification of all enterococcal spp. which allowed performing PCR at the same time in a single PCR instrument.

In conclusion, we have determined the usefulness of the multiplex PCR method that was optimized in this study for identification of *van* genes in *E. faecium* and *E. faecalis*. This method appears to be convenient, rapid and simple for identification of the VRE species with the glycopeptide resistance genes in the clinical laboratory.

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